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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Abstract

The potential of 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 (70Gy) of host (pest) was exercised to avoid any pest population build-up from the host larvae that could miss inadvertently EPN infection. The infective juveniles (IJs) derived from radio-sterilized host exhibited 67.3h to induce host mortality, 132h for IJs incubation, 87.8% parasitization and 98.9 IJs harvesting per mg host body weight, that indicated almost similar parasitizing behaviour of these IJs as control. The findings indicated the suitability of 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 through biocontrol strategy.

Keywords Entomopathogenic nematodes, host irradiation, biocontrol agents, pest management, Spodoptera litura, Steinernema thermophilum.
Introduction

The cotton leafworm *Spodoptera litura* (Fabricius) (Lepidoptera : Noctuidae) is a dreadful cosmopolitan pest which infests over hundred host plants including soybean, tobacco, cotton, cabbage and chickpeas\(^1\). The outbursts of *S. litura* has become very frequent lately in Asian regions, due to insecticide resistance\(^2-4\). Amongst all the ecologically sound tactics which are applied for the control of *Spodoptera* spp., biorational and biological control techniques are pre-eminent. One of such potential biological control pest management tactics is by using entomopathogenic nematodes\(^5,6\). In recent times, the application of entomopathogenic nematodes (EPNs) from the families *Steinernematidae* and *Heterorhabditidae* have become very prominent, due to their safety to humans, immense reproductive potential, and specificity \(^7-9\). The EPNs mostly attack the pests present in soil\(^10\). The infective juveniles (IJs) enters into the insect body via natural openings and release their symbiotic bacteria into the insect hemocoel. The bacteria multiplies into the insect, releases various toxins and causes the death of the insect within 48-72h\(^11\). The potential of EPNs as a biological control agent on noctuid lepidopteran insects has been studied broadly by applying *Steinernema* spp. against *Spodoptera* spp.\(^12-15\), *Helicoverpa zea*\(^16\). *S. 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 the management of insect pests\(^19\). The nuclear techniques have a great relevance in the biological control of pests in different means, 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 techniques like disruption of pheromone\(^21\), host plant resistance\(^22\), entomopathogens\(^23\) and natural enemies\(^24\).
Insects infected with entomopathogenic nematodes could be released, and the pest suppression could be achieved finally by the progeny IJs which would be released from the insect cadavers\textsuperscript{25}. EPNs are capable of surviving in the harsh and dry conditions for a long period of 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\textsuperscript{26,27}. It has been stated that EPNs can discover and rapidly kill their host in 48-72 hours after entering into them\textsuperscript{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 host followed by exposure to IJs, then they should be transferred to the field before they face mortality. In this manner the IJs emerging out of the infected cadavers get released into the environment and they further seek out new hosts in the field to multiply and maintain their population\textsuperscript{25}. 

The present study was attempted to study the parasitization performance of EPNs, \textit{Steinernema thermophilum} on radio-sterilized lepidopteran pest, \textit{Spodoptera litura} (as host) and ascertain the viability of \textit{S. thermophilum} derived from the radio-sterilized host, \textit{S. litura} in order to assess the potential of this sterilized lepidopteran pest as feasible host to transport the viable parasitoid, \textit{S. thermophilum} safely into the field in a safe mode.

2. Materials and Methods

2.1 Maintenance of insects

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

**2.2 Maintenance of *Steinernema thermophilum* entomopathogenic nematodes**

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

**2.3 Irradiation of insects**

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), Ministry of Defence, Delhi, India, with Cobalt-60 source with dose rate 0.509-0.483 KGy/min. 70 Gy dose was employed for the radio sterilization of last instar *S. litura* larvae.

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

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

0-1 day old sixth instar *S. litura* larvae (normal and irradiated 70 Gy) were placed individually in glass petri dishes (50×15mm) lined with double layer of Whatman filter paper. The freshly harvested IJs (1-2 week old) were used in all the experiments for inoculation. 25-30 IJs per larvae were inoculated and the petri dishes were sealed with parafilm to avoid any infection. The larvae were kept at 27± 2°C, 75± 5% relative humidity. The host larvae were observed every 2-3 hour to check the morbidity and mortality time. Morbidity was assessed as a primary
behavioural response of the larvae caused due to release of bacterial toxins into the hemolymph of the host resulting into septicemia after the infection of EPNs. It included slow response of the larvae to a probe (gently touching them with a pair of forceps), lethargic nature and lag in the resumption of body posture when it was turned upside down. Larvae which died due to infection became slightly flaccid and dark brown in color, which was not observed in natural mortality. After the death of the larvae, the cadavers were transferred into White traps (prepared with petri dishes-90 mm diameter). The IJs started releasing after 5-6 days of the death of host as the resources inside the host body started depleting. The time taken by the IJs to release from the host cadavers was also recorded as incubation time. The IJs coming out of the cadavers were harvested daily and the total harvesting profile of IJs, harvesting/mg body weight of host and the harvesting period were recorded in each condition. Average reading of each cohort of 10 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 percent parasitization.

