CHAPTER- III

3 MATERIALS AND METHODS

3.1 Collection and mass multiplication of *S. litura*

Spodoptera litura eggs were collected from host crops like castor & cabbage from Padra location (Gujarat, India). Excised plant parts harbouring *S. litura* eggs were transported in plastic containers to the insectary/laboratory and transferred immediately into diet rearing trays separately. It is essential to transfer eggs, especially from plant parts of crops for ease of handling. All the egg masses which were collected from the field were kept separately to avoid cross contamination of different pathogen. There was a chance of parasitisation of *Spodoptera litura* eggs by any parasitic wasp because in field condition it was very much possible. After hatching neonate larvae started feeding on artificial diet.

The segregation of larvae was done for easy handling& five neonates per cell were transferred, on 16 & 32 cell rearing trays containing semi-synthetic diet. A soft camel hairbrush was used to gently transfer neonates on to the cell of rearing trays having diet. Two-day old first instar larvae are pale cream opaque in appearance and are referred to as 'white stage'.

Figure 1 Castor field showing damage of the insect pest

Figure 2 Collection site of *S. litura* at Padra,

Figure 3 Insect pests observed at castor field

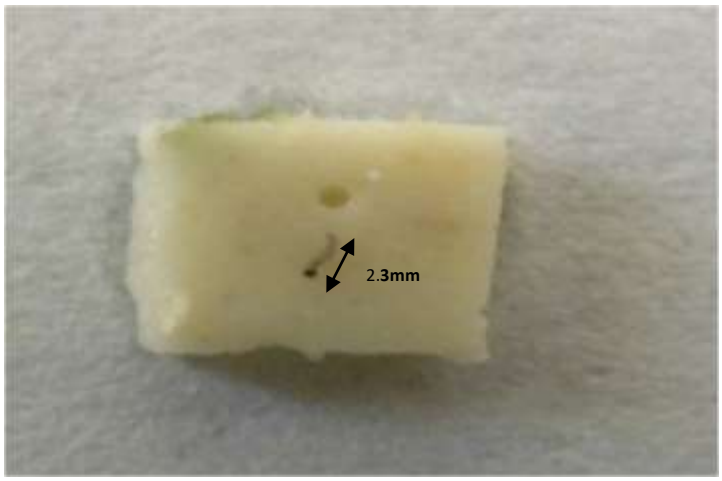

Figure 4
1st instar larva on artificial diet

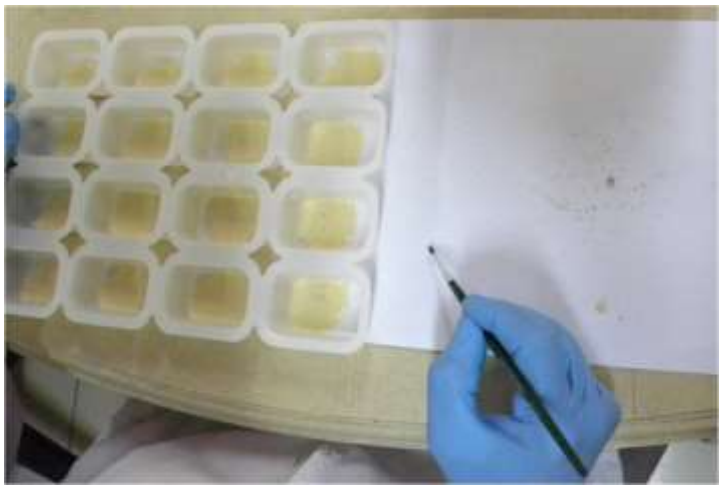

Figure 5 Transferring of neonates on artificial diet

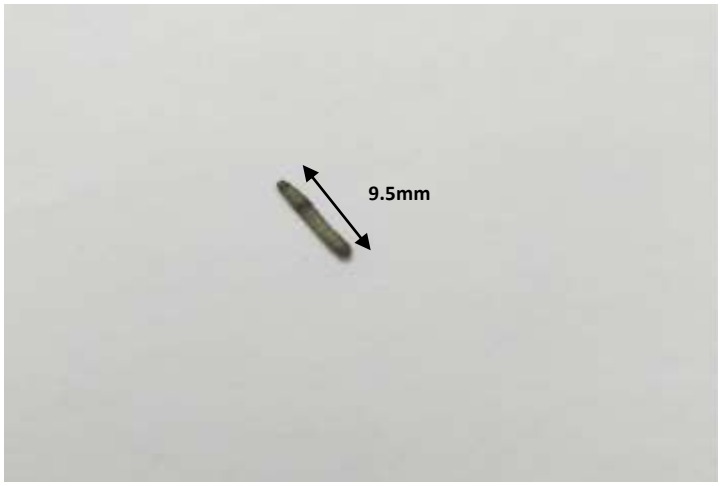

Figure 6 2nd instar larva

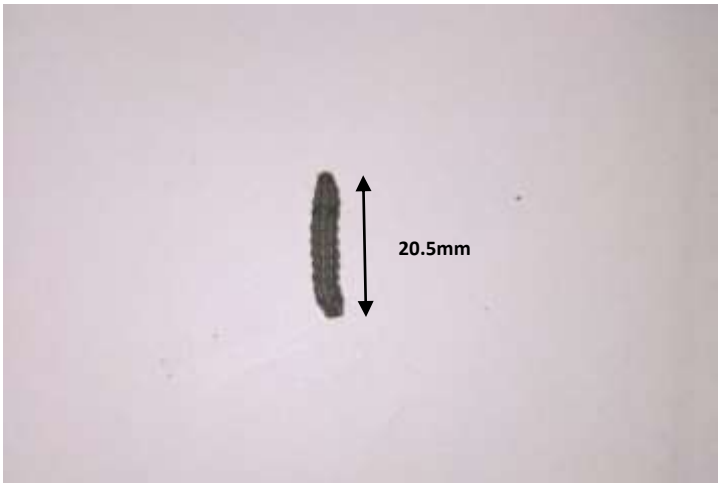

Figure 7 3rd instar larva

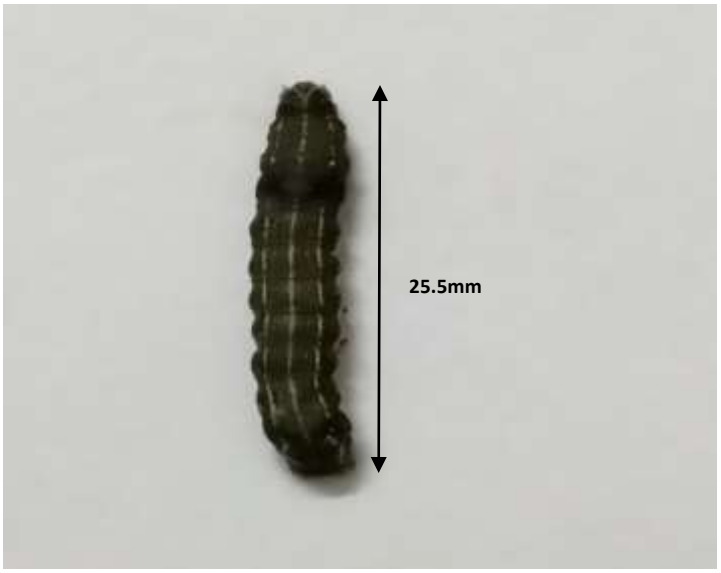

Figure 8 4th instar larva

Four days after hatching, the larvae reached early second instar and were transferred into individual cells. Late second instar and older stages are cannibalistic and start eating each other if left together without food. Moulting plays very important role as it indicates transformation of one instar from another instar. 3rd instar stage was more voracious feeder as compared to 1st and 2nd instar larvae. Hence most of the studies related to insecticide testing was done on 3rd instar stage only. For avoiding any pathogenic infection due to excreta regular transferring of larvae to fresh diet was performed. If the diet and rearing conditions are optimum and well suited for larval growth, the larvae reach third instar on the sixth day after hatching. Third instar larvae were sorted & transferred on to fresh diet. Each diet-rearing tray was covered with a semi-permeable thin

