Chapter 2

Materials and Methods
Materials and Methods

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2.1 The model pest of the study

The present investigations were carried out on a polyphagous insect pest, *Spodoptera litura* (Fabricius), noctuid in nature (Order: Lepidoptera; Family: Noctuidae) and commonly known as the tobacco caterpillar or common cutworm. A culture of this moth was maintained in the laboratory as per the specifications based on quasi mass rearing of insects having sustained quality with respect to proper growth and adult viability.

2.2 Maintenance of insect culture

The culture of *S. litura* was initiated in the insectary with a few pairs of freshly emerged adults procured from the agricultural fields of Indian Agricultural Research Institute (IARI), New Delhi. Mass rearing of this insect was established in an insectary (11x15ft.). As this insect is vulnerable to the attack of microorganisms, the insectary was sterilized with 10% formaldehyde fumigation prior to establishing the culture. The room was illuminated with fluorescent light. Ambient environmental conditions like, 27±1.0°C temperature, 75.0±5.0% relative humidity, and 12h light:12h dark, photoperiod regimen were maintained.

2.2.1 Adult pairing

The male and female adults were paired in groups in specially designed cages made of perspex and nylon. The cage of 20 x 20 x 20 cm size was used for rearing. The cages were specially designed with all the four walls made of perspex sheet, out of which the front side of the cage was fitted with 30cm long nylon net for convenient manipulation of moths. The two facing side-walls of the cage were fitted with nylon net windows to provide sufficient aeration (Fig. 2.1). Cotton swabs soaked in 10-15% honey solution (w/v) were placed in small plastic containers on which adults were fed. These swabs were replenished every day. A fresh castor (*Ricinus communis*) leaf with its petiole dipped in water contained in a glass vial was introduced in the cage to provide a natural environment to the female moths for oviposition. The eggs were generally laid in clusters of about 100-400 eggs on the ventral surface of the leaves and sometimes on the nylon/perspex walls of the cage. The eggs were collected with the help of a fine hair brush (No.0) in small plastic containers (7 x 7 x 5cm). The eggs
Fig. 2.1: Cages used to pair adults for insect rearing and experiments (cage size 20 x 20 x 20cm)
Fig. 2.1
were treated with 0.2% sodium hypochlorite (NaOCl) or 0.1% formalin solution, followed by a rinse with distilled water for proper surface sterilization.

### 2.2.2. Rearing of *S. litura* on Natural diet

In order to have a continuous supply of a large number of quality insects a stock culture of *S. litura* was maintained on leaves of the castor plant, *Ricinus communis* that served as the natural diet of the insects.

**Rearing of Egg stage:** A tender and fresh castor leaf was kept in the plastic containers having eggs on the third day of incubation to provide immediate food accessibility to the newly hatched caterpillars. The newly hatched larvae were tiny, about 1-2mm in length. The larvae were transferred to glass jars (15cm dia. x 20cm ht.) lined with filter paper at the base. Initially, about 50-100 larvae were placed in each jar but as the larvae grew in size they were gradually thinned-out to avoid overcrowding and infection. Tender and soft castor leaves were provided as food to the larvae.

**Rearing of Larval stage:** The larval stage of *S. litura* comprised of six instars of which the later larval instars were voracious feeders. The body of the larva was distinctly divided into head, thorax and abdomen. The abdomen consisted of ten segments. The functional legs were present on the ventro-lateral aspect of the thoracic segment. Five pairs of abdominal prolegs were also located on the abdominal segments 3-6 and 10. The larvae grew in size with each moult.

The growing larvae were transferred to clean and sterilized glass jars and provided with fresh castor leaves every day. The rearing jars were cleaned every day to remove all the excreta, dead and infected larvae and the exuviae. The jars were washed and rinsed in 2.0% formalin solution to avoid infection.

The larvae became fully-grown in about 15-17 days, reaching a length of 40-50mm. The mature caterpillar was stout and smooth, dull-grayish and blackish-green with yellow dorsal and lateral stripes. The yellow lateral stripe was bordered dorsally with a series of semi-lunar black marks. The head capsule was black in colour with a typical inverted “V” mark on it.
The 6th (last) instar larvae fed voraciously for 2-3 days. This phagoperiod was followed by cessation of feeding, and the larvae entered the ‘wandering stage’ wherein the larvae showed random movements. Subsequently with an extensive purging-out from the alimentary canal, the larvae entered the pre-pupal stage. These insects became lethargic, contracted in size and acquired somewhat ‘comma-shaped’ body structure. The pre-pupal stage lasted for about 20-30h terminating by a moult into the pupal stage.

**Rearing of pupal stage:** The freshly moulted pupae were pale green in colour that gradually changed to dark brown as a result of tanning of the cuticle in a span of about 4h after mouling (Seth *et al.*, 1997). A pupa measured about 16-18mm in length and 5-6mm in breadth. Female pupae were slightly larger than the male pupae.

After proper sclerotization of the pupae, followed by hardening of pupal skin hard enough to be handled manually, the pupae were sexed according to position of the gonopore which was located on the 8th abdominal sternum in case of female pupae and on the 9th abdominal sternum in male pupae. The two-day-old pupae thus formed were collected and surface-sterilized by rinsing in 3.0-4.0% formalin solution and subsequently with water. Pupal period lasted for 7-8 days after which the adults eclosed. The female moths were observed to emerge one day earlier than the male moths.

**Rearing of adult stage:** The adults were stoutly built. The adults had about 2.5cm wing span. The females were larger than the males. The males were distinguished by the presence of a steel-gray shiny patch at the outer corner of the forewings whereas in females the patch at the corresponding area had a slightly different configuration, which was pale-straw to golden in colour and was easily distinguishable from that of the male. The moths remained quiet during the daytime, but when disturbed could exhibit flight in a zigzag manner. Life span of adult moths ranged from 9-11 days. Reproductive pairing of the adults was resumed for continuation of the culture. The whole life cycle took about 27-30 days. Various developmental stages and their characteristic features are shown in **Fig. 2.2 and Table 2.1**.
Table 2.1: Morphology and Biology of *Spodoptera litura*  

