CHAPTER THREE
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MATERIALS AND METHODS:

In the present chapter details of materials used and then techniques employed for different experiments of this investigation are dealt here with under following heads-

INSECT:

3.1A. Source:

Male and female *Diacrisia obliqua* Walker (Lepidoptera: Arctiidae) were collected in the second week of August, 2003 and the same were brought to the laboratory for maintaining their stock for different experiments. The larvae and adults employed in laboratory studies were obtained from this stock which was maintained throughout the tenure of the present investigation.

3.1B. Marks of Identification:

Moths are medium sized with a wing expanse of about 50 to 66 mm. The wings are pale buff coloured. Abdomen of moth is crimson with black spots. Males and females are usually identical in appearance. The female is bigger than male. The abdomen of female is wider and stumpy. Adults mate soon after emergence.

3.1C. Host plants and damage:

*D. obliqua* is a polyphagous insect. Moths feed on sesameum, linseed, safflower, castor, jute sunflower, potato, tomato, several seeds and
cause mild to severe loss. The hairy caterpillars feed on leaves, buds and flowers of different host plants. In case of severe infestation, the plants may be completely defoliated.

3.1D. Life Cycle of *Diacrisia obliqua* Walker:

A female lays about 280-450 eggs during her life period. The eggs are deposited in clusters. The eggs are small, spherical and pale or yellow in colour and then gradually turn deep yellow. The egg stage lasts for 2-4 days. The newly hatched larva is dark grey with yellow bands on the body, measuring about 2 mm. in length. The full grown larva measuring 28 to 40 mm in length is hairy orange with head black and the last two segments black. The caterpillar march from field to field in large numbers causing destruction. It moults four times to make the number of instars five. The larval period lasts for 13 to 28 days. The larva, having attained the full growth, pupates in soil or on the ground in a silken cocoon. The pupa is reddish brown measuring upto 2 cm in length. Adult emerges out from the pupa in 4 to 10 days, depending on the season. The life cycle is completed in 26 to 45 days.

3.2 LABORATORY STOCK OF THE MOTH:

A stock of the moth was maintained in the laboratory to ensure its regular supply of different developmental stages in a large number for different studies during the investigation. For this purpose, the moth was reared in large number, generation after generation.
Moths, males and females obtained from field & were maintained in glass chimneys provided with twenty per cent sugar solution with *Crotalaria* leaves for oviposition. Eggs obtained from them were kept as such for hatching.

On their hatching, larvae obtained were reared on tender *Crotalaria* leaves, in large petridish to grow in groups of twenty per petridish upto second instar stage. The food supply was maintained twice a day in view of evaporation of water from leaves. Precaution was taken to avoid infection or contamination. After the second instar stage the larvae were reared in groups of five in petridishes. For these larvae also, the food supply was maintained as described above till the pupation. When larvae acquired full growth and stopped feeding, they were transferred in pneumatic troughs having about 10 to 15 cm thick soil on their bottoms. The larvae pupated either on the surface of the soil or little below the surface of the soil. Pupae, thus obtained were kept for emergence of moths. On emergence, adults were maintained as already described above for obtaining eggs. From larvae of these moths, next generation was reared as described above and in the same way the insect was reared generation after generation and the continuous supply of this moth throughout the whole tenure of the investigation was ensured.

3.3 BIOCHEMICALS USED:

The following commercial preparations of *B. thuringiensis* whose efficacy as controlling agents has already been evaluated among different
insects by different economic entomologists were employed against *Diacrisia obliqua* in this study.

Name of commercial preparations of *B. thuringiensis* used in this investigation are mentioned below-

1. **Dipel** wettable powder containing $25 \times 10^9$ viable spores per gram of final product of *B. thuringiensis* var. *kurstaki* (Serotype 3 a, b strain HD-1).

2. **Thuricide** H.P. wettable powder containing $30 \times 10^6$ viable spores of *B. thuringiensis* var *kurstaki* (serotype 3a, b strain HD-1) per gram of final product.

3. **Bactospeine** containing $1 \times 10^8$ viable spores of *B. thuringiensis* (serotype-1) per gram of final product.