plastic lid. The semi-permeable thin plastic lid prevents escape of neonate larvae. The rearing trays are incubated at 27 $\pm$ 2°C and 70 + 5 % R.H. in insectary. Ideally moths raised from the field-collected larvae must be single paired and the resulting progeny pooled together to represent the larval collection. This was necessary to avoid overestimating or underestimating resistance due to polyandry, polygamy in mass mated *S. litura*. Diseased or parasitized larvae were discarded immediately. To avoid disease spreading through the colonies, especially with NPV or stunt viruses, field collected material were quarantined for at least one generation before integrating into the colony.

Late fifth instar larvae stopped feeding just before pre-pupal stage and started wandering in search of a site for pupation. The larvae started shrinking from both the ends and try to hide themselves inside diet forming cave like structure to simulate field like situation. Firstly, they turned greenish and slowly start turning brown. During early stage pupae were not disturbed. Care was taken to ensure that the pre-pupae were not injured or disturbed. Pre-pupae construct a pupation cell and pupate in 2-3 days. At a right stage, they were transferred into container (15 cm dia x 8 cm h) containing tissue paper at the base. Each container can accommodate 10-15 pupae.

Figure 9 Different stages of *S. litura larva*

The fully-formed pupae were removed from the container 4-5 days later. Pupae were surface sterilised with 1% sodium hypochlorite solution, after which they were washed with distilled water, wiped with soft tissue paper and placed in container. Pupae were kept at 25-27°C and 10:14 hour light:dark photoperiod. Male moths were light jade-green while females are light brown in colour. Moths generally emerge shortly after midnight and mate 2-3 days after emergence. The moth pairs were held individually in small jars of 20 cm x 25cm (diameter x height), kept at 27 $\pm$ 2°C and RH 75-80%. Jars were covered on the top with black muslin cloth. Cotton swabs, soaked in a solution containing 5 % each of honey and sucrose were suspended along the walls of the jars and changed three times a week. Oviposition starts a day after mating and continues for 6-8 days. If moths were over-fed or under-fed, longevity and fecundity were severely affected. It was preferable to starve the moths for about 12 hours after emergence. It was also important to change the diet (5% honey + 5% sucrose in water swabs) frequently to prevent fermentation, which may lead to moth mortality. Under ideal conditions each moth lays about 500- 2000 eggs. It's important to maintain a humidity of 70-75% during hatching, to prevent neonates from desiccating. Eggs were light yellow in colour when freshly laid and turn brown to black 2-3 days later, just before hatching. Infertile eggs turn brown and shrivel and can be easily differentiated from fertile eggs at 10 x magnification. Soon after hatching, the larvae were transferred on to semi synthetic diet. Brushes and forceps that were used to transfer larvae should be periodically disinfected with a 2% sodium hypochlorite solution.

Precautions in *Spodoptera litura* rearing

1. Laboratory cultures of *Spodoptera litura* were successfully established and reared continuously for at least 10-14 generations without any problem, as adequate care was taken to maintain proper rearing conditions. The following steps helped in successful maintenance of the laboratory cultures.
2. It became very important to clean and sterilise all equipment and containers using hypochlorite, autoclaving, exposure to sunlight and or germicidal lamps before re-use. Diet rearing trays, moth chambers, forceps and brushes were regularly decontaminated in 5% sodium hypochlorite and rectified spirit. The working bench surfaces and floor of the rearing room was regularly cleaned and disinfected with rectified spirit and 5% hypochlorite.
3. *Spodoptera litura* cultures can be very difficult to maintain if proper care is not taken to ensure clean sanitation and regular disinfection of cultures and culturing conditions. Some of the most problematic diseases are NPV (Nuclear Polyhedrosis Virus), microsporidian protozoa- *Nosema*spp, fungus - *Aspergillus* spp. and a range of bacteria. NPV infected larvae appear creamy white in the terminal stages and liquefy rapidly thereby spreading the virus. Nosema affected larvae stop feeding, lose weight, have a shrivelled cuticle, appear dark and stay still unless prodded. The terminal stages were similar to that of NPV infection, where the body liquefies to release the microsporidian particles. Bacteria and fungi spoil the diet and can cause persistent problems in cultures.
4. One of the best ways to get rid of larvae harbouring latent infection of HaNPV (nuclear polyhedrosis virus) and *Nosema* spp. was to retain only larvae that reach the third instar stage by the 6th day after hatching. HaNPV and *Nosema* infected larvae have a slow growth rate and the slow growing larvae must be discarded. This helps in keeping cultures healthy. Diseased and dead larvae were disposed immediately by removing the entire rearing tray without opening it in the culture room. Eggs and pupae were regularly surface sterilised.

