MATERIALS AND METHODS
CHAPTER – III

MATERIAL AND METHODS

The present investigation on evaluation of microbial pesticides against gram pod borer *Heliothis armigera* and wilt causing fungi of chickpea in Chhattisgarh region was carried out at Raipur during 2000-2004. The materials used and the methods followed in various experiments are described below:

3.1 Geographical Location

Raipur is situated between 21.16° North latitude and 81.36° East longitude with an altitude of 289.56 meters above mean sea level.

3.2 Climate

The climate of Raipur is dry sub humid to semi arid with the average annual rainfall of 1280 mm mainly concentrated from middle of june to september with occasional shower in winter. The maximum temperature goes as high as 46° during the summer months and minimum as low as 6° C during the winter months.

3.3 Test organisms

The test insect was gram pod borer *H.armigera* (Hub.) larvae and Wilt complex fungi *Sclerotium rolfsii* Sacc, *Fusarium oxysporum* f. sp. ciceri (Padwick) Snyd. & Hans. and *Rhizoctonia solani* (Taub.).
3.4 Population Dynamics of Gram pod borer

For the study on population dynamics weekly observation on larval population and number of parasites, live cocoon, number of fungal diseased larvae, recorded in the field on the chickpea during rabi season 2000-2001.

3.4.1 Seasonal larval activity on chickpea

The data was recorded on seasonal changes in larval population of chickpea pod borer *H. armigera* along with activity of its larval parasite (*C. chloridae*) and larval infection by fungus, white muscardine disease (*Beauveria bassiana*), during 2000-2001 Rabi season.

3.4.2 Effect of temperature

Temperature effect on larval population of chickpea pod borer studied with reference to larval activity affected at maximum and minimum temperature.

3.4.3 Effect of Relative humidity

Relative humidity (RH) was related to larval population. to reflect response of the insect to effective humidity range.

3.4.4 Effect of rainfall

During the population dynamics study rainfall was measured to detect whether this factor triggered the growth of insect pest.

3.4.5 Seasonal activity of natural enemies of larvae of *H. armigera*

Activity of larval parasite *Campoletis chloridae*, *Beauveria bassiana* and natural infection of *Heliothis* larvae by nuclear polyhedrosis virus was studied.
3.5 Experimental details under field conditions for management of gram pod borer.

3.5.1 Layout of Field Experiment

A field experiment was laid out at Labhandi farm of Indira Gandhi Krishi Vishwavidyalaya, Raipur with gram (*Cicer arietinum* var. JG-74) as a test crop in a field. The experiment had 30 plots, one for each of 10 treatments (9 + 1) with three replication arranged in a randomized block design. The crop was sown on 12th October 2001 at row to row distance of 30 cm. The crop received basal fertilizer dose in ratio 20:50:30. Observations were recorded on randomly selected 10 plants/plot. Number of larvae per plant after 3, 5, 7, 10 and 15 days from treatment.

The experimental layout is shown in Fig 3.1

<table>
<thead>
<tr>
<th>Rep 1</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
<th>T6</th>
<th>T7</th>
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<tbody>
<tr>
<td>Rep 2</td>
<td>T5</td>
<td>T9</td>
<td>T4</td>
<td>T6</td>
<td>T8</td>
<td>T2</td>
<td>T3</td>
<td>T10</td>
<td>T7</td>
<td>T1</td>
</tr>
<tr>
<td>Rep 3</td>
<td>T8</td>
<td>T3</td>
<td>T1</td>
<td>T9</td>
<td>T6</td>
<td>T10</td>
<td>T4</td>
<td>T7</td>
<td>T2</td>
<td>T5</td>
</tr>
</tbody>
</table>

T1- HNPV @ 300 ml/ha, T2- HNPV @ 400 ml/ha, T3- HNPV @ 600 ml/ha, T4- HNPV @ 800 ml/ha, T5- HNPV @ 1000 ml/ha, T6- HNPV @ 1200 ml/ha, T7- Endosulfan @ 1500 ml/ha, T8- HNPV @ 1200 ml/ha + jaggery, T9- HNPV @ 1200 ml/ha + boric acid, T10 - Control

Fig 3.1 Schematic layout of the field experiment.
3.5.2 Insecticidal HNPV spray mixture preparation