### 3.4. CONCENTRATIONS OF BIO CHEMICALS USED:

The commercial preparation of *B. thuringiensis* namely Dipel, Thuricide HP and Bactospeine were obtained in pure form, and stock solution used in the present investigation was prepared in distilled water. One gram of Dipel, Thuricide HP and Bactospeine were mixed separately in 100 ml of distilled water which gave the concentrations of one per cent, from this, other concentrations were prepared by serial dilution method.

Two per cent skimmed milk powder was added to bacterial suspension for improving the adhering quality of the bio-chemical. The
concentrations were applied against *D. obliqua* in the studies included 0.05, 0.10, 0.50, 0.75 and 1.0 per cent.

3.5. METHODS OF APPLICATION OF Bt. INSECTICIDES:

The experimental insects were treated with different concentrations of bacterial preparations by following two methods.

3.5A. Leaf dip method:

In this method of treatment small and uniform size of leaves of host plant were treated with each concentration of particular bacterial preparation by leaf dip method.

3.5B. Topical method:

In this method of treatment about 2 hr old adults were exposed to a thin film of residue of a concentration of a particular bacterial preparation. For obtaining the thin film of bacterial preparation as residue, about 10 ml of a concentration of bacterial preparation was poured in a petridish (10 cm dia.) and the petridish was tilted in different ways to spread the bacterial preparation on the whole floor area of the petridish and its raised periphery. This led to the formation of a thin film of a concentration of bacterial preparation in the petridish as residue. Adults were left in petridishes having thin film of the bacterial preparation for 24 hours. The petridishes were covered by thin muslin cloth to prevent the escape of the adults. Such treated adults were employed in the different experiments as described later on.
3.5C. Control Experiment:

Control experiments were also set up to evaluate and compare the findings of different experiments. In control feeding experiment the same procedure as of feeding treatment was adopted but the *Crotalaria* leaves that were given to the larvae were not treated in the bacterial solution. They were simply dipped in two per cent skimmed milk solution. In the topical control experiment only 2 per cent skimmed milk solution was poured in the petridish. Rest of the operations and observations were done in similar manner for different aspects, as taken in the feeding and topical treatments.

3.6. DESIGNES OF STUDIES:

Studies presented in this thesis were conducted experimentally under laboratory conditions of temperature and relative humidity. These studies were carried on under following headings:

A. The effect of bacterial preparations on growth.

B. The effect of bacterial preparations on development.

C. The effect of bacterial preparations on fecundity and fertility.

D. Sex specific sterility effect of bacterial preparations on sexes.

E. Study of the compatibility of *B.thuringiensis* with chemical insecticides.

The above mentioned aspects were studied as under:
3.6A. Effect of bacterial preparations on growth:

This aspect was studied in terms of accumulation of biomass in larva at regular intervals and acquisition of biomass in both pupa and adult and was evaluated as under-

3.6 A.1. The influence of bacterial preparations in larva pupa and adult:

This was studied under two different conditions of treatment (leaf dip method and topical method). In both conditions, the adult insects were treated with a strength of bacterial preparation by leaf dip method and topical method. The influence of a bacterial preparation on biomass accumulation in larva under both treatments was studied as follows.

3.6A.1a. The influence of bacterial preparations on biomass accumulation in larva under treatment by leaf dip method.

This was studied by employing larvae on the leaf treated with different concentrations. The influence of a bacterial preparation on the larval growth under this treatment was studied by five experiments, one for each strength, each consisting of three replicates. Twenty larvae (1/2-1 hr old) per replicate were reared on tender leaves of C. juncea till the 16th days of development. The weight of these larvae was recorded on the 5th, 10th and 15th day of their larval duration. These records were obtained with reference to each strength of each bacterial preparation. The experiment designed to determine the influence of a bacterial preparation were accompanied by a control experiment.
3.6.A.1b. The influence of bacterial preparations on biomass accumulation in larva under treatment by topical method:

The larvae obtained from the adult treated with different strengths of the bacterial preparations by the topical method were employed for evaluation of their growth. The influence of the bacterial preparations on the growth was determined with reference to identical five strengths of each bacterial preparation exactly on the above mentioned pattern and the related records were obtained with reference to them.

