CHAPTER 4

MATERIALS AND METHODS

4.1 MATERIALS

All the chemicals and media used for laboratory studies were analytical grade.

4.1.1 Solid substrates

Solid substrates like pearl millet, maize, cow pea, rice bran and wheat bran were purchased from local market at Chengalpattu and press mud and bagasse were collected from Thiruthani Co-operative Sugar Mill.

4.1.2 Byproducts of sugar industry

The main byproducts of sugar industry are (i) bagasse (ii) press mud (iii) molasses

4.1.2.1 Bagasse

The fibrous residues of the cane stalk after crushing and extraction of the juice consists of water, bagasse and relatively small quantities of soluble solids.

4.1.2.2 Press mud

The impurities of the cane juice are precipitated either through sulphatation or carbonation process. The amount of the filtermud present in cane and
its composition vary with the locality, variety of cane, milling efficiency method of clarification etc. It is a soft, spongy, amorphous, dark brown material containing sugar, fibre and coagulated colloids. It is such source of organic carbon and contains a good proportion of nitrogen, phosphorus, calcium, iron and manganese.

4.1.2.3 Experimental sites

The study sites were selected based on the increasing infestation of white grubs which was observed by Chief Cane Officers, Cane Assistant and cane growers of Dharamapuri, Subramaniya Siva, Vellore and Salem Cooperative Sugar mills of Tamil Nadu, India. The experiments were conducted at four different sites. The first site was located in Thimiri, Vellore Co-operative Sugar mill. The second site was located in Palacode, Dharmapuri Co-operative Sugar mill. Third site was located in Rasipuram, Salem Co-operative Sugar mill and the fourth site was located in Subramaniya Siva Co-operative Sugar mill in Tamil Nadu. All the sites were located in farming areas.

4.1.3 Collection of host insects

Uniform sized third instar larvae (Head capsule of width 7.8 mm) of *H. serrata* F (Scarabidae: Coleoptera) were collected from Salem, Dharmapuri, Vellore and Bannari Amman Sugar mills and reared in plastic boxes (30x20x10cm) containing moistened soil with sugarcane root bits. These grubs were used for laboratory bioassay in assessing the virulence of the isolates of the *M. anisopliae*.

4.1.4 Source of fungal culture

*B. bassiana* was isolated from the mycosed cadaver of the pathogen collected from Department of pathology, Tamil Nadu Agricultural University, Coimbatore. *B. brongniarti* and *M. anisopliae* used for the laboratory bioassay during 2008, 2009 collected from PDBC, Bangalore. During 2010, 26 isolates were isolated form six different locations of Tamil Nadu. Among the 26 isolates, six strains were identified as *M. anisopliae*. 
4.2 METHODS

4.2.1 Isolation of *B. bassiana* from mycosed cadaver of the insect

*B. bassiana* infected specimen of insect was collected from Dr Manimegalai, Associate Professor, Department of plant pathology, Tamil Nadu Agricultural University, Coimbatore. A part of the insect cadavers that showed signs of fungal growth was touched with a sterile needle and small amount of fungus material surface sterilized with 70 per cent ethanol and washed with three changes of sterile distilled water and then transferred into sterile Petri dishes with Sabourauds dextrose agar medium. The plates were incubated for 7-10 days.

White cottony colonies identified as *Beauveria bassiana* and were transferred to Potato dextrose agar slants.

4.2.2 Isolation of entomogenous fungi from soil by insect baiting method

Soil samples were collected at 20 cm depth in triplicate from six locations of agricultural lands. Samples were homogenized and sieved in 1mm sieve. 40 g of 1mm sieved soil sample taken in 90 mm petriplates and 10 *Galleria melonella* larvae were buried in the soil. Petri plates were sealed with para film and incubated in dark for 15 days. After 15 days larvae were recovered from the soil, surface sterilized with 1 per cent sodium hypochlorite and later the sterilizing agent was removed by washing with three changes of sterile distilled water. Larvae were placed in Sabourauds dextrose agar medium. Plates were incubated for 10 days at 25± 2º C Colonies of *Metarhizium sp* were identified and transferred to SDA slants.

In order to maintain the virulence of the fungi, they were inoculated in their respective hosts once a year, reisolated from the host insects, and brought into pure culture.
4.2.3 Maintenance and multiplication of fungal culture

The cultures of *B. bassiana* *B. brongniarti* and *M. anisopliae* were maintained by periodic sub-culturing in slants / Petri dish in SDA.