The infected Gram pod borers were collected in dark bottles and were kept in cool place for decay upto 7 days. The diseased larvae was macerated in blender. Polyhedra was allowed to settle at the bottom as white layer. Sediment was collected and suspended in distill water. Mixture was centrifuged for 1 min. at 600 round per minute. Supernatant contained polyhedral bodies which was further centrifuged for 5 minute at 2500 rpm. Pellets containing polyhedral bodies were collected. Thus a solution with 100 larval Equivalent (L.E.) was prepared. Different concentrations varying from 300 ml/ha to 1200 ml/ha of NPV was prepared. Knapsack sprayer was used for spraying. Two spraying were given first on 26th November 2001 and second on 23rd January 2002 by spraying solutions of respective insecticides. The crop in the control treatment plots was simultaneously sprayed with water. The insecticidal spray mixture was prepared freshly at the site of application every time for every individual treatment. During the application the sprayer was rinsed thoroughly with water i.e. after the application of each treatment.

3.5.3 Observations of Field Experiment

Observations were recorded on randomly selected 10 plants/plot on –

- Number of larvae per plant before and after 3, 5, 7, 10, 15 days from treatment.
- Number of dead larvae per plant before and after 3, 5, 7, 10, 15 days from treatment.
- Number of eggs per plant before and after 3, 5, 7, 10, 15 days from treatment.
- Number of cocoon per plant before and after 3, 5, 7, 10, 15 days from treatment.
- Average plant height at full podding stage.
- Number of branches per plant at full podding stage.
- Number of pods per plant at full podding stage.
- Number of damaged pods per plant at full podding stage.
- Grain yield at harvest.

3.6 Host Specificity and safety of Nuclear Polyhedrosis Virus (NPV) to beneficial pests of chickpea (*Cicer arietinum* L.)

The NPV inoculum was multiplied by feeding third and fourth instar larvae of *H.armigera* with virus contaminated chickpea leaves. The purified, concentrated suspension of polyhedra isolated from the dead, diseased larvae of *H.armigera* was used as infective material as described by Backwad and Pawar (1981).

The fungus inoculum obtained was isolated and multiplied as per Kiraly *et al.* (1974b). Then the spores were transferred in to a conical flask contained through a double layer sterile cheese cloth and filtrate was used as stock suspension. A standard haemocytometer (Neubaur's improved double ruling, Germany) was used for counting the polyhedra under phase contrast microscope.

Adults of aphid predators viz., *Coccinella septumpunctata* Linn. (Coccinellidae), *Menochilus sexmaculatus* Fab. (Coccinellidae), *Chrysoperla carnea* Steph were fed with aphids dipped in viral and fungal suspensions for 24 hours and healthy aphids were provided thereafter. Adult predators of the same age group provided with healthy aphids constituted untreated control and served as a check on the NPV activity.

Parasitoid viz., *Campoletis chloridae* was provided with viral and fungal infected larvae of *H.armigera* as host material.
The experiment was conducted with 3 replications consisting of 60 larvae. The second instar larvae of *H. armigera* served as control and as check on the viral and fungal activity. Observations were recorded on the larvae mortality till pupation.

3.7 Isolation of *Trichoderma* species from soils & plants of different parts of Chhattisgarh.

Soil and plant samples were collected from Districts of Rajnanandgaon, Durg, Mahasamund and Raipur of Chhattisgarh state for isolation of *Trichoderma*.

**ISOLATION TECHNIQUES**

**Isolation from Rhizosphere (Dilution plate method)**

1. Rhizosphere soil was separated from 5-6 root samples with the help of a brush in a Petri plate.

2. 10 g of rhizosphere soil added in 90 ml sterile water blank and shaken for 15 minutes on a magnetic shaker.

3. Serial dilutions of (w/w) $10^{-2}$ to $10^{-6}$ prepared.

4. 1 ml each of dilution $10^{-2}$ to $10^{-6}$ transferred to sterile petri plates.

5. Melted and cooled PDA poured in petri-plates.

6. The plates incubated at $25^\circ$C for 5-7 days.

7. Isolated colonies were then transferred into PDA slants.

**Isolation from Rhizoplane:**

1. Serial washings (10-20) were given to the above roots with sterilized water until clear root surface was exposed.
2. Washed roots plated on the specific medium.

3. The plates incubated for 5-7 days at 25°C

3.8 Physico-chemical characteristic of wilt complex affected soils

Samples were specially collected from chickpea fields suffering from wilt complex diseases from Villages of Dharsiwa, Arang, Mahasamund, Bhansoj, Berla, Kumhari, Ahirwara and Baghbahra blocks of the state. All these samples were examined for pH, Ec (dSm⁻¹), Organic carbon (%), available N and K to find out relationship between physico-chemical & biological characteristic of wilt complex affected soils. Organic carbon was estimated by Walkey and Black's rapid titration method (Jackson, 1967). Available Nitrogen by modified Kjeldal’s method and Potassium was estimated by Flame photometric method (Jackson, 1967).