The experiments for a bacterial preparation were accompanied by a control experiment also.

3.6.A.II. Effect of bacterial preparations on weight acquisition by pupae and adults:

This aspect was studied by applying the bacterial preparations by leaf dip method and topical method to adults. The adults were treated by topical method while larvae by leaf dip method.

3.6A.IIa. Effect of bacterial preparations on weight acquisition by pupae & adults under leaf dip treatment:

Sixty larvae were selected at random from the laboratory stock and were treated with a strength of bacterial preparation by leaf dip method. These treated larvae were maintained in glass chimneys for the development. After that one glass chimney housed one pair of adult. When oviposition occurred, eggs obtained were kept on moist filter paper for their hatching. Sixty larvae of such
eggs were selected at random and divided into three groups of twenty larvae, each group constituted a replicate. The larvae of the replicate were reared on tender leaves of host plant until they pupated. The pupae thus obtained when acquired 4 to 6 hr age, were weighed and their weight was recorded. These pupae were kept for emergence of adults. On emergence and after having the discharge the meconium the adults were weighed after one hour and their weight was recorded. The aforesaid study was conducted with reference to each strength of every bacterial preparation tested and records identical to above mentioned were obtained.

3.6. All.b. Effect of bacterial preparations on weight acquisition by pupae and adults under topical treatment:

Twenty pairs of adults selected at random from the rearing stock and were treated with a strength of a bacterial preparation for 24 hr in petridishes and thereafter, they were maintained in glass chimneys for oviposition. The eggs deposited were kept for hatching and the sixty larvae of such eggs were selected indiscriminately. These larvae made three replicate and were reared to obtain their pupae and adults. The pupae when 4 to 6 hr old were weighed and their weight was recorded. The adults were also weighed, about one hr old, after discharge of meconium and subsequently their weight was recorded.
3.6B. EFFECT OF BACTERIAL PREPARATIONS ON DEVELOPMENT:

The effect of different strengths of all the considered bacterial preparations on development of *Diacrisia obliqua* was studied in response to their application to moth by leaf dip method and topical method as described below:

3.6B.1 Effects of bacterial preparations on development under leaf dip method of treatment:

This was studied experimentally with immediately hatched larvae obtained from females fed on a strength of a bacterial preparation were employed experimentally. This was tested by one experiment designed separately for each strength of bacterial preparation. Twenty such larvae per replicate were reared on tender leaves of host plants until their pupation, number of larvae pupated, their developmental duration and survival were recorded. These pupae were kept to obtain adults. On emergence of adults, the number of adults emerged and their pupal period were recorded besides these, the sex ratio of adults was also recorded. The experiment was further extended to record the life duration of males and females. For this purpose males and females were maintained individually date wise in glass chimneys on daily supply of twenty per cent sugar solution till their natural death and on their expiry, their longevity was recorded. Besides, for the purpose of comparison a control experiment was also set for each strength of bacterial preparation.
3.6B.II. Effect of bacterial preparations on development under topical method of treatment:

This was studied experimentally with newly hatched larvae of adults which were already forced to contact their residue film of a strength of each bacterial preparation. It was determined in one experiment which consisted of three replicates. Twenty such larvae were reared on leaves of *C. juncea* till their pupation and when they pupated, the number of pupae obtained and the larval period were recorded. The pupae were kept in glass chimneys date wise and when moths emerged from them, their number and pupal period were noted. Besides, this the sex ratio was also recorded. The experiment was further extended for recording the life span of male and female moths. For this purpose males and females were maintained individually date wise in glass chimneys on daily supply of twenty per cent sugar solution till their natural death and on their expiry, their longevity was recorded.

Besides the above records under different methods of application of bacterial preparations, the record pertaining to net mortality was obtained as suggested by Abbot (1925) as follows:

\[
\text{% Net mortality} = \frac{\text{% Mortality in test} - \text{% Mortality in normal}}{100 - \text{% Mortality in normal}} \times 100
\]

3.6C. Effect of bacterial preparations on reproduction:

The reproduction in *D. obliqua* under influence of different bacterial preparations was studied under two headings ---
1. Effect of bacterial preparations on reproductive periods and fecundity.