5. Regular exposure to scales over long periods may lead to respiratory allergic reactions in laboratory staff. It was advisable to remove scales every day using vacuum cleaners if available. It is preferable to wear dust mask and apron to prevent inhalation of allergenic particles or their contact with the skin.

3.2 Diet preparation for *S. litura*

Different ingredients required for preparing artificial diet which contributes larval developments are as under.

Table 1**:** List of ingredients for preparing artificial diet

Sr. No.	**Ingredients**	**500ml**
1	Wheat germ	25 gm
2	Chickpea flour	75 gm
3	Sorbic acid	0.75 gm
4	Ascorbic acid	2.50 gm
5	Methyl-p-hydroxy benzoate	2.30 gm
6	Formaldehyde 5%	12 ml
7	Becosule	8 ml
8	Propionic acid	1.8 ml
9	Yeast	28 gm
10	Agar agar	10 gm

Larval diet preparation procedure

1. Measured quantities of chickpea flour, wheat germ, sorbic acid, ascorbic acid, methyl paraben, propionic acid, becosule syrup & formaldehyde were added into a large bowl. 250 ml of pre-boiled warm water, was added and stirred thoroughly to mix well. We call it these mixtures as part A of diet.
2. The instant dried yeast was dissolved in 250 ml water and boiled for 5 minutes, after which agar was added, dispersed well and boiled for 5min.

3. Yeast and agar solutions, were mixed boiled for5 minutes and added to the bowl containing other diet ingredients. Blender was used to mix them well. We call it these mixtures as part B.
4. Part B was mixed into part A, mix thoroughly with the blender.
5. The hot diet was transferred into soft plastic squeeze-bottles, close with lids having spouts trimmed to 1 cm, and dispensed the diet into wells of multi-cell trays.
6. The rearing trays can be stored at 4-8°C for a week.

Figure 10 Weighing of ingredients for diet preparation

Precaution while diet preparation

1. All the ingredients were measured accurately
2. Measuring cylinders were kept on a stable surface while measuring water and check the lower meniscus/ upper meniscus at eye level only
3. Utmost care was taken while handling microwave and hand blender
4. It was taken care that hand blender did not get overloaded. If it was getting over heated, there was a pause for 2 minutes resume the work. If overheating continued, blender was repaired keep stirring the mixture so that it does not burn at the bottom of vessel.

3.3 Good laboratory practices

1. Usage of Lab coat during all lab work, hand gloves during diet preparing.
2. Maintenance of hygiene by discarding waste material and by avoiding contamination.
3. Regular work on daily basis as per proper schedule was done like newly hatched larvae taken on fresh diet, transferring of larvae from old to freshly prepared diet, separation of pupae, preparation of oviposition pot.
4. All the records were kept updated taking into account the number of larvae hatched, number of larvae transferred, number of oviposition pots prepared, number of adults emerged, approximate hatching observed etc. There records played a very important role to confirm the good health of culture.

3.4 Experimental design

Different factors like biotic and abiotic were studied on life cycle of *Spodoptera litura*. For easy understanding flow chart was prepared as under.