3.9 Preliminary screening experiments for selection of location specific effective Trichoderma sp. against wilt complex causing fungi (Interaction study: Under dual culture).

To know the antagonistic relationship between Trichoderma sp. and wilt / root rot causing fungi (Fusarium, Sclerotium and Rhizoctonia), dual culture technique was employed. A 7-day old culture of wilt / root rot causing fungi was used for inoculation in combination with six local isolates of Trichoderma viride on PDA medium under in vitro conditions. Each treatment were replicated thrice including control and incubated at 25 ± 1°C. These plates were regularly observed at 24, 48, 72 and 96 hrs for their vegetative growth and the final growth area (sq. mm.) of wilt / root rot causing fungi and Trichoderma isolates were recorded at 96 hrs of incubation.
Ratio of T/F, T/S and T/R was obtained by dividing the growth area of *Trichoderma* with the growth area of *Fusarium, Sclerotium* and *Rhizoctonia*. Similarly, the percent inhibition of wilt / root rot causing fungi due to different local isolates of *Trichoderma viride* were also determined using following formula:

\[
\text{Per cent growth inhibition} = \frac{\text{Growth of wilt / root rot causing fungi in control plate} - \text{Growth of wilt / root rot causing fungi in presence of *Trichoderma* isolates}}{\text{Growth of wilt / root rot fungi in control plates}} \times 100
\]

3.10 Efficacy of local isolates of *Trichoderma viride* in management of wilt complex causing fungi of chickpea by Standard Blotter Method

Healthy surface sterilized seeds (1:1000 HgCl\(_2\)) of gram cv. "Vijay" were artificially infected with 7-day old culture of pathogenic fungi, *F. oxysporum* f.sp. *ciceri, R. solani* and *S. rolfsii*. The infected seeds were then inoculated with *T. viride* isolates. Five seeds (One seed in the centre and four at the periphery: ISTA, 1985) were placed on moist blotter papers and kept in a sterilized Petri dishes. Un-inoculated seeds were considered as control. The seeds were examined for per cent germination, incidence of wilt complex fungi, plumule and radical lengths.

3.11 Testing of *Rhizobium* as bio-control agent of wilt causing fungi (*Fusarium oxysporum*)

Twenty isolates of Gram *Rhizobium* were tested for their antifungal property against *Fusarium oxysporum* on agar plates (Martin medium without streptomycin and rose bengal) using dual culture technique (Buonassisi et al., 1986). These rhizobial isolates were also tested for their inhibitory property against the mycelial growth of the above pathogenic fungi in liquid media (Omar & Abdalla, 1998).
Further the promising rhizobial isolates were characterized for their stress tolerant behaviour.

a). Thermal tolerance study: Selected isolates of Gram *Rhizobium* were subjected to 45, 50 and 55°C for 10-30 minutes at water bath. After thermal shocks when broth culture cool down up to room temperature, each isolate (loopful) was inoculated into petri plates containing Yeast Extract Mannitol Agar (YEMA) medium with four replications. Control was also maintained for comparison. All the inoculated petri plates were incubated in BOD incubator at 28±2°C (Benson, 1990). Observations were recorded for survival of Rhizobium up to 10 days.

b). Acidity tolerance study: Selected rhizobial isolates were inoculated separately on YEMA medium having adjusted pH 5.0, 5.5, 6.0 and 6.5. Four replications were maintained along with control. Observations for survival of *Rhizobium* were started after 48 hrs of incubation.

3.12 Glass house experiment for interaction study among root nodule bacteria, local/national checks of *Trichoderma* and wilt complex causing fungi of chickpea

Experiments conducted under controlled conditions for interaction study with root nodule bacteria, wilt complex causing fungi & *Trichoderma* sp. Six local isolates and four national strains of *Trichoderma* were compared in these experiments (Table:3.1). Vertisols of village Saddu containing wilt complex causing fungi was used as inoculum. Subsequently germination %, plant height, % wilt incidence, fresh weight, dry weight and nodulation were assessed.
Table 3.1: Treatment Details of the Glass House Experiment