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

2.5 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 the adult moths developed from normal and radio-sterilized host. 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.

3. Results
3.1 Radio-sterilization of host larvae, *S. litura*

The adults developed from irradiated sixth instar larvae (70Gy) were significantly affected. The percentage of pupa formation was significantly decreased in case of irradiated larvae as compared to control larvae. In control condition, the percent pupa formation was found to be 93.7% while in irradiated condition it was reduced to 40.6% (Table 1). The egg viability was nil when the adults derived from irradiated larvae were crossed, in comparison to the control where egg viability was found to be 91.4%. It confirmed the complete sterilizing potential of 70Gy gamma dose. (Table 1).

3.2 Influence of host radiation on IJs infectivity potential

When *S. thermophilum* IJs were released on normal host larvae, the morbidity time was found as 24h and mortality time was recorded as 62h. The time taken by the IJs to release from the host cadavers (incubation time) was also recorded as 136.8h. Total harvesting of IJs per host was in the range of 29020-34529 IJs. The harvesting could also be expressed as 121.9 IJs/mg host body weight and the harvesting period was recorded as 8-10 days (Table 2).

In response to infection of *S. thermophilum* IJs on irradiated host, the morbidity and mortality time were reduced about 27% as compared to control (Normal IJs vs Normal host). Due to radiation stress, the total harvesting of IJs/host was also affected and it was decreased to the range of 16321-20416 IJs, however these IJs were in sufficient number to further parasitize the other host larvae (the potential pests) in the field since these EPNs can multiply their number readily, possess great reproductive capability and are extremely virulent. The harvesting period of these IJs was about 6-7 days (Table 2).

3.3 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 timing for morbidity and mortality (Table 2), and exhibited no significant change in parasitization response as compared to control (normal IJs vs normal host) (Figure 1), although the total IJs harvesting from the infected host was about 20% less as compared to control (Table 2).

While comparing the IJs performance in three regimens (i) N-IJs vs N-host, (ii) N-IJs vs Irr-host, (iii) F1-IJs (from Irr-host) vs N-host, the morbidity and mortality timings were significantly influenced in the ‘N-IJs vs Irr-host’ regimen (*F*<sub>2,12</sub> = 65.6*, *p* < 0.0001 for morbidity timing; *F*<sub>2,12</sub> = 48.9*, *p* < 0.0001 for mortality timing). Similarly the incubation time of IJs was decreased in the ‘N-IJs vs Irr-host’ regimen as compared to the incubation period expressed by N-IJs and F1 IJs (derived from irradiated host) on normal host (*F*<sub>2,12</sub> = 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 regimens (*F*<sub>2,12</sub> = 1.04, *p* = 0.372) indicating that *S. thermophilum* was almost equally capable of infecting the normal and irradiated host larvae and host irradiation didn’t affect the parasitization potential of IJs of this EPN species (Figure 1).

There was significant effect on the total harvesting potential of IJs from the host cadavers in different experimental regimens (*F*<sub>2,12</sub> = 98.96*, *p* < 0.0001). The IJs harvesting was more influenced in 70 Gy irradiated host than control (the IJs harvesting exhibited by ‘N-IJs vs N-host’), and ‘F1IJs (from Irr-host) vs N-host’ regimens, 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 as compared to control (ANOVA, *F*<sub>2,12</sub> = 28.25*, *p* < 0.0001).

4. Discussion
In the present study *S. thermophilum* was selected as model entomopathogenic nematode species because it was isolated from India and exhibited a larger size in comparison of other EPN species. The pathogenicity of *S. thermophilum* has been tested previously against *Galleria mellonella* (Lepidoptera), *Aplosonyx chalybaeus* (Coleoptera), *Athalia lugens* (Hymenoptera), *Helicoverpa armigera* and *Spodoptera litura* (Lepidoptera). Previous studies have shown that *S. litura* can serve as a potential host for different *Steinernema* species. The infective potential of EPNs is influenced by external factors (temperature, moisture means of application etc.), internal (host specific) factors, and few host specific chemical cues which employ a crucial role in finding the appropriate host by EPNs.

Stress induced due to irradiation has been suggested to be used for the radio-genetic control of lepidopteran family pests. Advancement has been made to this tactic by integrating it with other ecologically safe pest management strategies such as biological and parabiological control. The suppression of lepidopteran pests can be enhanced by using host irradiation for augmenting parasitoids transport, quality and production, in conjunction with radiation mediated Sterile Insect programmes including F1 sterility technique.