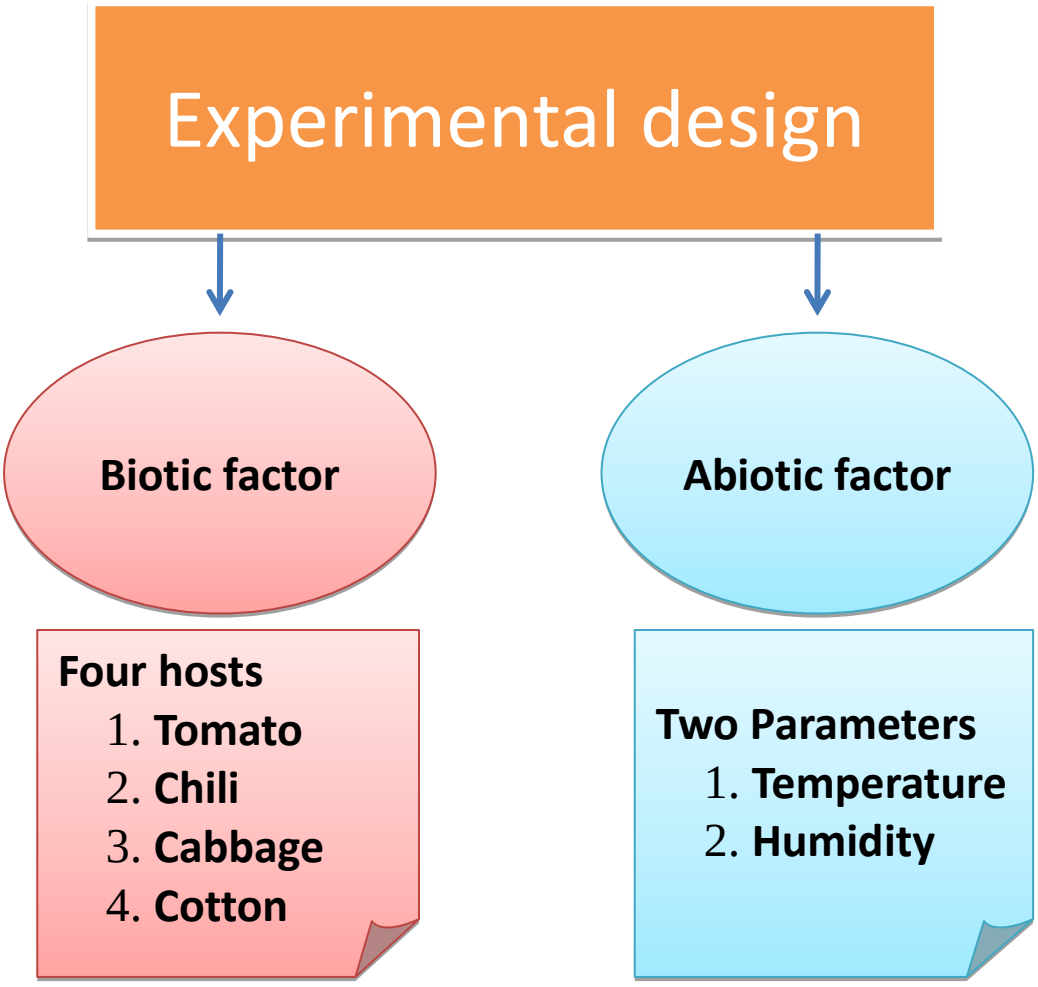

Figure 11 Experimental design

Influence of different hosts on life cycle of *S. litura*

The insect & plants interact with each other and this type of interaction is called as insect host relationship. By feeding different hosts insect shows some behavioral changes through metamorphosis and which can be reflected in insect life cycle. So to check the influence of different host on life cycle of *S. litura* four different host were selected. Selection of the host is based on the economical importance. The hosts which were selected mentioned in below table.

Table 2: Different host plants tested against *S. litura*

Sr.no.	Common name	Scientific name	Family
1	Tomato	*Solanum lycopersicum*	Solanaceae
2	Chili	*Capsicum annuum*	Solanaceae
3	Cabbage	*Brassica oleracea*	Brassicaceae
4	Cotton	*Gossypium*	Malvaceae

Plant rising for the experiment

Healthy plants were raised by giving proper attention on regular basis. In case of tomato, chili and cabbage sowing was done in 84 cell nursery tray and after 15-20 days transplanting was done in plastic pots having 10cm diameter and 10cm height. For raising cotton plants direct sowing done in plastic pots having 10cm diameter and 10cm height. Watering and fertilizer application was done on regular basis to get healthy leaves for the experiment.