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Stages</th>
<th>Climatic conditions</th>
<th>Diet/Habitat</th>
<th>Duration</th>
<th>Colour</th>
<th>Length/ Size (cm)</th>
<th>Length of head capsule (µm)</th>
<th>Weight (mg)</th>
<th>Other characteristics features</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Egg</td>
<td>Non feeding stage (but 80% RH require)</td>
<td>Tender leaves of castor (It just scrap the surface of leaves)</td>
<td>2-5 day</td>
<td>Green</td>
<td>0.06±0.02</td>
<td>Not measurable</td>
<td>0.037±0.003</td>
<td>Eggs are spherical, laid in cluster of 100-300 under ventral side of leaves covered with hairy scales. Fecundity is adversely affected by high temp. &amp; low humidity.</td>
</tr>
<tr>
<td>2.</td>
<td>L₁</td>
<td>12h dark : 12h light 70±5% Relative humidity 27±2°C temperature</td>
<td>Tender leaves</td>
<td>2-3 days</td>
<td>Green colour with black head capsule</td>
<td>0.18±0.04</td>
<td>3.43±0.02</td>
<td>0.79±0.02</td>
<td>Distinct black band appear on the 1&lt;sup&gt;st&lt;/sup&gt; abdominal segment. Scrap the chlorophyll of the leaf and also make small holes.</td>
</tr>
<tr>
<td>3.</td>
<td>L₂</td>
<td>12h dark : 12h light 70±5% Relative humidity 27±2°C temperature</td>
<td>Tender leaves</td>
<td>2-3 days</td>
<td>Creamish or yellowish green</td>
<td>0.26±0.05</td>
<td>4.58±0.11</td>
<td>2.58±0.04</td>
<td>Black band at 1&lt;sup&gt;st&lt;/sup&gt; abdominal segment clearly visible.</td>
</tr>
<tr>
<td>4.</td>
<td>L₃</td>
<td>12h dark : 12h light 70±5% Relative humidity 27±2°C temperature</td>
<td>Leaves</td>
<td>2-3 days</td>
<td>Darker blackish green</td>
<td>0.88±0.01</td>
<td>6.53±0.10</td>
<td>15.19±0.25</td>
<td>‘V’ mark appears on the head capsule.</td>
</tr>
<tr>
<td>5.</td>
<td>L₄</td>
<td>12h dark : 12h light 70±5% Relative humidity 27±2°C temperature</td>
<td>Leaves</td>
<td>2-3 days</td>
<td>Light green</td>
<td>1.48±0.02</td>
<td>11.08±0.08</td>
<td>99.93±2.45</td>
<td>‘V’ mark appears on the head capsule.</td>
</tr>
<tr>
<td>S.No.</td>
<td>Stages</td>
<td>Climatic conditions</td>
<td>Diet/Habitat</td>
<td>Duration</td>
<td>Colour</td>
<td>Length/Size (cm)</td>
<td>Length of head capsule (µm)</td>
<td>Weight (mg)</td>
<td>Other characteristics features</td>
</tr>
<tr>
<td>-------</td>
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<td>-----------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>6.</td>
<td>L₅</td>
<td></td>
<td>Leaves</td>
<td>2-3 days</td>
<td>Grey</td>
<td>2.18±0.03</td>
<td>24.23±0.24</td>
<td>256.4±2.87</td>
<td>Smooth skinned with a pattern of red, yellow and green lines.</td>
</tr>
<tr>
<td>7.</td>
<td>L₆ 0d</td>
<td>12h dark : 12h light</td>
<td>Leaves</td>
<td>1 day</td>
<td>Black</td>
<td>2.96±0.02</td>
<td>30.12±0.22</td>
<td>415.84±6.14</td>
<td>They are black with three thin yellow lines: One is the middle and one each side. A row of black run along each side, and a conspicuous row of dark triangles decorate each lateral side</td>
</tr>
<tr>
<td>8.</td>
<td>L₆ 1d</td>
<td></td>
<td>Leaves</td>
<td>1 day</td>
<td>Black</td>
<td>3.95±0.05</td>
<td>34.52±0.16</td>
<td>6.34.78±13.9</td>
<td>They become light or grey or brown in colour, all lines become dull. Lateral lines start to disappear.</td>
</tr>
<tr>
<td>9.</td>
<td>L₆ 2d</td>
<td></td>
<td>Leaves</td>
<td>1 day</td>
<td>Blackish grey</td>
<td>4.21±0.03</td>
<td>35.76±0.76</td>
<td>1002.61±10.33</td>
<td>Lateral lines completely disappear.</td>
</tr>
<tr>
<td>10.</td>
<td>Wandering stage</td>
<td></td>
<td>Leaves</td>
<td>1 day</td>
<td>Blackish grey</td>
<td>3.06±0.04</td>
<td>33.05±0.27</td>
<td>450.81±9.47</td>
<td>Decreases in weight due to exudation.</td>
</tr>
<tr>
<td>S.No.</td>
<td>Stages</td>
<td>Climatic conditions</td>
<td>Diet/Habitat</td>
<td>Duration</td>
<td>Colour</td>
<td>Length/Size (cm)</td>
<td>Length of head capsule (µm)</td>
<td>Weight (mg)</td>
<td>Other characteristics features</td>
</tr>
<tr>
<td>-------</td>
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<td>-------------</td>
<td>---------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>11.</td>
<td>Prepupa</td>
<td>Leaves for moisture</td>
<td>1 day</td>
<td>Dark or black</td>
<td>2.03±0.02</td>
<td>33.58±0.20</td>
<td>309.14±6.45</td>
<td></td>
<td>They cease motions, go to soil for pupation.</td>
</tr>
<tr>
<td>12.</td>
<td>Pupa</td>
<td>Moisture/Soil with moisture</td>
<td>7-8 days</td>
<td>Reddish Brown</td>
<td>♂: 1.89±0.07</td>
<td>Not measurable</td>
<td>♂: 280.08±5.23</td>
<td>♂: 305.12±4.02</td>
<td>Pupate without cocoon, tip of abdomen with two small spines ♂ gonopore on 8th sternum ♂ gonopore on 9th sternum</td>
</tr>
<tr>
<td>13.</td>
<td>Adult (Female)</td>
<td>Honey solution (15-20%)</td>
<td>9-11 d</td>
<td>Brown</td>
<td>Length: 1.98±0.01 Width: 3.70±0.02</td>
<td>28.04±0.29</td>
<td>134.09±2.99</td>
<td></td>
<td>1. Adult moth is brown with a complex pattern of cream streaks criss-crossing the forewings. 2. Hind wings are silvery white. 3. Females have white band from open to the inner margin of each forewing.</td>
</tr>
<tr>
<td>14.</td>
<td>Adult (Male)</td>
<td>Honey solution (15-20%)</td>
<td>9-11d</td>
<td>Dark brown</td>
<td>Length: 1.95±0.02 Width: 3.61±0.03</td>
<td>25.72±0.23</td>
<td>114.22±2.03</td>
<td></td>
<td>Males have silvery band from apex to the inner margin of each forewing.</td>
</tr>
</tbody>
</table>
Fig. 2.2  Life cycle of *Spodoptera litura*
Fig. 2.2
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Monitoring pupae and adult for healthy culture: The pupae and adults were closely monitored for the presence of any morphological deformities and such individuals were removed from the insect culture. Various characteristic features of pupae viz. temporally synchronized pattern of sclerotization, tanning and melanization, symmetry in sclerotized cephalic and thoracic appendages, abdominal segments, and growth in terms of weight and size were observed to assess the quality in the insect population. Pupae with any minor deviation marked other than the normal features described above, might be apparently normal looking pupae but the adults eclosed out of these pupae were reported to show poor viability and reproductive performance (Seth et al., 1997).

Asynchronous rearing of the insect was conducted to have desired life stage at any particular time for experimental purpose, as per the need.

2.2.3 Rearing of *S. litura* on Semi-synthetic diet

Insects used for experimental purpose were reared on semi-synthetic diet developed in the laboratory (Sharma 1999, Seth and Sharma 2001). The semi-synthetic diet mainly consisted of a ground seed source suspended in an inert carrier of aqueous agar-agar along with other nutritive materials. Appropriate amounts of proteins, carbohydrates and lipids along with vitamins and minerals were incorporated in the diet. In addition, microorganism inhibitors were included in the diet to prevent microbial infestations.

2.2.3a Preparation of semi-synthetic diet

Agar was mixed to 750ml autoclaved de-ionized water. In another container, all the ingredients referred in part (B) were taken and mixed into 400ml of de-ionized water and 6.25ml of 4M KOH was added to it. Corn oil, linseed oil, sinigrin and formaldehyde (part C) were also added to the solution of ingredients. Then dissolved agar was mixed well to this and finally the ingredients mentioned in part (D) were added to the mixture when it got cooled to about 70°C to avoid denaturation of vitamins (Table 2.2). The prepared diet was poured out in the plastic containers having a foil lining and was left to settle. When the diet cooled down to room temperature, it was stored at 4°C in a refrigerator.
Table 2.2: Constituents of semi-synthetic diet used to rear *S. litura*

<table>
<thead>
<tr>
<th>Set</th>
<th>Ingredients</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td>Agar</td>
<td>25.0g</td>
</tr>
<tr>
<td></td>
<td>De-ionized water</td>
<td>750ml</td>
</tr>
<tr>
<td>(B)</td>
<td>Casein</td>
<td>44.0g</td>
</tr>
<tr>
<td></td>
<td>Gram flour</td>
<td>93.50g</td>
</tr>
<tr>
<td></td>
<td>Wessons salt</td>
<td>12.50g</td>
</tr>
<tr>
<td></td>
<td>Cholesterol</td>
<td>1.25g</td>
</tr>
<tr>
<td></td>
<td>Yeast</td>
<td>19.0g</td>
</tr>
<tr>
<td></td>
<td>Methyl-p-hydroxybenzoate</td>
<td>1.25g</td>
</tr>
<tr>
<td></td>
<td>Sugar</td>
<td>39.0g</td>
</tr>
<tr>
<td></td>
<td>Sorbic acid</td>
<td>2.0g</td>
</tr>
<tr>
<td>(C)</td>
<td>Corn oil</td>
<td>2.50ml</td>
</tr>
<tr>
<td></td>
<td>Linseed oil</td>
<td>2.50ml</td>
</tr>
<tr>
<td></td>
<td>10% HCHO</td>
<td>5.50ml</td>
</tr>
<tr>
<td></td>
<td>4M KOH</td>
<td>6.25ml</td>
</tr>
<tr>
<td></td>
<td>De-ionized water</td>
<td>400ml</td>
</tr>
<tr>
<td></td>
<td>Sinigrin (1.0%)</td>
<td>3.53ml</td>
</tr>
<tr>
<td>(D)</td>
<td>Antibiotic and vitamin mix</td>
<td>7.50g</td>
</tr>
<tr>
<td></td>
<td>Choline chloride</td>
<td>1.25g</td>
</tr>
<tr>
<td></td>
<td>Vitamin E</td>
<td>0.20g</td>
</tr>
<tr>
<td></td>
<td>Vitamin mixture (Roeche Co.)</td>
<td>2.0g</td>
</tr>
<tr>
<td></td>
<td>Chloremphenicol</td>
<td>2.0g</td>
</tr>
<tr>
<td></td>
<td>Streptomycin</td>
<td>2.0g</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
<td>2.0g</td>
</tr>
<tr>
<td></td>
<td>Ascorbic acid</td>
<td>80.0g</td>
</tr>
</tbody>
</table>

2.2.3b Rearing of insects

Freshly hatched larvae were transferred to a plastic chamber (8 x 8 x 6cm) having a 1.0cm thick strip of diet. About 100 larvae were transferred in each such chamber and these larvae were allowed to feed and grow in a gregarious manner. After 4-5 days when the larvae reached 3rd instar, these larvae were placed individually in glass specimen tubes (2.5cm dia. x 10cm ht.) containing small cubes (2 x 2 x 2cm) of diet.
late instar larvae (L₆), diet was increased to cubes of about 4 x 4 x 2cm. These tubes were loosely plugged with cotton to provide proper aeration to larvae and to avoid their escape out of the tube. Diet in these tubes was replenished every 3rd day (Fig. 2.3). The wandering stage larvae were allowed to pupate inside the diet. These pupae were collected after 48-72h of pupa formation from the diet because by that time the pupae were sclerotized enough to resist handling pressure. The adults that eclosed out of these pupae were kept for reproductive pairing.

2.2.4 Handling techniques to control microbial contamination in culture of S. litura

Following precautions were carried out to check the microbial infection during the rearing of insects:

1. The insectary was fumigated with 10% formalin for disinfection before introduction of insects for quasi-mass rearing.
2. Glassware and plastic containers were washed with detergent, followed by rinsing in 5.0% formalin and then water, and finally oven drying at about 70°C.
3. The castor leaves were washed before use with a dilute solution (0.001%) of potassium permanganate followed by a wash with water.
4. Autoclaved de-ionised water was used to prepare semi-synthetic diet.
5. Eggs were surface sterilized with sodium hypochlorite (0.1-0.2%) or formalin (1.0-2.0%).
6. Larvae were transferred to semi-synthetic diet under sterilized conditions using laminar flow cabinet.
7. Larvae were cleared of the excreta and diet particles sticking to their body surface before replenishing fresh diet in the vials.
8. The excreta and leftover leaves were removed regularly from the insect rearing jars.
9. As the larvae in the late wandering and the early pre-pupal stages were observed to exude and release moisture, addition of fresh diet was avoided at this stage to check infection.
Fig. 2.3: Rearing of *Spodoptera litura* on semi-synthetic diet

(a) 4\textsuperscript{th} instar larvae (L4) being reared individually in glass specimen tubes (2.5 cm dia x 10 cm. ht.) containing cubes (2x2x2 cm) of semi-synthetic diet

(b) Last instar larvae (L6) being reared individually in glass specimen tubes (2.5 cm dia x 10 cm. ht.) containing cubes (4x4x4 cm) of semi-synthetic diet
Fig. 2.3
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10. Pupae were surface sterilized with sodium hypochlorite (1.0%) or formalin (3.0-4.0%).