2. Effect of bacterial preparations on fertility and incubation period.

3.6.C.1. Effect of bacterial preparations on reproductive periods and fecundity:

   The pre-oviposition and oviposition periods and the number of eggs laid by a female were studied separately by applying bacterial preparations to larvae and adults as described under.

3.6.C.1a. Effect of bacterial preparations on reproductive periods and fecundity under leaf dip method of treatment:

   Ten males and ten females were obtained indiscriminately from the earlier treated stock. The females were maintained individually with a male in glass chimney on daily supply of twenty per cent sugar solutions, for oviposition. When these females laid eggs for the first time, pre-oviposition period was recorded. The females were maintained till they laid last egg and after that their oviposition period were recorded and their total number of eggs was counted. The above study was made separately for each strength of all the tested bacterial preparations and above mentioned records were obtained for them. A control experiment was also set for each bacterial preparation.
3.6C. 1b. Effect of bacterial preparations on reproductive periods and fecundity under topical method of treatment:

Ten females along-with ten males were selected at random from the laboratory stock. Both males and females were compelled to contact a thin film of strength of bacterial preparation for 24 hrs. Thereafter, each female was maintained in a glass chimney with a male on twenty per cent sugar solution. They were kept as such for egg laying and when the first egg laid, the pre-oviposition period was recorded. The females were maintained till the deposition of their last egg, after which the oviposition period was recorded. The total number of eggs laid during the oviposition period was recorded. The above mentioned study was conducted separately for all concentrations of the tested bacterial preparation and the above mentioned records were obtained for them. Besides, a control experiment was also designed for each bacterial preparation.

3.6c.II. Effect of bacterial preparations on fertility and incubation period:

The influence of bacterial preparations on fertility and incubation period in *D. obliqua* was studied with reference to leaf dip method and topical method as follows:
3.6C.Ila. Effect of bacterial preparations on fertility and incubation period of *Diacrisia obliqua* under leaf dip method of treatment:

Ten females and ten males, each 1 hr old were selected indiscriminately from the earlier treated laboratory stock. These moths were maintained as pairs in glass chimneys with twenty per cent sugar solution. Each pair constituted a replicate. The eggs from each replicate collected daily and kept date wise on moist filter paper. On hatching of the eggs, the number of eggs hatched and their incubation period were recorded. The above study was undertaken with reference to different strengths of all the tested bacterial preparations and the above mentioned records were obtained. A control experiment was set for every bacterial preparation and similar records were maintained.

3.6C.Ilb. Effect of bacterial preparations on fertility and incubation period of *Diacrisia obliqua* under topical method of treatment:

For the study, ten males and ten females were drawn indiscriminately from the laboratory stock. These moths were compelled to contact thin residue film of a strength of bacterial preparations for 24 hrs and thereafter these were maintained as pairs in glass chimneys with twenty per cent sugar solution; each chimney had one pair of moth. Each moth pair made a
replicate. Eggs from each replicate were collected daily and kept date wise on moist filter paper. On hatching of the eggs, their viability and incubation period were recorded. The experiment for each bacterial preparatoin was accompanied by a control experiment.

Besides the above mentioned records, the records pertaining to the reduction in the fecundity, net sterility and control over reproduction were also obtained as described below:

The reduction in the fecundity was calculated following the formula of Chamberlain (1962) as detailed below:

\[
\text{% Reduction in fecundity} = \frac{\text{Eggs laid in normal} - \text{Eggs laid in test}}{\text{Eggs laid in normal}} \times 100
\]

The sterility was calculated following the formula of Abbot (1925) as detailed below:

\[
\text{% Net sterility} = \frac{\text{% Sterility in test} - \text{% Sterility in normal}}{100 - \text{% Sterility in normal}} \times 100
\]

The control over the reproduction was calculated by following the formula of Chamberlain (1962) as detailed below:

\[
\text{% Control over reproduction} = \frac{\text{Eggs hatched in normal} - \text{Eggs hatched in test}}{\text{Eggs hatched in normal}} \times 100
\]

3.6D. Sterility effect of bacterial preparations on sexes:

Studies described at serial 3.6A did not project the sex specific influence of the tested bacterial preparations. In order to determine this, the following study was carried by monitoring matings between treated female and
untreated male and between untreated female and treated male. This was studied with reference to each strength of all the tested bacterial preparations separately by two experiments, each consisting of three replicates. All experiments were set as described in 3.6C. Experiments were also accompanied by control experiment.