4.2.4 Identification of *M. anisopliae*

The colony of *M. anisopliae* appeared white when young, but as the conidia mature, the color turned to dark green. The conidiophores were branched, and the initial conidium’s were produced by simple abstrictions at the distal end of the conidiophores. A chain of conidia was formed on each conidiophore with the youngest conidia being adjacent to the conidiophores. The mass of spore chains became so dense and coheres with each other to produce prismatic masses of columns of spore chains. Other strains of *M. anisopliae* formed different colored colonies.

4.2.5 Molecular characterization of *M. anisopliae*

4.2.5.1 DNA extraction

Isolates were grown in SDAY/4 broth 5-7d on in orbital shaker set at 125 rpm and 25°C. The tissue was removed from the broth rinsed twice with sterile water, filter dried, frozen at 80°C and lyophilized. Approximately 50 mg of lyophilized mycelium was ground into powder with the fast prep tissue homogenizer (MP biomedical, Irvine California). The pulverized tissue was lysed with 900 µL of lysis buffer (2m Nacl, 0.4% w/v deoxycholic acid, 1.0% w/v polyoxyethylene ether) and incubated 10 min at 55°C. Cellular byproducts were extracted with 750 µL of chloroform: isoamylalcohol (24:1) and centrifuged to separate the aqueous and particulate phases. The 700 µL of the cleared solution containing DNA was removed, placed in a clean tube and mixed with equal portion of 6M guanidium isothiocyanate. DNA was bound to 40 µL of equal volumes of diatomaceous earth
and flint glass powder. The bound DNA was resuspended twice in ethanol (75%), dried and eluted in sterile distilled water incubated 5min at 55°C [404].

4.2.6  PCR amplification and nucleotide sequencing

Partial sequence of three nuclear protein coding genes were amplified and sequenced for their study. They include the 5´intron-rich region of the translation factor 1-alpha (EF-1α intron region). The primers used for amplification and sequencing was 5´ef-1 α intronregion: EF1T (ATGGGTAAAGGARGACAAGAC) and EF2T (5´-GGAAGTACCAGTGATCATGTT).

4.2.7  Sequence alignment and phylogenetic analysis

The partial 5´ tef sequence of isolates M. anisopliae has been deposited in the GenBank database. The retrieved gene sequences were compared with other fungal sequences by using NCBI BLAST search for their pair wise identities, further, the phylogenetic tree was constructed with MEGA 4.0 software (http:www.megasoftware.net) by using the UPGMA neighbor-joining (NJ) method with 1000 replicates as bootstrap value and NJ belongs to the Distance – Matrix Method (DMM).

4.2.8  Studies on the growth characteristics of six isolates of M. anisopliae

4.2.8.1  Suitability of different solid substrate media for the conidia production growth of six isolates of M. anisopliae.

Seven different solid substrates namely pearl millet, maize, cowpea, rice bran, wheat bran, press mud + yeast extract (1%), and bagasse + yeast extract (1%) were tested for the sporulation and conidia production by M. anisopliae. 100 g of each of substrate except rice bran, wheat bran press mud and bagassse were soaked in sterile distilled water for 1- 2 h in 500 ml sterilizable bottles and washed
with fresh water. Rice bran, wheat bran, press mud and bagasse were moistened with sterile distilled water. All the substrates were sterilized in autoclave at 121°C at 15 lb pressure for 30 min. After cooling each of substrate was inoculated with 1x10^6 spores of individual isolates of *M. anisopliae*.

For assessing the conidial count homogenous conidial suspension was prepared by adding Tween 80 (0.02 %) as wetting agent to get uniform spore suspension. Spores were further extracted by passing through muslin cloth. The filtrate was diluted and final volume was made upto 100 ml by the addition of sterile distilled water. After serial dilution, spore count was recorded using double ruled Neubauer haemocytometer. The number of conidia produced per g of the solid substrate was calculated using the formula:

\[
x / 40 \times 400 \times 10 \times 1000 \times \text{dilution factor}
\]

Where \( x \) = mean number of conidia observed per 40 square. The conidia produced per g of medium were calculated for 15th days after inoculation. Each treatment was replicated three times.