2.2.5. Life cycle of *S. litura*

The life cycle of *S. litura*, from egg to adult was observed to pass through larval, pre-pupal and pupal stages (Fig. 2.2 and Table 2.1). The male and female moths were observed to attain sexual maturity about one day after emergence. Mating occurred during scotophase. Eggs were laid on the lower surface of the castor leaves. In addition some eggs were also deposited on the walls of the cage. The eggs were deposited in clusters of 100–400 each. On an average a mated female laid 1800-2200 eggs in its life span. The eggs were arranged in rows and the egg mass was generally covered with buff-coloured hair shed from the anal tuft of the female moth. Each egg was round and ridged, pearly white when freshly laid but turned darker afterwards. The eggs’ incubation period was about 3–5 days.

There were six larval stages in *S. litura*. The newly hatched caterpillars were tiny (averaging about 1.0mm in length), blackish-green in colour. The body of the larva was distinctly divided into head, thorax and abdomen. The abdomen consisted of ten segments. The functional legs were present on the ventro-lateral side of the thoracic segments. Five pairs of abdominal prolegs were also located on the abdominal segments 3-6 and 10. The larvae grew in size with each moult. The mature last instar caterpillar was stout and smooth, dull-grayish and blackish-green in colour with pale white dorsal and lateral stripes. The head capsule was black with a typical inverted ‘V’ mark over it. The last instar caterpillars stopped feeding and secreted fluid from the body resulting in contracted size. These larvae became lethargic and acquired somewhat ‘comma’ shaped body forms. This stage lasted for 1-2 days and it was termed as the pre-pupa, which moulted to form the pupa.

Freshly formed pupae were pale green in colour and with gradual melanization they turned dark reddish-brown in colour with shiny surface (Fig. 2.2). The skin covering the wing pads was found to be darker in colour than rest of the body within 4h of pupa formation. Abdomen of pupa had dark spiracles at the lateral sides and centrally placed inwardly curved spines on the last segment. The pupa measured about 16-
18mm in length and 5-6mm in breadth. Female pupae were slightly larger than the male pupae. In addition, the fresh weight of female pupae was greater than that of male (Seth et al., 1997).

Identification of sexes was possible at the pupal stage. The pupae were examined minutely to find out any external feature(s) to identify the sexes. In both the sexes, the last three abdominal segments (8th-10th) were immovably articulated to form a large cone. These segments were partially fused, having a longitudinal mid-ventral furrow close to the apex. The furrow was laid at the position of imaginal anus and was surrounded by a raised circular area. In the female pupae, this area was smaller and positioned near the apex, while in males it was more prominent, large and positioned slightly away from the apex. The genital pore was situated mid-ventrally on the ninth segment in case of males, while in females it was present on the eighth segment. The anal pore in both the cases was situated at the tenth segment. In females either side of genital pore had an inverted ‘V’ shaped depression that extended up to the tenth segment. The distance between the anal and genital pore in females was greater than in the males. The pupal period lasted for about 7-8 days after which the adults eclosed.

The adults were stoutly built. The adults had about 2.5cm wing span. The females were slightly larger than the males. The males were distinguished by the presence of a steel-gray shiny patch at the outer corner of the forewings, whereas, in females the patch at the corresponding place was pale-straw to golden in colour. The moths remained quiet during the day, but when disturbed exhibited flight in a zig-zag manner. Life span of adult moths was 9-11 days. The whole life cycle took about 38-40 days (Table 2.1).

2.3 Irradiation of insects

Irradiation of the insects was conducted in the Radiobiological unit of the Institute of Nuclear Medicine and Allied Sciences (INMAS), Ministry of Defence, Delhi-110054 using the 60Co source, placed in the Gamma cell-5000 (Gamma –5000 irradiator, BRIT, BARC, Trombay). The dose rate of the radiation was about 2.74-1.18kGy/hr during the experimental period. The unit of gamma radiation used was Gray (Gy) (1Gy=100rads). On the basis of initial studies by Seth and Sehgal (1993), sub-sterilizing doses of 100Gy and 130Gy were selected for ‘F1 inherited sterility’ in the
present studies. In certain cases, the sterilizing doses of 200-250Gy and sub-lethal(higher) doses of 300-400Gy were also used for experimental evaluation in concern with of F₁ sterility technique. 0-1day male adult was the stage irradiated for behavioural and physiological response of treated moth and its F₁ progeny. For biochemical studies, 1-2day sixth intar larvae (L₆) were irradiated with dose range of 0.5-200Gy, and 0-1day first instar larvae (L₁/neonates) irradiated with 100Gy in order to evaluate radiation induced changes/damage/repair in the intrinsic system of insect using a Co⁶⁰ source (GC-5000 for doses 10Gy onwards and teletherapy unit for 0.5Gy). Irradiation of insect was carried out at room temperature.

2.4. Study of Behavioural characteristics associated with F₁ sterility technique

Different bio-characteristics were studied to assess the quality of irradiated insects and their F₁ progeny to be employed for F₁ sterility technique.

2.4.1. Flight assay in Spodoptera litura

The male flight ability of F₁ progeny males derived from sub-sterilized male moths, S. litura in presence of normal females, was tested in two modes in ‘Flight assay chamber’(a perspex cage of size, 45 x 45 x 60 cm) having a black hollow cylinder (25.4cm dia, 30.5cm ht.) placed inside (Fig 2.4). In Mode-I, the male pharate adults were placed inside the black cylinder and female pharate adults outside the cylinder within the flight assay chamber; whereas in Mode-II, the female pharate adults were placed inside the black cylinder and male pharate adults outside the cylinder, within the flight assay chamber. Each replicate for testing flight capacity comprised of a group of 24 irradiated (I) F₁ males and 12 normal(N) females and 15 replicates were taken for each regimen. The eclosion performance of these male and female adults from late stage pupae(pharate adults) was ascertained, followed by their flight capacity. The male and female insects that were positioned at side-walls/roof at 12-14 hr after eclosion, due to their flight activity were also recorded. Finally the male flight ability of irradiated male insects (I♂ or F₁♂) was judged in terms of their performance towards mating success. Male flight ability was tested with a population of insects having sex ratio as ♂: ♀:: 2:1, since, the insects at 2:1(♂:♀) sex ratio were found to exhibit reasonably proper reproductive performance.
Fig. 2.4: ‘Flight assay chamber’ (a perspex cage of size, 45 x 45 x 60 cm) having a black hollow cylinder (25.4 cm dia, 30.5 cm ht.) placed inside.
Fig. 2.4.
2.4.2 Mating Behaviour of *Spodoptera litura* in different sex ratio

Mating behaviour was evaluated in terms of mating frequency, mating success and mating propensity in the P\textsubscript{1} and F\textsubscript{1} male paired with female in different sex ratio. The mating behaviour of irradiated (I) male *Spodoptera litura* and its progeny (F-1) when crossed with normal counterparts, was studied. The mating status was determined in terms of mating frequency, mating success and re-mating tendency observed during 72hrs pairing of each specified cross. The mating behaviour was assessed in case of different sex ratios for each specified cross (♂:♀ :: 1:1, 2:1 and 5:1). The mating performance was evaluated in different size cages for different ratios, (i) 20 x 20 x 20 cm\textsuperscript{3} cage for evaluating 12 -15 pairs in sex ratio ♂:♀ :: 1:1, (ii) 20 x 20 x 25 cm\textsuperscript{3} cage for evaluating 12 -15 females paired in sex ratio ♂:♀ :: 2:1, (iii) 25 x 25 x 30 cm\textsuperscript{3} cage for evaluating 12 -15 females paired in sex ratio ♂:♀ :: 5:1. The size of the cage was selected keeping in view the minimum space availability of ca. 200 cm\textsuperscript{3} per insect.

The mating behaviour was studied by pairing 0-1 day old virgin moths as follows: (i) Normal males x Normal females (ii) P\textsubscript{1} males x Normal females, (P\textsubscript{1} - irradiated as 0-24h old adult moths with 100-130Gy), and (iii) F\textsubscript{1} male moths derived from cross of irradiated P\textsubscript{1} male x Normal females. F\textsubscript{1} male moths crossed in three combinations viz. (i) F-1♂ x N♀, (ii) N♂ x F-1♀ and (iii) F-1♂ x F-1♀ i.e. self crossed.

The mating success i.e. per cent females mated out of total pairing moths was recorded by observing the presence of spermatophore(s) in the bursa copulatrix of females to study the effect of gamma doses and moths’ physiological status on the mating ability of the moths. Further, the mating frequency i.e. ability of mating to remate was evaluated by the total number of spermatophores in the mated females. Remating propensity was evaluated by recording the number of mated female having more than one spermatophore.