3.7. Compatibility of Insecticides with Dipel:

On the basis of screening different commercial formulations of *B. thuringiensis*, Dipel was found most effective. Therefore, compatibility of insecticides was studied with Dipel only.

To study the compatibility of insecticides with Dipel against the larvae of *D. obliqua*, the two experiments were set. First experiment comprises the evaluation of insecticides while the second experiment was set for the evaluation of insecticides of combination with the pathogen. Six commercial insecticides namely; Endosulfan, BHC, Quinalphos, malathion, Fenvelerate and Cypermethrin were tested for their relative toxicity by the following technique.

Five concentrations of each insecticide were taken and the leaf dip method as described earlier was adopted for the leaf treatment. For determining the relative toxicity of insecticides, small, uniform leaves of castor were treated with different concentrations of insecticides and five days old larvae, starved for twelve hours, were released for twenty four hours. Thereafter, fresh untreated leaves were provided to them for feeding. The larval mortality was recorded after
24, 48, 72, 96 and 120 hours of treatment. The data, thus obtained after 120 hours, were utilized for the assessment of relative toxicity of insecticides tested.

Similarly, in the second experiment, a constant sub-lethal concentration of Dipel (0.05%) was mixed to each concentration of insecticide tested and bioassay was made against the test larvae as described earlier. The data noted on larval mortality were subjected to statistical analysis for working out the LC$_{50}$ value of each insecticide in combination with Dipel.

The toxicity index was also worked out for insecticides and their combination with Dipel by taking the LC$_{50}$ of Malathion as unit. The toxicity index was calculated with the help of following formula;

$$\text{Toxicity index} = \frac{\text{LC}_{50} \text{ of insecticide A}}{\text{LC}_{50} \text{ of insecticide B}}$$

Where A = LC$_{50}$ of Malathion

B = LC$_{50}$ of other tested insecticide

With each set of the experiment a control experiment was also set, where the larvae were treated with distilled water.

3.8. STATISTICAL ANALYSIS:

Various statistical analysis mentioned below have been applied to study the nature and relationship between variables, to know the reliability and precision in the results obtained, to test the significant difference between the observed and corresponding expected values and to predict the estimated values of effectiveness for a given value of concentration.
3.8a. Standard error:

Standard error is used for estimating the errors which are likely to be there in the average of the values obtained in the difference replicates of experimental treatments. The standard error has been calculated with the help of the following formula.

\[ SE = \frac{SD}{\sqrt{n}} \]

Where,

\( n \) = Number of observations.

S.D. = Standard deviation.

Standard deviation was calculated by the following expression.

\[ S.D. = \sqrt{\frac{\sum (X - x)^2}{n}} \]

Where,

\( x \) = observation of variate values.

\( X \) = Arithmetic mean of the observation.

\( n \) = Number of observations.

3.8b. Significance test:

The significance test was done to reveal, whether the differences in the results obtained at the different levels were due to errors of sampling or there existed real differences between the treatments.
(a) Chi Square test ($x^2$ test):

For testing the independence or association between the effectiveness and concentrations, $x^2$- test was also used. The heterogeneity of the data were tested maximum at 5 per cent probability level.

3.8.c. Regression equation:

Regression is the measure of the average relationship between two or more variables in terms of the original units of the data. Regression lines between probit mortality/reduction in larval growth and log concentration was determined to predict the estimated value of effectiveness at the given value of log concentration. (Finney 1952).

3.8.d. Determination of E.D. 50:

Different E.D.$_{50}$ values were calculated with the help of the regression equation to know the concentration/dose causing, 50% mortality/reduction in food consumption/reduction in larval growth.

3.8.e. Relative efficacy:

Relative efficacy was calculated by dividing the different values by suitable standard value.