### 4.2.8.2 Effect of different solid substrate on the radial growth of six isolates of *M. anisopliae*

Seven different solid substrates namely pearl millet, maize, cow pea, rice bran, wheat bran, press mud + yeast extract (1%) and bagasse + yeast extract (1%) were tested for the radial growth of *M. anisopliae*. 100 g of each of substrate except rice bran, wheat bran, press mud and bagasse were soaked in sterile distilled water for 1-2 h in 500 ml sterilisable glass containers and washed with fresh water. Rice bran, wheat bran, press mud and bagasse were moistened with sterile distilled water. All the substrates were cooked and filtrate was taken. Individual solid substrate agar media were prepared, sterilized in autoclave at 121°C at 15 lb pressure for 30 min. After cooling each of substrate was inoculated with 5 mm cut disc of the individual isolates of *M. anisopliae*. Each treatment was replicated three times.
4.2.8.3 **Effect of different solid substrate on the biomass yield of six isolates of *M. anisopliae* strains**

Seven different solid substrates namely pearl millet, maize, cow pea, rice bran, wheat bran, press mud + yeast extract (1%) and bagasse + yeast extract (1%) were tested for the biomass yield of *M. anisopliae*. 100 g of each of substrate except rice bran, wheat bran press mud and bagassse were soaked in sterile distilled water for 1-2 h in 500 ml sterilisable glass containers and washed with fresh water. Rice bran, wheat bran, press mud and bagasse were moistened with sterile distilled water. All the substrates were cooked and filtrate taken. Individual solid substrate broth media prepared, sterilized in autoclave at 121°C at 15 lb pressure for 30 min. After cooling each of substrate was inoculated with 1x10^6 conidia of the individual isolates of *M. anisopliae*. Each treatment was replicated three times.

4.2.9 **Determination of protein content in six *M. anisopliae* isolates**

Protein was estimated according to Bradford method (1976) using crystalline bovine serum albumin as standard.

4.2.10 **Determination of chitinase activity in six *M. anisopliae* isolates**

Briefly 1x10^8 ml^-1 of culture filtrate of conidia of each species was incubated in 1 ml of 0.7 per cent acid swollen chitin and 1 ml of 50 mM acetate buffer pH 5.0. and incubated at 50°C for 1 h. The reaction was terminated by adding 0.1 ml of 0.08 M potassium tetra borate, pH 9.2 to 0.5 ml of reaction mixture and then boiled in water bath for 3 min. Then 3 ml of diluted *p*-diethyl amino benzaldehyde (*p*-DMAB, Sigma Chemicals, USA) was added and incubated at 37°C for 15 min. The GlcNAc produced was estimated at 585 nm in a Spectrophotometer (Hitachi, Japan). Buffered substrate was used as control (Reisig et al., 1994).
4.2.11 Effect of environmental factors on growth of efficient isolates of *M. anisopliae* JQ013738 and JQ013739

4.2.11.1 Effect of temperature on spore production

The sterilized wheat bran were inoculated with $1 \times 10^8$ conidia of efficient strains JQ013738 and JQ013739 *M. anisopliae* separately and incubated at 15, 20, 25, 30, 35 and 40°C in B.O.D. incubator. The conidia produced per g of the substrate were calculated 15 days after incubation. Each treatment was replicated four times and experiment was conducted in CRD.

4.2.11.2 Effect of relative humidity on growth of the fungus

Five different relative humidity levels viz 60, 70, 80, 90, 100 per cent were maintained. The conidia produced per g of the solid substrate were calculated 15 days after incubation. Each treatment was replicated four times.

4.2.11.3 Effect of pH on spore production of the fungus

The influence of various pH levels on spore production of the solid substrate was studied by adjusting the pH of solid substrate to 4, 5, 6, 7, 8 and 9 with 1 N sulphuric acid or 1 N sodium hydroxide, inoculated with $1 \times 10^8$ conidia of the fungus, JQ013738 and JQ013739 *M. anisopliae* and incubated for 15 days. The conidia produced per g solid substrate were calculated 15 days after incubation. Each treatment was replicated three times and experiment was conducted in CRD.
4.12 LABORATORY BIOASSAY STUDIES

Laboratory bioassay was conducted during August 2008, 2009 and 2010.