2.4.3 Sequential mating for evaluation of sperm mixing and reproductive success

Effect of multiple mating was studied on the sperm utilization pattern, which would influence fertility, by conducting sequential mating of females with sub-sterilized or sterilized male moths, and normal moths. Sequential matings of virgin female with N♂ and I♂ /or F1♂ were studied in various regimens, i.e., (i) N♀x N♂x N♂, (ii) N♀x
N♂x I♂ , (iii) N♀x I♂ x N♂,(iv) N♀x I♂ x I♂. The first mating was performed between 0-1 day old moths, and second of previously paired female mating with another category of freshly emerged male moths was allowed after a gap of 24-36hr after the first mating of the female. Similar pattern of sequential matings was conducted for F₁ progeny males(F₁ ♂). In each regimen of sequential mating crosses, the reproductive parameters viz., mating success, re-mating propensity, fertility or egg viability after first mating and second mating were ascertained. The evaluation of freshly emerged adults (24 ♂ vs 12♀) constituted each replicate for assessing mating success, percent remating propensity and fertility for the untreated males and females (control). The success of the second mating was verified by examining the female moths for the presence of a second spermatophore.

2.4.4. Effect of female age on its receptiveness towards irradiated male and their F₁ progeny

The effects of female age at the time of first mating on the reproductive potential of freshly emerged male irradiated with 100-130Gy and their F₁ progeny, was studied by allowing female of three different age groups viz. 0-1day, 2-3day and 5-6day old to mate with male moths for a period of 72hr. Evaluation of freshly emerged male moths and female moths of different age groups (16♂ vs 8♀) constituted each replicate for assessing mating success and remating propensity. The number of replicates were 12-15. The effect of age of female moth on its receptivity with respect to irradiated P₁ male and its F₁ male progeny was assessed in terms of mating behaviour (percent mating success and percent remating propensity) of treated male moths and translated in terms of sperm transfer and resulting viability(fertility) of the eggs laid. Mating was confirmed by the presence of spermatophore and remating success was recorded by presence of more than one spermatophore within bursa copulatrix, and computed as % rematings in females out of total matings. Further, the reproductive potential was determined by fertility of eggs oviposited by different age groups of female, inseminated by irradiated male moth (100Gy,130Gy) and their F₁ progeny moth.

2.4.5 Pheromone Test

Attractiveness of F₁ males of sub-sterilized male parents was ascertained towards pheromone traps in the field cages: The orientation of F₁ males of sub-sterilized male
parents were assessed towards the pheromone traps in the field cages (~ 9ft x 6ft x 7ft ht). A cohort (22-30) of 0-1 day old untreated male moths (unmarked and marked with fluorescent paint on thorax), were released and evaluated for their attractiveness towards pheromone traps that were obtained from PCI(Pest Control India Pvt Ltd). This pheromone trap (Fero-T™), consisted of a funnel base, a canopy and a transparent sleeve (Fig. 2.5). The pheromone trap had pheromone lure (Spodo-lure) that was fixed to the canopy, which was later placed over the funnel. Further, 0-1 day old, F₁ male moths (derived irradiated male parent) were released and evaluated for their attractiveness towards pheromone traps (Fero-T™). Experiment was replicated ten times. Number of males captured in pheromone trap were observed at 1hr, 6hr, 24hr and 48hr. Total number of insects trapped in pheromone trap and ratio of marked and unmarked insect were computed.

2.5. Study of Physiological characteristics associated with F₁ sterility technique

Various physiological characteristics related to competitiveness of P₁ and F₁ insects studied were detailed under following sub-sections.

Preparation of Reagents used for physiological studies

I) Belar’s saline a insect physiological buffer

Belar’s saline stain was prepared as per method given in Flint and Kressin (1969). NaCl (6.0g), KCl (0.02g), CaCl₂ (0.29g), Na₂CO₃ (0.29g) were taken and added in distilled water to make 1 litre.

II) Lacto-aceto-orcein (LAO) a DNA specific stain

The LAO stain was prepared by mixing 85% lactic acid and acetic acid (1:1), boiling it and finally adding synthetic orcein to make 2% lacto-aceto-orcein stain. This stain was cooled and filtered. About 2.0ml of glycerine was added to 100ml stain solution.

2.5.1 Sperm (Apyrene and Eupyrene) bundles production in testis

In order to assess the sperm production and their release pattern from testes, the adult male moths were dissected at early photophase(10-11AM) in Belar’s saline (Fig. 2.6).
**Fig. 2.5**: Pheromone test in *S. litura*

(a) Field cage (~ 9ft x 6ft x 7ft ht)

(b) Pheromone trap (Fero-T™)
Fig. 2.6: Male reproductive system of *Spodoptera litura* (Left: Pictoral arrangement of different reproductive parts of male-Source Etman and Hooper 1979; Right: reproductive male tracts after dissection)
Fig. 2.6
The numbers of sperm bundles in the testes were quantified in the various age-groups of adults (0–3 day old). The testis of each moth was removed and placed in a 0.5ml microcentrifuge tube and to this tube, containing the testis sample, 100µl of 2.0% lacto-aceto-orcein (LAO) as a specific DNA stain was added. The testis was macerated within the eppendorf tube and the contents were thoroughly mixed by gentle vortexing. An aliquot (5µl) from this diluted preparation was spread in longitudinal rows on a microscope slide and the number of sperm bundles was counted (magnification ×400) using an Olympus BX-60 microscope. Eupyrene sperm bundles was identified by their stained nuclei, whereas apyrene sperm bundles did not get stained. The average number of sperm bundles counted in 15 such 5µl aliquots of diluted testes extract from each sampled moth constituted one replicate. Ten to twelve replicates were taken for each developmental stage or time point. Sperm production at different age-groups (0-1, 1-2, 2-3day old) of virgin male moths was determined in the partially radio-sterilized males and in F₁ progeny males derived from sub-sterilized males, and compared with controls.

2.5.2 Sperm descent from testes and its circadian rhythm

Circadian rhythm of sperm descent from testes down in to the reproductive tract in radiation induced substerilized moths was conducted, as per the method described by Seth et al., (2002). For determining the descent of sperm from the testes into the male reproductive tract, various regions of the male tract, viz. upper vas deferens (UVD), seminal vesicle (SV), and the ductus ejaculatorius duplex (Duplex) were dissected out in the Belar’s saline. Detailed morphology of male reproductive parts is given in Fig. 2.6. These regions of the male reproductive tract, along with their contents, were carefully separated out on a microscope slide.

For the assessment of eupyrene sperm (bundles), 50µl Lacto aceto orcein (LAO) was added to each of the reproductive tract portions, and 5µl aliquot was taken for each reading with mean of five such aliquot observations constituting each replicate for each tract portion. Eupyrene bundles were quantified by observing under microscope using 400x magnification.
For the assessment of apyrene sperm, the sample of ingredients (sperm + secretions) from each reproductive tract portion was diluted in Belar’s saline (500µl for UVD and 500µl for SV, and 1500µl for duplex). 2µl aliquot was taken for each observation of counting individual apyrene sperm (as apyrene sperm bundles dissociated shortly after release from testes), with five aliquot observations constituting each replicate for a specific tract portion. Individual Apyrene sperm were quantified by observing under microscope using 400x magnification.

This experiment was performed in virgin male moths at 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} day of emergence of both normal and treated insects. The numbers of eupyrene bundles and individual (dissociated) apyrene sperm were quantified in different parts of reproductive tract at two particular times of the day, at 10–11 AM (during photophase) and at 10–11 PM. (during scotophase), in order to ascertain the sperm descent profile and confirm their descent rhythm in this lepidopteran pest under the influence of gamma irradiation. This was the first attempt to quantify number-wise, the dissociated (loose) apyrene sperm in different parts of the reproductive tract in respect of methodology unlike in the earlier report (Seth et al., 2002) where apyrene density was determined scale-wise. Sperm decent and its rhythmic pattern were studied in substerilized male moths irradiated as 0-1 male moth with 100-130Gy and their subsequent F\textsubscript{1} progeny.

2.5.3. *In vitro* sperm activation bioassay

Since, sperm activation may act as a tool to assess the viability of irradiated moths and their F\textsubscript{1} male moths. The effect of gamma irradiation on sperm activation in irradiated males (P\textsubscript{1} generation) and F\textsubscript{1} progeny of P\textsubscript{1} male moths was studied. Experiments were performed on 2-3 day old virgin adults due to accumulation of spermatozoa in large numbers in the duplex, along with adequate secretions in the prostatic part by this time.

Following parameters were observed for assessing spermatozoa activation:

i)  
*Time of initiation of sperm activity*

ii)  
*Time of termination of sperm activity*
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iii) **Duration of sperm activity** (from initiation to termination of sperm activity)

iv) **Percent active sperm:** For studying % sperm activation, each replicate at one particular time point constituted mean of 10 readings (10 different visual fields on the microscopic slide having 5µl aliquot of incubated mixture of sperm and activator) from each insect. It was replicated 25 times.

v) **Intensity of sperm activation:** For assessing intensity of sperm activity, each replicate at one particular time point constituted mean of 10 readings (of individual sperm from different visual fields) on the microscopic slide having 5µl aliquot of incubated mixture of sperm and activator from each insect, and each data comprised of two phases (i) computing the number undulations per sec, (ii) transforming the number of undulations into scale from 0-4, viz. nil undulation – ‘0’, 1-5 undulations/sec – ‘1’(+), 6-10 undulations/sec – ‘2’(++), >10 undulations/sec – ‘3’(+++), > 15 undulations/sec – ‘4’(++++). Each regimen observation was replicated 25 times.