In the light of this, the feasibility of *in-vivo* transport of the EPN, *S. thermophilum* within host larvae of a serious pest *Spodoptera litura* (against which this EPN species was found to act promisingly as a biocontrol agent) was ascertained in the present study. *S. thermophilum* is best suggested for parasitizing the hosts present in the soil with low mobility. Generally, for *in vivo* transport of EPNs, the inoculation of host by IJs is done in large number and the infected host larvae are transported to the field immediately so that IJs can be released in the specific agro-field in time (in view of limited 4-5 days of incubation) so as to encounter the pest (host) larvae in the field. It is believed 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. Several studies have
also suggested that the application of entomopathogenic nematodes in infected cadavers was more efficient than the application of these IJs in aqueous suspensions\textsuperscript{26,43-45}.

In order to avoid any pest population build-up due to inadvertent missing of host inoculation by EPNs to the host (i.e., pest larvae), the host irradiation was considered to exercise safe transport of IJs of \textit{Steinernema thermophilum} to the field.

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

In conclusion, the study indicated that releasing EPN, \textit{Steinernema thermophilum} via radio-sterilized infected hosts might have an immense potential for their use as potential parasitoids to control noctuid pest, \textit{S. litura}. These findings added support to the use of irradiated host, \textit{S. litura} larvae for the mass production of \textit{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 pest population. This study also reflected the reasonable parasitization and proliferation potential of \textit{S. thermophilum} carried in vivo within radio sterilized host. It also indicated that host irradiation didn’t influence the bio-efficacy of this EPN species which could
be used in inundative mode or even in inoculative mode due to IJs substantial bio-efficacy and proliferation capacity. Further studies on using the radio-genetic and biological control techniques together for the management of *S. litura* are in progress.

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**Conflicts of interest:** The authors declare no conflicts of interest.

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

<table>
<thead>
<tr>
<th>Host stage irradiated</th>
<th>Dose given</th>
<th>Pupa formation (%)</th>
<th>Malformed pupa&lt;sup&gt;1&lt;/sup&gt; (%)</th>
<th>Adult emergence (%)</th>
<th>Malformed adult&lt;sup&gt;2&lt;/sup&gt; (%)</th>
<th>Egg viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L6</td>
<td>0Gy</td>
<td>93.7±2.5a</td>
<td>7.4±0.87a</td>
<td>86.2±4.1a</td>
<td>3.9±0.53a</td>
<td>91.4 ± 3.1a</td>
</tr>
<tr>
<td>L6</td>
<td>70Gy</td>
<td>40.6±2.1b</td>
<td>34.9±1.7b</td>
<td>06.1±0.52b</td>
<td>3.2±0.23a</td>
<td>0b</td>
</tr>
</tbody>
</table>

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 constituted of cohort of 25 L6 with daily observations on growth and metamorphosis.; <sup>1</sup> % malformed pupa was computed out of total pupae formation. <sup>2</sup> % malformed adult was computed out of total adults eclosed. For egg viability, the hatchability of the eggs laid due to self cross of the adults derived from treated L6 was observed; the percentage data was transformed for biostatistics.
Table 2. Infective performance of entomopathogenic nematode (EPN) *Steinernema thermophilum* on sixth instar larvae of *Spodoptera litura*.

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Morbidity time (h)</th>
<th>Mortality time (h)</th>
<th>Incubation time (h)</th>
<th>Harvesting/mg body wt.</th>
<th>Total Harvesting</th>
<th>Harvesting Period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal IJs vs Normal Host</td>
<td>24.53± 0.8</td>
<td>62.48± 2.41</td>
<td>136.8± 4.9</td>
<td>121.9± 6.8</td>
<td>31837±121.1</td>
<td>9.2± 0.374</td>
</tr>
<tr>
<td>Normal IJs vs Irradiated (70 Gy) host</td>
<td>17.88±0.62</td>
<td>45.43± 2.1</td>
<td>109.6± 3.5</td>
<td>71.41± 4.1</td>
<td>18154± 820</td>
<td>6.6± 0.244</td>
</tr>
<tr>
<td>F1 IJs (from irradiated host) vs Normal host</td>
<td>24.35±0.82</td>
<td>67.36± 1.5</td>
<td>132.0± 4.6</td>
<td>98.97± 4.5</td>
<td>25486± 890</td>
<td>7.6± 0.244</td>
</tr>
</tbody>
</table>

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 10 host larvae constituted one replicate for morbidity, mortality, incubation time and harvesting parameters.

0-1 day old L6 of *S. litura* were exposed to 1-2 weeks old IJs at a dose rate of 25-30 IJs/host for 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 10 host larvae constituted one replicate for morbidity, mortality, incubation time and harvesting parameters.
Figure legend

**Figure 1.** Parasitising 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 percent parasitization parameter.
Figure 1