4.12.1 Laboratory bioassay of three entomopathogenic fungi against *H. serrata* (2008)

The third instar larvae of *H. serrata* were collected from infested sugarcane fields from Vellore Cooperative Sugar mill, Ammundi, Tamil Nadu. Third instar grubs, which were placed in moist soil in individual plastic boxes, and thirty larvae taken for each treatment, treated with 30 ml fungal conidial suspension of *B. bassiana, B. brongniarti* and *M. anisopliae* at $3 \times 10^9$ concentrations separately in Petri plates, transferred to individual plastic boxes, and the remaining suspension dispensed in each box. Control larvae treated with sterile distilled water with Tween (0.01%) alone. The grubs were fed with sugarcane roots. The grubs were examined for mortality on 5th, 10th and 15th day after treatment.

The dead grubs were collected and placed in moist filter paper in Petri plates and observed for fungal growth. Mortality due to fungal infection was determined by microscopic examination. The mortality due to mycosis was recorded from 5th day onwards and continued up to 15 days and the cumulative mortality data were subjected to probit analysis.

4.12.2 Laboratory bioassay of three doses of *M. anisopliae* fungi against *H. serrata* (2009)

Third instar grubs, which were placed in moist soil in individual plastic boxes, and thirty larvae taken for each treatment, treated with 30 ml fungal conidial suspension at different concentrations ($3 \times 10^6, 3 \times 10^8, \text{ and } 3 \times 10^9$) separately in petri plates, transferred to individual plastic boxes, and remaining suspension dispensed in each box. Control larvae treated with sterile distilled water with Tween (0.01 %) alone. The grubs were fed with sugarcane roots. The grubs were examined daily for
mortality from 5\textsuperscript{th} day onwards up to 20 days. The dead grubs were collected and placed in moist filter paper in petri plates and observed for fungal growth. Mortality due to fungal infection was determined by microscopic examination. The mortality due to mycosis was recorded from 5\textsuperscript{th} day onwards and continued up to 15 days and cumulative mortality data were subjected to probit analysis.

4.12.3 Preparation of fungal inoculums for laboratory and field studies during 2010

For laboratory and field studies, conidia were produced in the solid substrate wheat bran. Conidia were produced in bags filled with 500 g wheat bran and 100 ml distilled water. The wheat bran filled bags were first autoclaved for 30 min (121°C). Thereafter, 10 ml of a spore suspension containing $1 \times 10^8$ conidia ml\(^{-1}\) were added under sterile conditions to each bag. Bags were kept for three weeks under dark conditions in an incubator at 25 ± 2°C. Spores were air dried for three days before harvesting through sieve. Then spores were formulated in either an oil based formulation or an aqueous conidial suspension to compare their efficacy on white grub mortality.

4.12.4 Efficacy of the six isolates against \textit{H. serrata} in the laboratory bioassay

For each of the isolates the following treatments were tested with three replicates.

T1: Oil formulation of the isolate JQ013738

T2: Oil formulation of the isolate JQ013739

T3: Oil formulation of the isolate JQ013740
T4: Oil formulation of the isolate isolates JQ013741

T5: Oil formulation of the isolate isolates JQ013742

T6: Oil formulation of the isolate isolates JQ031714

T7: Conidial suspension of the isolate JQ013738

T8: Conidial suspension of the isolate JQ013739

T9: Conidial suspension of the isolate JQ013740

T10: Conidial suspension of the isolate JQ013741

T11: Conidial suspension of the isolate JQ013742

T12: Conidial suspension of the isolate JQ031714.

T13: Water control (with 0.01% Tween 80)

T14: Oil control (with 0.01% Tween 80)

Two different controls consisting of sterile distilled water and Tween 80 mix and the sterile oil mix were also prepared. Third instar grubs were immersed in the different treatments for 1 min, before being blotted on paper towel. For each treatment 30 third instar grubs were treated and three replicates were maintained. The grubs after treatment were transferred to plastic boxes and observed for mortality on 5th and 10th day after treatment. The dead grubs were examined daily from 3rd day onwards and dead individuals transferred to clean petri plates lined with moist filter paper. All Petri dishes were kept in an incubator at 25± 2°C. The percent mortality due to mycosis was evaluated in each treatment and compared. The total number of grub mortality due to fungus over a period of 14 days period was recorded.
4.13 FIELD TRIALS

Field experiments were conducted during September 2008, 2009 and 2010.