2.5.3a **Reagents used in sperm activation in vitro assay**

Following chemicals procured from Sigma Chemical Co., USA were used in the sperm activation experiments:

i) HEPES (N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid)

ii) Ammonium bicarbonate (NH₄HCO₃)

iii) Bovine serum albumen (BSA)

2.5.3b **Protocol for in vitro sperm activation assay**

Effect of endogenous factors on activation of sperm was studied in the unirradiated controls, irradiated male, and in their F₁ progeny. In vitro sperm bioassay was standardized (Shepherd 1974) and performed to assess the influence of gamma doses on sperm motility. For this, the sperm and activator were prepared separately as detailed below,

2.5.3c **Preparation of sperm**

Adult male moths (2-3day old) were immobilized by keeping them at 4°C in the refrigerator for 2-5 minutes. The moths were then dissected in Belar’s saline in a
petriplate. The ductus ejaculatorius duplex (Fig 2.6) was transacted with the help of a forceps just proximal to the prostatic part and accessory glands and rinsed in 100µl of HEPES-KOH buffer at pH 7.0. The duplex was then transferred to 50µl of 0.3M HEPES-KOH buffer at pH 7.0 containing 20mg/ml BSA (bovine serum albumin). The ends of the duplex were gently cut and its contents were thoroughly mixed with the HEPES-KOH buffer. Sperm secretions in HEPES-KOH buffer were then transferred to 4.0cm squares of parafilm placed on a wet filter paper bed in a petriplate (5.0cm). Petriplate was covered and incubated at room temperature (ca. 25°C).

2.5.3d Preparation of the activator

The prostatic part (Ductus ejaculatorius simplex; Fig 2.6) from 2-3day male moths was transacted with the help of a forceps and rinsed in 0.5ml of ammonium bicarbonate-acetic acid buffer at pH 7.0. Each prostatic part was then kept in 40µl of ammonium bicarbonate-acetic acid (0.03M NH₄HCO₃-CH₃COOH) buffer at pH 7.0. The secretions were then collected, after giving transverse cuts to the prostatic part (ductus ejaculatorius simplex) at some places, with the help of capillary pipette. The buffer containing the secretions was centrifuged at 6000rpm at 4°C for 10min. The supernatant was collected on 4.0cm square of parafilm kept on a wet filter paper bed in a petriplate (5.0cm dia.). Petriplate was covered and incubated at room temperature (ca. 25°C).

2.5.3e Sperm activation assay

The sperm activation was assayed by mixing equal volume of sperm (40µl) and activator (40µl), for the observation of temporal profile of sperm activation. The sperm and activator were mixed thoroughly with the help of a micro-pipette and then incubated at room temperature. 5µl aliquot of this incubation mixture was taken on a glass slide and covered with coverslip. The observations were conducted at 5min, 15min and 30min interval under microscope using 600x magnification.

2.5.3f Effect of virgin and mated male on the sperm activation

i) Sperm Activation in virgin male moth: Effect of sub-sterilizing (100Gy and 130Gy), sterilizing (200Gy and 250Gy) and higher doses/sub-lethal doses (300Gy and 400Gy) was examined on percent sperm activity and degree of intensity of
active sperm in 2-3day virgin male parent irradiated as 0-1day male moths. Further, the effect of sub-sterilizing doses on percent active sperm and intensity of sperm activity in 2-3day virgin P₁ and their F₁ progeny derived from male parent irradiated as 0-1day male moths was examined in order to evaluate the sperm competence in view of reproductive performance underlying ‘inherited sterility technique’.

ii) **Sperm Activation in mated male moth:** Effect of gamma irradiation on sperm activation of mated male moth was determined through the *in vitro* analysis of percent sperm activation and degree of intensity of sperm activation in view of multiple mating observed in *S. litura*. Treated 0-1day male moth was allowed to mate with normal female for 72hr (2-3day) and occurrence of mating was confirmed by presence of spermatophore in female. Unlike in virgin male adult, radiation doses up to 250Gy were administered to parent male moths as mating was not observed to occur (no spermatophore found in bursa copulatrix) in 300-400Gy treated male moths. *In vitro* sperm activation in mated F₁ male adults from 100Gy and 130Gy treated male parents, was evaluated in view of sperm competence to be functionally important in inherited sterility, for management of *S. litura*.

**2.5.4. Sperm transfer to spermatheca of female by male**

The number of sperm transferred from irradiated male moths (P₁) and their ensuing F₁ progeny male was assessed in the spermatheca of untreated females mated with these irradiated male moths (P₁ and F₁). Mating success was recorded by presence of spermatophore in female after 12-24 hr (one night) of pairing. After 12-24hr of mating (*Fig. 2.7a*). The eupyrene sperm bundles were found to be dissociated, appearing thick and smooth thread like structure as compared to individual apyrene sperm which was thin and wavy in appearance (*Fig. 2.7b*). Sperm transferred by irradiated P₁ and their F₁ male progeny was studied by counting the sperm (collectively apyrene and eupyrene sperm) in spermatheca of female after 12-24 hr (one night) of mating with 0-1day male moth. These parameters were correlated with percent fertility resulted from sperm transfer in spermatheca.

For determining transfer of eupyrene and apyrene sperm from male to female reproductive tract, viz. spermatheca (*Fig. 2.7a,b*) were dissected out in Belar’s saline.
Fig. 2.7a.: Female reproductive system of Spodoptera litura (mated female contain spermatophore inside bursa copulatrix which was dissected to confirm the occurrence of mating.
Fig. 2.7a.
Fig. 2.7b.: Morphology of individual sperm in spermatheca of mated female, *S. litura*

(a) Apyrene sperm

(b) Eupyrene sperm
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Fig. 2.7b:
For assessment of sperm no. transferred, sample (sperm+secretion of male ejaculate transferred to female) spermatheca was diluted in Belar’s saline up to 100µl with 2µl aliquot for each observation of counting apyrene and eupyrene dissociated (individual) sperm, and mean of five such aliquot observations constituting each replicate. Each regimen observation was replicated 25 times.

2.6. Study of Biochemical characteristics associated with F₁ sterility technique

Biochemical characteristics to relate the intrinsic system of insect with the reproductive viability of P₁ and F₁ insect were studied in detail under following modes.

2.6.1. Oxidative stress and Antioxidant Defence System

2.6.1a Radiation treatment:

Radiation doses administered to L6 (P₁ generation) 1-2 day old were 0.5Gy, 10Gy, 50Gy, 100Gy and 200Gy. Irradiated P₁ larvae were incubated for four hours following irradiation. For F₁ 100Gy and 130Gy larvae, the male adults were irradiated with 100Gy and 130Gy and crossed with normal female and their corresponding F₁ progeny were reared up to L6 (F₁ generation).

2.6.1b Sample Preparation

For insect gut, the insects selected for dissection were weighed and narcotized by chilling at −20 °C for 8–10 min following which the gut was dissected out on an ice plate and blotted dry following removal of adhering fat body and malpighian tubules. Foregut and midgut (Fig. 2.8) contents were removed by flushing with ice chilled assay buffer. Then the tissues of foregut and midgut were collected in pre-weighed microcentrifuge tubes in ice and weighed gut tissue to make 20x dilution (w/v) with assay buffer and homogenized in assay buffer followed by sonication using a Vibrasonics 300 sonicator (Virtis Company, Gardiner, NY, USA) for 4 pulses of 15sec with 5 sec interval.
**Fig. 2.8:** Gut (Fore+midgut full of content) of sixth instar larvae (1-2 day) of *S.litura* used for antioxidant assay after removing content of gut
Fig. 2.8:
2.6.1c Protein Estimation

Bradford reagent (Sigma, USA) for protein estimation has been used to estimate protein concentrations for all the biochemical assays. Protein concentration in tissue sample was measured by Bradford’s Reagent mixed in 3:1 ratio (3 part was Bradford’s Reagent and 1 part was gut sample). Reaction mixture gave blue colour after keeping in dark for 15 min. Absorbance taken at 595 nm and quantified with a BSA standard. 50-100 µg protein was used for performing antioxidant assay.

2.6.1d Chemicals and instruments

All chemicals used in assays were obtained from Sigma Chemical Co. St. Louis, MO, USA. All spectrophotometric measurements were taken in a multiwell plate visible spectrophotometer (ECIL, India) unless otherwise indicated.

2.6.1e Lipid Peroxidation Assay: Assessment of Macromolecular Damage

Aldehydes are produced when lipid hydroperoxide break down in biological system. Malondialdehyde (MDA) is the most abundant aldehyde resulting from lipid peroxidation, and it is biochemically determined by thiobarbituric acid (TBA) concentration as described by Halliwell and Chirico (1993). TBARS (Thiobarbituric Acid Reactive Substances) assay was a end point spectrophotometric assay.

TBARS Assay Procedure

50 µL of 0.2% BHT (dissolved in ethanol) was added to 0.5 mL sample. BHT (Butylated hydroxytoluene) was added to minimize oxidation of lipids that might contribute as an artifact during sample processing and TBA reaction. The resulting reaction mix was equally divided and 1.5 mL of 0.44 M H₃PO₄ was added to each tube to maintain low pH. Tubes were incubated for 10 min at room temperature and 0.5 mL TBA reagent (6 g/L in ethanol solution) was added. Reaction mixture was incubated at 95°C for 30 min. After cooling the reaction mixture, a pink chromogen (allegedly a [TBA] 2-malondialdehyde adduct) was measured by taking absorbance at 532 nm.
Calculation of TBARS concentration

TBARS concentration was measured by dividing Optical Density at 532 nm / extinction coefficient (i.e. O.D. 532nm/ 156000 M⁻¹ cm⁻¹) and expressed as nmol TBARS/mg protein. Absorbance of sample was corrected with the absorbance of sample blank where instead of protein sample; the assay buffer (50mM Potassium phosphate buffer, pH 7.0) was used.

\[
TBARS\ concentration = \frac{\text{Optical Density at 532nm}}{156000M^{-1}cm^{-1}}
\]

2.6.1f Enzymatic Antioxidant assays

I) Superoxide Dismutase (SOD) Assay

Superoxide is one of the main reactive oxygen species (ROS) in the cell. Consequently, SOD serves a key antioxidant role by catalyzing the dismutation of the superoxide anion to \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \). There are three distinct types of SOD classified on the basis of the metal cofactor: the copper/zinc (Cu/Zn-SOD), the manganese (Mn-SOD) and the iron (Fe-SOD) isozymes.