Larval requirement and experiment set-up

S. litura egg masses were collected from castor plant and reared in laboratory. After completing one generation in laboratory newly hatched larvae were selected having age of 0-12 hours old for the experiment. 16 & 32 cell plastic trays were used for the experiments. Total of 10 larvae were tested per host. Newly emerged larva was kept singly with fresh leaf in individual cell. Excreta and left behind of leaves were cleaned daily to maintain hygienic condition. Fresh leaves were provided after every 24 hours. Careful observations were recorded on larval moult for seeing number of days taken to convert from one instar to another. When larvae moult into pupae than they were taken up into plastic container and sterilized with 10% sodium hypochlorite solution to avoid any contamination. Observed days taken for adult emerge from puparium. The life cycle of *S. litura* was observed under controlled condition i.e. 27±2°C Temperature & 70±5 % RH to nullify other conditions.

3.5 Morphometric changes when fed with different host plants

Along with life cycle study another experiment was conducted to see weight gain of 3rd instar larvae when fed with different hosts. For larval weight gain study, total of 8 larvae were taken. Before releasing larvae into container having host leaf, pre weighing was done for individual larvae and after 48 hours post weight was recorded.

Set-up to see host feeding preference

For that four 3rd instar larvae were taken into plastic container individually and provided with pre weighed different host leaves. Consumption of leaf was observed and after 48 hours post weight of leaves was recorded.

Influence of different abiotic factors on life cycle of *S. litura*

Abiotic factors like temperature and relative humidity were studied by keeping two different range (20°C Temp & 40% RH) considering optimum range (27±2°C Temp & 70±5 % RH). The selection of the particular temperature and humidity is based on literature survey. BOD will be utilized to regulate different abiotic factors. When testing temperature all the other factors were kept as normal, this will be done to see only impact of that factor which we were looking. In current study decrease in these two parameters were studied i.e. 7°C decrease in temperature & 30% decrease in humidity. For example is we have to see impact of temperature than in BOD set point was 20°C for temperature & 70 percent for relative humidity. If we have to study relative humidity than set point in the BOD was 27°C and 40 percent relative humidity. The duration of lifecycle was studied by observing whether it prolongs or become shorter due to fluctuation of these factors. Other growth parameters were observed by weighing different stages as well as consumption of leaf to predict food preference.

3.6 Statistical analysis and equations

The significance amongst different treatments was studied by different plots made through Minitab 19 software.

Other growth indexes were studied like larval growth index, pupal growth index and total growth index. For finding larval growth index two parameters were required. Firstly, how many larvae undergo pupation on the basis of which we calculate percent pupation and secondly, we need to know total larval period days. Total larval period was recorded by recording day on which larvae hatches to larvae goes into pupal stage.

Equation 1

$$\text{Larval growth index} = \frac{\%\ \text{Pupation}}{\text{Larval period (days)}}.$$

For finding pupal growth index two parameters required that is how many adults emerged from pupae and on the basis of that we calculate percent adult emergence and another we have to have total pupal period days. Pupal period was calculated by day on which pupa formed to day on which adult emergence seen.

Equation 2

$$\text{Pupal growth index} = \frac{\%\ \text{Adult emergence}}{\text{Pupal period (Days)}}$$

For finding total developmental index two parameters required that is how many larvae, pupae and adult survived throughout the life cycle and on the basis of that we calculate percent survival and another we have to have total developmental period in days.

Equation 3

$$\text{Total developmental index} = \frac{\%\ \text{Survival}}{\text{Total developmental period (Days)}}.$$

Equation 4

% efficiency of ingested food = [(D-C)/(A-B)] ×100

A=Pre weight of the leaf

B=Post weight of the leaf

C=Pre weight of larvae

D=Post weight of the larvae