4.13.1 Field efficacy of three entomopathogenic fungi under field conditions (2008)

Field trials were conducted to test the efficacy of the talc based formulations of three entomopathogenic fungal formulation against sugarcane white grub in Dharmapuri Cooperative Sugar mill, Tamil Nadu. The trials were conducted at Randomized Block Design with five treatments and five replications. Pretreatment count was taken in 1m$^2$ area. The total number of grubs ranged from 10-13. One kg talc based formulation with spore count of $7.8 \times 10^9$ per g was mixed with 50 kg well decomposed Farm Yard Manure and applied near the root zone of the infected field at 10-15 cm depth. The field was irrigated immediately after application. Mortality count taken after 15th and 20th days after application. The data were subjected to analysis of variance.

4.13.2 Field efficacy of different doses of fungi *M. anisopliae* (2009)

Three doses of *M. anisopliae* $8 \times 10^6$, $8 \times 10^8$, $8 \times 10^9$ spores per g were used in field trials in Randomized Block Design with four treatment and five replications. The field trials were laid out in Vellore Cooperative Sugar mills, Tamil Nadu. Pretreatment count was taken in 1m$^2$ area. The total number of grubs ranged from 10-12. The different treatments of the formulation mixed well with 50 kg well-decomposed farmyard manure, applied near the root zone of the cane at 10-15 cm depth, and irrigated immediately after application. A total two numbers of applications were made at 15 days interval. Mortality count was taken after 15 days after each application. The data were subjected to analysis of variance.
4.13.3 Field efficacy of conidial Suspension and oil formulation of six isolates of *M. anisopliae* (2010)

White grub infested sugarcane field in Vilapkkam village in Vellore Co-operative Sugar Mills, Tamil Nadu, India was selected as the test site for the field experiments with *M. anisopliae* where in the grubs were found to occur at a density of 15-20 grubs per m². The oil formulations and aqueous conidial suspensions were applied at $3 \times 10^{12}$ conidia per ha in 5 cm wide by 10 cm deep furrow. The raised soil beside the furrow was pushed back to cover the formulation or aqueous suspension followed by irrigation. Control plots were treated with sterile distilled water with Tween 80 (0.01%) or oil with Tween 80 (0.01%). Larval mortality was determined weekly for two weeks. The treatments were randomized and five true replicates were maintained. On 5th and 10th day after treatment, the grub population per square meter was assessed and mortality was recorded. The per cent mortality due to *M. anisopliae* was calculated for each treatment and compared.

4.13.4 Field efficacy of different formulation of efficient isolates JQ013738 and JQ013739 fungi *M. anisopliae*

Four different formulations of *M. anisopliae* i.e. talc, lignite, press mud and liquid formulations were used during the trials laid in Rasipuram village at Salem cooperative sugar mill during 2010. Field trials were conducted with five treatments and five replications in Randomized Block Design. Pretreatment count was taken in 1 m² area. The total number of grubs ranged from 10-12. The different treatments of the formulation i.e. talc, lignite and press mud mixed well with 50 kg well decomposed Farmyard manure and applied near the root zone of the cane at 10-15 cm depth and irrigated immediately after application. Mortality count was taken after 15 days after application. The data were subjected to analysis of variance.
4.13.5 **Assessment of sugarcane yield and quality parameters**

Sugarcane stalks were cut at ground level and cane weight was assessed. The cane stalks were cut at ground level at the time of harvest, cleaned and crushed immediately in a 3-roller power crusher giving about 60 per cent juice extraction. The juice was then strained through muslin cloth to remove suspended impurities and then used for further analysis. Brix was recorded in brix hydrometer and the correction was applied using the correction table depending on the juice temperature. The sucrose level of the cane juice was calculated following standard procedures of Meade and Chen (1985).

4.13.6 **Statistical Analysis**

Statistical analysis was carried out using SPSS 17.0 software (SPSS Inc. Chicago, IL, USA) and STATA 9.0 (College Stations, Texas, USA). Data were summarized as mean ± standard deviation (SD) and mean ± standard error of the mean (SEM). Turkeys test carried out for multiple comparisons of data. Corrected percent mortality was done using Abbot (1925). In the field experiment, mortality was corrected for the average number of larvae recovered from the untreated check. Data on the per cent mortality were subjected to analysis of variance (ANOVA).