SOD Assay Principle

Total Superoxide dismutase activity was measured by using tetrazolium salt (NBT) as described by Mishra and Fridovich, 1972. In this assay Xanthine and Xanthine Oxidase is used for Superoxide generation. Xanthine Oxidase generates superoxide as a consequence of the oxidation of their substrates. Superoxide ions (\( \text{O}_2^- \)) generated by xanthine oxidase (XOD) with concurrent conversion of xanthine to uric acid and hydrogen peroxide. Superoxide radical reacts with NBT and converts it to NBT-diformazan, which absorbs light at 560 nm. SOD reduces the superoxide ion concentration and thereby lowers the rate of NBT-diformazan formation. Schematic representation of principle involved in SOD activity assay is given in Fig. 2.9.

Chemical Requirements: Xanthine, Xanthine Oxidase, Catalase (to scavenge \( \text{H}_2\text{O}_2 \) that inhibit Cu/Zn SOD activity), BSA, Nitro Blue tetrazolium (NBT), Bathocuproine
disulphonic acid (BCDA is a Chelating agent that blocks interfering metals e.g. Cu), Potassium Phosphate

**Reagents and Buffers prepared:** 50mM potassium phosphate buffer (PPB) at pH=7.0, 10U/ml Catalase, 1.76mg/ml BSA (Bovine Serum Albumin), 2mM NBT (Nitro Blue Tetrazolium), 5mM Xanthine, mM BCDA, 10U/ml Xanthine Oxidase.

All reagents were prepared in 50mM Potassium phosphate Buffer (pH=7.0) except Xanthine which was prepared/dissolved in NaOH (1μM) solution.

**SOD Assay Procedure**

Enzymatic assay reaction was setup as follows:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Stock Conc.</th>
<th>Final Assay Conc.</th>
<th>Reagent Blank</th>
<th>Enzyme Blank</th>
<th>Unknown Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPB</td>
<td>50 mM</td>
<td>50 mM</td>
<td>455 μl</td>
<td>525 μl</td>
<td>425 μl</td>
</tr>
<tr>
<td>Catalase</td>
<td>10 U/ml</td>
<td>1.25 U/ml</td>
<td>120 μl</td>
<td>120 μl</td>
<td>120 μl</td>
</tr>
<tr>
<td>BSA</td>
<td>1.7 mg/ml</td>
<td>0.171 mg/ml</td>
<td>100 μl</td>
<td>100 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>NBT</td>
<td>2 mM</td>
<td>70 μM</td>
<td>-------</td>
<td>35 μl</td>
<td>35 μl</td>
</tr>
<tr>
<td>Xanthine</td>
<td>5 mM</td>
<td>0.125 μM</td>
<td>40 μl</td>
<td>40 μl</td>
<td>40 μl</td>
</tr>
<tr>
<td>BCDA</td>
<td>5 mM</td>
<td>62.5 μM</td>
<td>80 μl</td>
<td>80 μl</td>
<td>80 μl</td>
</tr>
<tr>
<td>Xanthine Oxidase</td>
<td>10 U/ml</td>
<td>1 U/ml</td>
<td>100 μl</td>
<td>100 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>Protein Sample soln.</td>
<td>-------</td>
<td>-------</td>
<td>100 μl</td>
<td>-------</td>
<td>100 μl</td>
</tr>
<tr>
<td>Total Volume</td>
<td>1000 μl</td>
<td>1000 μl</td>
<td>1000 μl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total reaction Volume was 1ml. Absorbance was taken at 560nm at every 20sec. for 5min in spectrophotometer immediately after sample addition. The extent of reduction in the appearance of NBT-diformazan was a measure of SOD activity present in an experimental sample.
Fig. 2.9: Schematic representation of principle involved in SOD activity assay
Materials and Methods

Fig. 2.9: NBT-diformazan Blue soln. -560nm
Materials and Methods

SOD Activity Measurement
One Unit of SOD enzyme decreases the rate of NBT reduction to 50% at 25°C at pH=7.0. SOD activity expressed in Unit/mg protein.

II) Catalase Activity Assay
Hydrogen peroxide is a harmful byproduct of many normal metabolic processes and can be produced by exogenous factors(e.g.radiation). Catalase is a homotetrameric ferriheme-containing enzyme and catalyses the conversion of hydrogen peroxide into water and oxygen.

Catalase Activity Assay Principle
Catalase activity was measured as described by Sinha (1972). This assay was based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H$_2$O$_2$ which was measured colorimetrically at 570nm. Therefore, Catalase assay was an end point spectrophotometric assay.

Catalase Activity Assay Procedure:
The reaction mixture consisted of 10 µl of protein sample and 100 µl of 0.2M H$_2$O$_2$ in 140µl of 50mM potassium phosphate buffer (pH=7.0), vortexed and kept for 1min. 500µl of 5% K$_2$Cr$_2$O$_7$: acetic acid (1:3 ratio) was added and it resulted in blue precipitation Reaction mixture was boiled for 10min and it produced green precipitation. Absorbance at 570 nm was measured in ELISA reader.

Catalase Activity Calculation
Using regression equation from standard catalase, activity of catalase in sample was calculated in terms of U/mg protein.

III) Ascorbate Peroxidase (APOX) Assay
Ascorbate Peroxidase catalyzes oxidation of ascorbic acid with concurrent reduction of H$_2$O$_2$ into water and converts ascorbic acid into dehydroascorbic acid.

APOX Assay Principle
APOX activity was measured as described by Asada (1984). This assay is based on the disappearance of ascorbate (measured by its absorbance at 290 nm) as it is oxidized to dehydroascorbic acid by H$_2$O$_2$. 
Chapter 2

APOX Assay Procedure

For enzyme activity assay, 100 µg protein sample was added to 1ml of 50 mM potassium phosphate (pH 7.0) containing 0.5 mM ascorbic acid and 0.1 mM H$_2$O$_2$. The reaction mixture consisted of 880µL of 50mM phosphate buffer containing 0.5mM ascorbic acid, 10µL of 10mM H$_2$O$_2$, 10µL of 50mM ascorbic acid and 100µL of protein sample. The rate of change in absorbance (decrease) at 290 nm was measured at each 20sec. for 3min. in spectrophotometer. Correction was made for the non-enzymatic ascorbate oxidation by H$_2$O$_2$ (1 nmol/min) and for measurement of ascorbate oxidase activity.

APOX Activity Calculation

A decrease of 0.01 absorbance units at 290 nm corresponds to 3.6 nmol ascorbate oxidized. One unit of APOX activity was defined as the amount oxidizing 1nmol ascorbate/min at 25°C and pH=7.0. Activity of APOX expressed as unit/mg protein.

2.6.1g. Non- Enzymatic Antioxidant assays

I) GSH/GSSG Estimation

Glutathione (gamma-glutamyleysteinylglycine or GSH) is a naturally occurring tripeptide with a free thiol group. GSH scavenge hydrogen peroxide and lipid hydroperoxides to water and the respective alcohol.

GSH/GSSG Assay Principle

Reduced and oxidized glutathione (GSH/GSSG) was measured by the method of Tietze (1969) by Griffith (1980). Determination of the GSH amount utilizes a carefully optimized enzymatic recycling method, using glutathione reductase, for the quantification of GSH. The sulfhydryl group of GSH reacts with DTNB (5,5’-dithiobis-2-nitrobenzoic acid, Ellman’s reagent) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB) that is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction which is in turn directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 412 nm provides an accurate
estimation of GSH in the sample. GSH is easily oxidized to the disulfide dimer GSSG. GSSG is produced during the reduction of hydroperoxides by glutathione peroxidase. GSSG is reduced to GSH by glutathione reductase and it is the reduced form that exists mainly in biological systems. Because of the use of glutathione reductase in the method for GSH assay, both GSH and GSSG can be measured and the assay reflects total glutathione. GSH is generated from GSSG by glutathione reductase, and reacts with DTNB again to produce 2-nitro-5-thiobenzoic acid. Therefore, this recycling reaction improves the sensitivity of total glutathione detection and the process cycles continuously until all of the DTNB is reduced or NADPH is consumed (Fig 2.10a).

**Reagents and Buffer required:** 0.3mM nicotinamide adenine dinucleotide phosphate (NADPH a cofactor of GR), 50U/ml Glutathione reductase, 6mM DTNB, 125mM Sodium Phosphate buffer( containing 6.3 mM EDTA) at pH=7.5, 1M 2-VP

All reagents (except 2-VP) were made in 125mM Sodium Phosphate buffer(containing 6.3 mM EDTA) at pH=7.5

**Protocol for Total glutathione (GSH+GSSG) assay**

(i) The reaction mixture consisted of 700 µl of 0.3 mM NADPH, 190 µl of protein sample/buffer(for blank)/GSH(for standard curve) and 10 µl of 50U/ml Glutathion reductase (GR)

(ii) Incubated at 30°C for 1 min.

(iii) 100µl of 6mM DTNB was added and shaken for 5 min. to initiate reaction

(iv) Absorbance at 412nm (yellow coloured compound) at every 60sec. for 5min was measured in spectrophotometer.