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Treatments</th>
<th>Symbols</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Soil inoculum**</td>
<td>T1</td>
</tr>
<tr>
<td>2.</td>
<td>Soil inoculum + Rhizo.</td>
<td>T2</td>
</tr>
<tr>
<td>3.</td>
<td>Soil inoculum + Rhizo. + Tr 1</td>
<td>T3</td>
</tr>
<tr>
<td>4.</td>
<td>Soil inoculum + Rhizo. + Tr 2</td>
<td>T4</td>
</tr>
<tr>
<td>5.</td>
<td>Soil inoculum + Rhizo. + Tr 3</td>
<td>T5</td>
</tr>
<tr>
<td>6.</td>
<td>Soil inoculum + Rhizo. + Tr 4</td>
<td>T6</td>
</tr>
<tr>
<td>7.</td>
<td>Soil inoculum + Rhizo. + Tr 5</td>
<td>T7</td>
</tr>
<tr>
<td>8.</td>
<td>Soil inoculum + Rhizo. + Tr 6</td>
<td>T8</td>
</tr>
<tr>
<td>9.</td>
<td>Soil inoculum + Rhizo. + TrNC 1***</td>
<td>T9</td>
</tr>
<tr>
<td>10.</td>
<td>Soil inoculum + Rhizo. + TrNC 2</td>
<td>T10</td>
</tr>
<tr>
<td>11.</td>
<td>Soil inoculum + Rhizo. + TrNC 3</td>
<td>T11</td>
</tr>
<tr>
<td>12.</td>
<td>Soil inoculum + Rhizo. + TrNC 4</td>
<td>T12</td>
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</tbody>
</table>

12 treatments with 6 replications and soils of village: saddu was taken as inocula under glass house expt.

***NC 1: *T. viride* (IIPR,Kanpur), NC 2: *T. viride* (IARI,N.Delhi), NC 3: *T. harzianum* (Parbhani) & NC 4: *T. viride* (TNAU,Coimbatore)

**Soil of Saddu used as inoculum.
Three surface sterilized seeds were sown in each pot on date 6.1.2002.

* Rhizo. indicates *Rhizobium* of gram

3.13 Evaluation of different growth media for biomass production of isolates of *Trichoderma*

Four broth media i.e. Potato Dextrose, Czapek's Dox, Richard's Broth and Rose Bengal were tested for growth and sporulation of five selected *Trichoderma*
viride isolates. 50 ml of each liquid medium was sterilized in 150 ml flask separately. Autoclaved flasks were inoculated with 5 mm mycelial bits of all the selected Trichoderma viride isolates separately and incubated at 27 + 1°C for 15 days. Mycelial dry weight was taken for all the isolates in all broth media. Sporulation was measured by Double Neubauer ruling Haemocytometer. Haemocytometer was cleaned in ethyl alcohol and dried. A drop of well suspended spore suspension was placed in the grid of haemocytometer. Cover glass was placed properly and preparation was allowed to stand for 2 minutes before counting so that spores settle on the bottom of the square. Spores were counted in the four corner squares and the centre square. Total number of spores were counted in all five squares and calculated as per the method by Baudoin (1990) as

\[
\text{No. of spores ml}^{-1} = \text{Number counted} \times 50 \times 1000
\]

3.14 Performance of Trichoderma and Rhizobium under natural field conditions at Village: Amaseoni and Saddu (Raipur)

3.14.1 Field experiment at Village - Amaseoni: Field experiment was conducted to evaluate different isolates of Trichoderma viride along with Rhizobium against wilt complex fungi during winter season, 2002-2003. Sowing was done on date 9th December, 2002. The details of the experiment are as:

**Experimental Details: Amaseoni**

- **Name of Farmer**: Shri Naresh Sahu
- **Plot size**: 70 sq. meter
- **Total area (approx)**: 2400 sq. meter
- **Variety**: JG-74
Dose: N:P:K: 20:50:30
Seed: Broadcast @ 100 Kg/ha
Fungicide (Bavistin): 2.5 gms /Kg Seed
Date of sowing: 9-12-2002
Date of harvesting: 28-03-2003

The experiment was conducted in vertisol. Four selected local isolates of *Trichoderma* were compared with three national checks. Uniform basal dose of NPK @ 20:50:30 kg ha\(^{-1}\) was added to all treatments except control plots. Healthy gram seeds cv. JG -74 were first treated with Bavistin (0.25%) followed by inoculation with promising local *Rhizobium* isolate of chickpea and respective inoculants except T1 (absolute control) and T2 (only basal dose of N:P:K).