**Protocol for Oxidized glutathione (GSSG) assay**

In GSSG assay, GSSG was selectively determined by assaying samples in which GSH was derivatized by 2-VP (2-vinyl pyridine). This procedure effectively blocks the existing GSH from entering the recycling assay and being quantified (Fig 2.10b). Added 10µl of 1M 2-VP (in ethanol) in 1 ml of protein sample and Incubated at 30°C for 1min. Same procedure was followed for assaying GSSG in 2-VP derivatized protein sample as it was in total glutathione.
Fig. 2.10: Reaction involved in GSH/GSSG assay

(a) Reaction involved in Total GSH assay

(b) Reaction involved in GSSG assay
**Materials and Methods**

GSSG + GSH

\[2 \text{ TNB} \quad (412\text{nm})\]

\[2 \text{ TNB} \quad (412\text{nm})\]

Yellow soln.

Fig. 2.10a:

GSSG + GSH

\[2 \text{ TNB} \quad (412\text{nm})\]

\[2 \text{ TNB} \quad (412\text{nm})\]

Yellow soln.

Fig. 2.10b:
Materials and Methods

Calculation of GSH/GSSG Concentration

Using regression equation from Standard Glutathione, Concentration of GSH/GSSG in sample was calculated in terms of μM/mg protein. Only GSH concentration (obtained by subtracting the GSSG conc. total GSH concentration). The GSH/GSSG ratio as an indicator of oxidative stress was also computed after estimation of total glutathione and oxidized glutathione.

2.6.2. DNA damage by Comet Assay

2.6.2a Radiation treatment

Radiation doses administered to L6 were 0.5Gy, 10Gy, 50Gy, 100Gy and 200Gy. For F-1 100Gy and 130Gy larvae, male adults were irradiated with 100Gy and 130Gy and crossed with normal female and their corresponding F-1 progeny were reared upto L6 1-2 day old. All the experiments were done after 3 incubation periods viz. 0hr, 2hr. and 4hr. following irradiation to determine the DNA damage.

2.6.2b. Chemicals required/used for comet assay:

1. 0.1% Agarose solution (made in PBS) for pre-coating of slide.
2. 0.75% Agarose solution (made in PBS) for cell (sample) embedding.
3. Neutral lysis buffer (pH 9.5):
   (i) 2.5% Sodium dodecylsulfate
   (ii) 1% Sodium sarcosinate
   (iii) 25mM EDTA
   (To make 500ml lysis buffer, added 12.5g Na-dodecylsulfate, 5g Na-sarcosinate and 25ml of 0.5M stock EDTA solution to 500ml of distilled water and pH maintained at 9.5)
4. TBE-Electrophoresis buffer (pH 8.4)
   (i) Tris base (Trizma base) 90mM
   (ii) Boric acid 90mM
(iii) EDTA 2.5mM

(To make 500 TBE buffer, added 5.46g of Tris base, 2.78g of Boric acid and 2.5ml of 0.5M stock EDTA to 500ml of distilled water and pH maintained at 8.4)

5. Propidium Iodide (for staining)

Stock solution: 1g/ml of PBS

Working solution: 50µg/ml PBS (25µM)

Note: Chemicals were stored at 4°C except agarose.

2.6.2c. Instruments required/used for comet assay

1. Multiphor II electrophoresis system (Pharmacia)
2. Fluorescence microscope (BX60, Olympus)
3. Comet: Komet 5.5 (Optimas image analysis software.)

2.6.2d. Sample preparation

Hemolymph was collected from cutting proleg of larvae in eppendorf containing few crystals of polythiourea (PTU) to avoid melanization of hemolymph and kept at ice. For 25-30 thousands cells of hemocytes, 50µl haemolymph was taken.

2.6.2e. Procedures for comet assay

Schematic representation of comet assay procedure is given in Fig. 2.11.

1. Pre-coating: Microscope slides were labeled with the help of diamond pencil and then kept on hot plate (45°C). These slides were pre-coated with 600µl of 0.1% agarose with the help of pipette and left on hot plate until it dried or for overnight.

2. Cell embedding: Cells embedding was done in agarose so that the DNA was immobilized for subsequent electrophoresis. For neutral comet assay, 50µl cells (hemolymph) were mixed in pre-warmed 600 µl of 0.75% agarose solution and layered on pre-coated slide. These slides were incubated at 4°C on cold plate (made by keeping temperature sensitive plate on ice layer) to solidify the gel for 10-15min. Cell lysis: After cell embedding, the cells were lysed to remove
membranes and histones from DNA. For neutral comet assay, these slides were in lysis buffer at room temperature for 15min. After that, slides were washed with distilled water for 2 times each for 5min.

3. **Electrophoresis**: The slides were put on electrophoretic unit and slides were kept in a dimension that cathode negative current (black color lead) went through slides to anode positive current (red color lead) as negatively charged DNA (fragmented) moved towards anode. Then the electrophoresis buffer (TBE, pH 8.4) was poured and electrophoresed at 12V for 5min. During electrophoresis any broken DNA fragments migrated farther than the super-coiled, undamaged DNA. After the cells electrophoresed, the slides were rinsed in distilled water at room temperature for 5min. The slides were dried at hot plate (45 °C) and then these slides were stored in humidified condition (at room temperature) for their image analysis.

4. **Staining**: The slide was rehydrated in distilled water for 10-15min and then stained with 100µl propidium iodide-25µM (PI) in dark condition for 1-2min. Again the slide was rinsed in distilled water to remove extra stain for 2-5min.

5. **Analysis/Observations**: After staining, the slide was observed under green fluorescence microscope at 600x magnification in which, the DNA resembled a comet with a brightly fluorescent head and tail whose length and intensity was determined by the level of DNA-strand breakage within the cells. After defining the comet area and tail area, comet software (Komet 5.5) automatically could give all parameters e.g. tail area, tail length, tail DNA, head DNA, olive tail moment and tail extent moment etc. In the present study, the DNA damage was determined in terms of tail DNA (Fig. 2.12). Number of comets observed were 50 from one larvae that constituted one replicate.

\[
\% \text{Tail DNA} = \frac{\text{Tail DNA Intensity}}{\text{Cell DNA Intensity}} \times 100
\]
Fig. 2.11: Schematic representation of comet assay protocol
Fig. 2.11:

Cells followed by treatment → Cell suspension mixed in agarose → Cells embedded on pre-coated slide with agarose → Treated with lysis buffer to remove histone & membrane → DNA rejoining-repair → Processed image → Analysis → Staining (25µM Propidium Iodide) → Electrophoresis (12V for 5min)
2.6.3. Transcript pattern of immune cascade enzyme and scavenging enzyme by Molecular analysis

2.6.3a Irradiation of Insects

Two life stages (i) first-instar larvae (L1) and (ii) 0-1 day old freshly eclosed male adults were selected and exposed to 100Gy (a gamma sub-sterilizing dose for F-1 sterility technique).

The irradiated neonates were reared to 3rd and 6th instar larvae, likewise the irradiated male adults were allowed to mate with normal females, and the resulting eggs were reared to 3rd and 6th instar larvae. For RNA isolation, the whole body tissue of the irradiated L-1, survivors proceeding to L3 stage and F-1 L-3 as well as hemocytes of L-6 survivors and F-1 L-3 were collected and stored in Trizol reagent (Invitrogen Corp., USA). Similarly, 6th instar larvae survived from irradiated L-1 and irradiation male adult moth, were subjected to microbial challenge. Subsequently, haemocytes of challenged and unchallenged larvae were collected and stored in Trizol reagent for RNA isolation.

2.6.3b Microbial challenge of Spodoptera litura Larvae

(I) Sub-lethal dose estimation of Escherichia coli K12

*Escherichia coli* strain, K-12 was inoculated in sterile tubes containing 2% LB broth (Luria Broth: 4g NaCl, 4g Tryptone, 2g Yeast Extract and 500ml) (Difco laboratories,
USA). The cultures were incubated at 37°C for 12 hrs with constant shaking at 200 rpm. The number of the injected bacteria was estimated by plating a known volume of injected suspension (1 x 10^9 cells/ml for K-12) on 2% LB-agar plates. An estimation of lethal and sub-lethal dosage was obtained (Table 2.3).

Table 2.3: Sub-lethal dose estimation of bacteria for transcript analysis of microbial challenged *Spodoptera litura*

<table>
<thead>
<tr>
<th>Different doses of <em>E. coli</em> K-12 cells</th>
<th>Effects on <em>S. litura</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>250 million</td>
<td>Death at 4hr.</td>
</tr>
<tr>
<td>50 million</td>
<td>Death at 4hr.</td>
</tr>
<tr>
<td>25 million</td>
<td>Death at 4hr.</td>
</tr>
<tr>
<td><strong>5 million</strong></td>
<td><strong>Improper feeding</strong></td>
</tr>
<tr>
<td>2.5 lakh</td>
<td>Improper feeding</td>
</tr>
<tr>
<td>1 lakh</td>
<td>Improper feeding</td>
</tr>
<tr>
<td>50,000</td>
<td>Improper feeding</td>
</tr>
<tr>
<td>25,000</td>
<td>Improper feeding</td>
</tr>
<tr>
<td>10,000</td>
<td>Improper feeding</td>
</tr>
<tr>
<td>5,000</td>
<td>Improper feeding</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Different doses of <em>Photorhabdus</em> cells</th>
<th>Effects on <em>S. litura</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>50,000</td>
<td>Death within 24hr.</td>
</tr>
<tr>
<td>5,000</td>
<td>Death within 24hr.</td>
</tr>
<tr>
<td>1,000</td>
<td>Death within 24hr.</td>
</tr>
<tr>
<td>500</td>
<td>Death within 24hr.</td>
</tr>
<tr>
<td><strong>100</strong></td>
<td><strong>Death within 24hr.</strong></td>
</tr>
</tbody>
</table>

(II) Sub-lethal dose estimation of *Photorhabdus luminescens*

*Photorhabdus luminescens* sub spp. *akhurstii* (Strain K-1) was grown in LB broth at 28°C for 12 hrs on a rotary shaker at 180 rpm. The number of the injected bacteria was estimated by plating a known volume of injected suspension (1 x 10^7 cells/ml for *Photorhabdus*) on 2% LB-agar plates. An estimation of lethal and sub-lethal dosage was obtained (Table 2.3).
(III) Procedure for Microbial Challenge to S. litura 6\textsuperscript{th} instar larvae

One ml of overnight grown cells (E. coli K12 or Photorhabdus luminescens) were harvested, washed with sterile Ringer’s solution (8.6 g NaCl, 0.4 g KCl, 0.4 g CaCl\textsubscript{2}, 0.2 g NaH\textsubscript{2}CO\textsubscript{3} for 1 L solution) and resuspended in 1 ml Ringer’s solution. Five microlitre of Ringer’s solution containing approximately 5 million cells of K12 or 100 cells of Photorhabdus luminescens were injected directly into the hemolymph of S. litura larvae (6\textsuperscript{th} instar, 1-2 day old). This was done by piercing through the proleg using a microinjector (KPS 210, KD scientific, Newhope, PA, USA) with Hamilton syringe. Five microlitre of Ringer’s solution alone was also injected which served as control. Post-injection larvae were held individually on diet and kept at 25°C. Hemocytes were collected 6 hrs. post-infection and resuspended in Trizol for RNA isolation.

2.6.2c. Isolation of S. litura hemocytes

Sixth-instar larvae of S. litura (1-2 day old) were used for hemolymph collection. The larvae were chilled on ice for 15 min to render them immobile and then pierced in their prolegs with a sterile needle. The hemolymph was collected in prechilled anticoagulant buffer (0.78% Citric acid, 0.76% NaCl, 0.76% EDTA and 0.4% NaOH). The hemolymph was properly resuspended in anticoagulant buffer. Hemocytes were separated by centrifugation at 700 x g for 5 min at 4°C. The pellet containing hemocytes was resuspended in Trizol reagent (Invitrogen Corp., USA) and processed for RNA extraction. Alternatively, the haemocytes were snap frozen in liquid nitrogen and stored at -70°C until further use.

2.6.2d. Isolation of total RNA from hemocytes/whole body tissue

Total RNA was extracted from hemocytes using the Trizol reagent according to the manufacturer’s instructions. Hemocytes or whole larvae were homogenized properly in Trizol reagent with a motor driven hand-held homogenizer using DEPC-treated sterile grinder tips. Whole body tissue of neonates and 3\textsuperscript{rd} instar larvae were homogenized in 1 ml of Trizol per 50-100mg of total body weight. The pellet containing hemocytes was lysed in 1 ml of Trizol by repetitive pipetting. The lysate was centrifuged at 12,000 x g for 10 min at 4°C to remove the insoluble material. The
resulting pellet contained extracellular membranes, polysaccharides and high molecular weight DNA while the supernatant contains RNA. The clear supernatant was transferred to a sterile tube and allowed to stand for 5 min at 15 to 30°C (or room temperature) to permit complete dissociation of nucleoprotein complexes. This was followed by the addition of 0.2 ml of chloroform per ml of Trizol and mixed by vigorous shaking. Phase separation was done by centrifugation at 12,000 x g for 15 min at 4°C. The upper aqueous phase containing RNA was collected in a fresh tube. The RNA was precipitated by the addition of 0.5 ml of isopropanol per ml of Trizol, mixed and incubated at room temperature for 10 min. The mixture was centrifuged at 12,000 x g for 10 min at 4°C to pellet the RNA. The pellet was washed with 75% ethanol, air-dried and dissolved in DEPC-water by heating at 60°C for 10 min.

2.6.3e. Spectrophotometric estimation of total RNA
Quantification and purity of RNA in solution was determined by measuring the absorbance at 260 nm. The purity of RNA was determined by taking the $A_{260}/A_{280}$ ratio.

2.6.3f. DNAse Treatment
The total RNA was extracted from haemocytes and whole body tissue and it was checked for genomic DNA contamination by PCR using gene-specific primers (Table 2.4). Contaminating genomic DNA was removed by DNase Amplification grade (RNase-free; Invitrogen). The enzyme was diluted to obtain a stock of 1U/µl using dilution buffer [20 mM Tris-Cl (pH 7.5), 50% glycerol; 120 mM MgCl$_2$ in DEPC-water to obtain a final volume of 1 ml]. One µg of total RNA was treated with 1U of DNase at 37°C for 30 min followed by heating at 65°C for 10 min to inactivate the enzyme.

2.6.3g. Semi-quantitative RT-PCR (qPCR)
RT-PCR was done using one-step RT-PCR kit (Qiagen) according to manufacturer’s instructions. One µg of total RNA (DNA-free) was used as template for reverse transcription. The PCR conditions included reverse transcription at 42°C for 30 min using Omniscript and Sensiscript reverse transcriptases. Inactivation of these enzymes and initial activation of HotStar Taq DNA Polymerase was done at 95°C for 15 min.
This was followed by a 3-step PCR cycle with denaturation at 94°C for 30 sec; annealing at 52°C for 30 sec; extension at 72°C for 30 sec for 32 cycles concluded with a final extension at 72°C for 10 min.

The gene specific primers chosen for RT-PCR are listed in Table 2.4. β-actin was used as an internal control to show equal amount of total RNA was used. PCR products were visualized by on 1% agarose gel. The pattern of gene transcription that was assessed by carrying out the reaction in triplicate for each instar, which invariably showed the same result.

**Table 2.4: List of Primers used for semi-quantitative RT-PCR and Real–time PCR in Spodoptera litura**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward and Reverse Primers (5’— 3’)</th>
<th>Amplicon Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prophenoloxidase (slppo)</td>
<td>Slppo RTF1&lt;br&gt;TTT GAT GAA CGT AAC TTG G&lt;br&gt;Slppo RTR1&lt;br&gt;GCT GGC GCT GCC TAC CAA CTC</td>
<td>300 bp</td>
</tr>
<tr>
<td>Prophenoloxidase-activating enzymes (slppae)</td>
<td>Slppae RTF1&lt;br&gt;CGA ATT CCC CTG GGC TCT&lt;br&gt;Slppae RTR1&lt;br&gt;CAG CCA ATC TGT CTG ATA GAT AAC</td>
<td>342 bp</td>
</tr>
<tr>
<td>Superoxide dismutase (slsod)</td>
<td>Slsod RTF1&lt;br&gt;CAT GGG TTT CAT GTG CAC GA&lt;br&gt;Slsod RTR1&lt;br&gt;GCC TTG GCC GAA ATC ATC AG</td>
<td>260 bp</td>
</tr>
<tr>
<td>Catalase (slsod)</td>
<td>Slcat RTF1&lt;br&gt;GAT CCT CGT GGA TTT GCT GT&lt;br&gt;Slcat RTR1&lt;br&gt;TGT GCC TGT AAC CAT CTG GA</td>
<td>253 bp</td>
</tr>
<tr>
<td>β-actin (control)</td>
<td>β-actin RTF1&lt;br&gt;CAG ATC ATG TTT GAG ACC TTC AAC&lt;br&gt;β-actin RTR1&lt;br&gt;GHC CAT CTC YTG CTC GAA RTC</td>
<td>300 bp</td>
</tr>
</tbody>
</table>
2.6.3h Real-Time PCR

Real-time PCR was done with Quantitect SYBR Green RT-PCR kit (Qiagen) using the iCycler iQ system (Bio-Rad). The Primer 3, web-based tool at [http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi](http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) was used to design gene specific primers, ensuring the length of the PCR product was nearly 300 bp.

Each 25 µl reaction mixture contained 2 µl RNA template (100-500 ng; DNA-free), 12.5 µl of 2X Quantitect SYBR Green RT-PCR Master mix, 10 picomoles of each forward and reverse primer and 0.25 µl of Quantitect RT enzyme mix and RNase-free water. Real time cycler conditions included a preliminary reverse transcription at 48°C for 30 min, an initial activation step at 95°C for 15 min and 40 cycles of denaturation at 94°C, annealing at 52°C and extension at 72°C for 30 sec each. The final step included gradual temperature increase from 50°C to 94°C at the rate of 1°C/10 sec to enable melt-curve data collection.

A non-template control (NTC- to check the cross contamination of sample) and β-actin reference was run corresponding to each instar. Equal amount of RNA was taken in all the samples and Real-time PCR was performed in triplicates for all the samples, control and reference.

The threshold cycles \((C_T)\) were recorded for each gene and β-actin transcripts for each experiment. The sample with lowest \(C_T\) value i.e., with highest expression serves as a calibrator in these charts and rest of the samples are calculated relative to the calibrator. Difference between \(C_T\) of the reference gene, β-actin and the gene of interest was determined and the relative abundance of the transcript was calculated using Comparative \(C_T\) method using the formula \(2^{-\Delta\Delta C_T}\) (Pfall 2001).

2.7. Photomicrography

Photomicrography was conducted using Olympus camera (U-CMAD3) mounted on Olympus BX60 microscope, Magnus Pro-Live camera (software), Sony Mavica digital camera (MVC- FD7), and high density (DSC-H9) digital camera as per the requirements of the experiments.
2.8 Statistical analysis

The data obtained in the above experiments were usually replicated ten times; and any variation in replicate number has been specified at an appropriate place in the text. The data were subjected to appropriate analyses of variance viz. one way analysis of variance (ANOVA). Percentage data was transformed using arcsine $\sqrt{x}$ value before ANOVA, but data shown in tables are back transformations. $P \leq 0.05$ level was considered significant. LSD post test was then performed to determine significant differences among the different treatments (Snedecor and Cochran 1989).