Standard methodologies were followed for analyses. The observations on plant height at 30, 45, 60 and 75 DAS, plant population at 30 DAS, nodulation at 45 DAS, wilted plants per plot at 60 DAS and dry weight g/plant at 45, 60 DAS were recorded. At harvest, grain and straw yield (q/ha) were also recorded. The final observations were statistically analyzed.

Wilted/root rot infected plants were collected from village Amaseoni and examined for associated fungi under laboratory conditions. Diseased portion of the plant was washed with tap water. Small pieces of infected portion adjoining the healthy area was cut with the help of sterilized blade. Ten cut bits of an infected plants were placed on PDA plates with the help of forceps 1-2 cm apart. The Petri plates were incubated at 28°C in BOD incubator. These Petri plates were examined after 2-4 days of incubation depending upon the growth of fungus appearing from the infected plant bits/pieces. The percent incidence of each of these pathogens i.e. *Fusarium, Sclerotium* and *Rhizoctonia* was determined from their frequencies.
Table: 3.2 Treatment details of experiments conducted at Village Amaseoni and Saddu during winter 2002-2003 and 2003-2004

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Treatments</th>
<th>Symbols</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td>Absolute control</td>
<td>T₁</td>
</tr>
<tr>
<td>2.</td>
<td>N : P : K (20 : 50 : 30)</td>
<td>T₂</td>
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<tr>
<td>3.</td>
<td>N : P : K + Rhizobia Gram 119 (5 g/kg seed)</td>
<td>T₃</td>
</tr>
<tr>
<td>4.</td>
<td>T₃ + Trichoderma Local isolate No. 1 (5 g/kg seed)</td>
<td>T₄</td>
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<tr>
<td>5.</td>
<td>T₃ + Trichoderma Local isolate No. 2</td>
<td>T₅</td>
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<tr>
<td>6.</td>
<td>T₃ + Trichoderma Local isolate No. 5</td>
<td>T₆</td>
</tr>
<tr>
<td>7.</td>
<td>T₃ + Trichoderma Local isolate No. 6</td>
<td>T₇</td>
</tr>
<tr>
<td>8.</td>
<td>T₃ + Trichoderma viride (TNAU)</td>
<td>T₈</td>
</tr>
<tr>
<td>9.</td>
<td>T₃ + Trichoderma viride (Kanpur)</td>
<td>T₉</td>
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<tr>
<td>10.</td>
<td>T₃ + Trichoderma viride (IARI)</td>
<td>T₁₀</td>
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3.14.2 Field experiment at Village – Saddu: In the next winter season, 2003-2004, field experiment was conducted at village saddu with same treatments as in the previous year (Table :3.2 ). Sowing was done on date 29th November, 2003. The details of the experiment are as:

**Experimental Details: Saddu**

Name of Farmer : Shri Narayan Sen

Plot size : 12 x 4.8 sq. meter = 57.6 sq.m.

Total area (approx.) : 1900 sq. meter

Variety : JG-74

Dose : N:P:K::20:50:30
Seed : Broad Cast @ 100 Kg/ha
Fungicide (Bavistin) : 2.5 gms /Kg Seed
Date of sowing : 29-11-2003
Date of harvesting : 25-03-2004

Standard methodologies were followed for analyses. The observations and recording of data was similar as followed in winter experiment 2002-2003 at village Amaseoni.

3.15 Source of material:

Whenever required, the glassware’s and chemicals of standard trade mark were used during the course of investigation. The following instruments/materials used in present studies were:

1. Autoclave for sterilization
2. BOD incubator for incubation
3. Compound microscope/ Phase Contrast Microscope
4. Hot air oven for glassware sterilization
5. Forceps, needles, blade, cork borer, inoculation needle
6. Growth chamber
7. Laminar flow for isolation
8. Spirit lamp
9. Water bath
10. Electronic Digital Balance

Cleaning and sterilization of materials:

Prior to use, all the glassware’s were cleaned with detergent powder and finally washed with tap water and or distilled water. The dried glassware’s were
sterilized in hot air oven at $180^\circ$C for two hours. The forceps and other metallic instruments were sterilized by dipping them in alcohol and heating over the flame before use. Sterilization of the media was done by autoclaving at 1.02 kg/cm$^2$ pressure for 20 minutes. The plastic plates were sterilized with alcohol and air dried before use.

3.16 Statistical Analysis

Spade programme was used to carry out statistical analysis i.e. C.D., Standard Error Mean etc.

Randomized Block Design was used when experiment conducted in field, while Completely Randomized Design was used when performed in laboratory.