MATERIALS AND METHODS

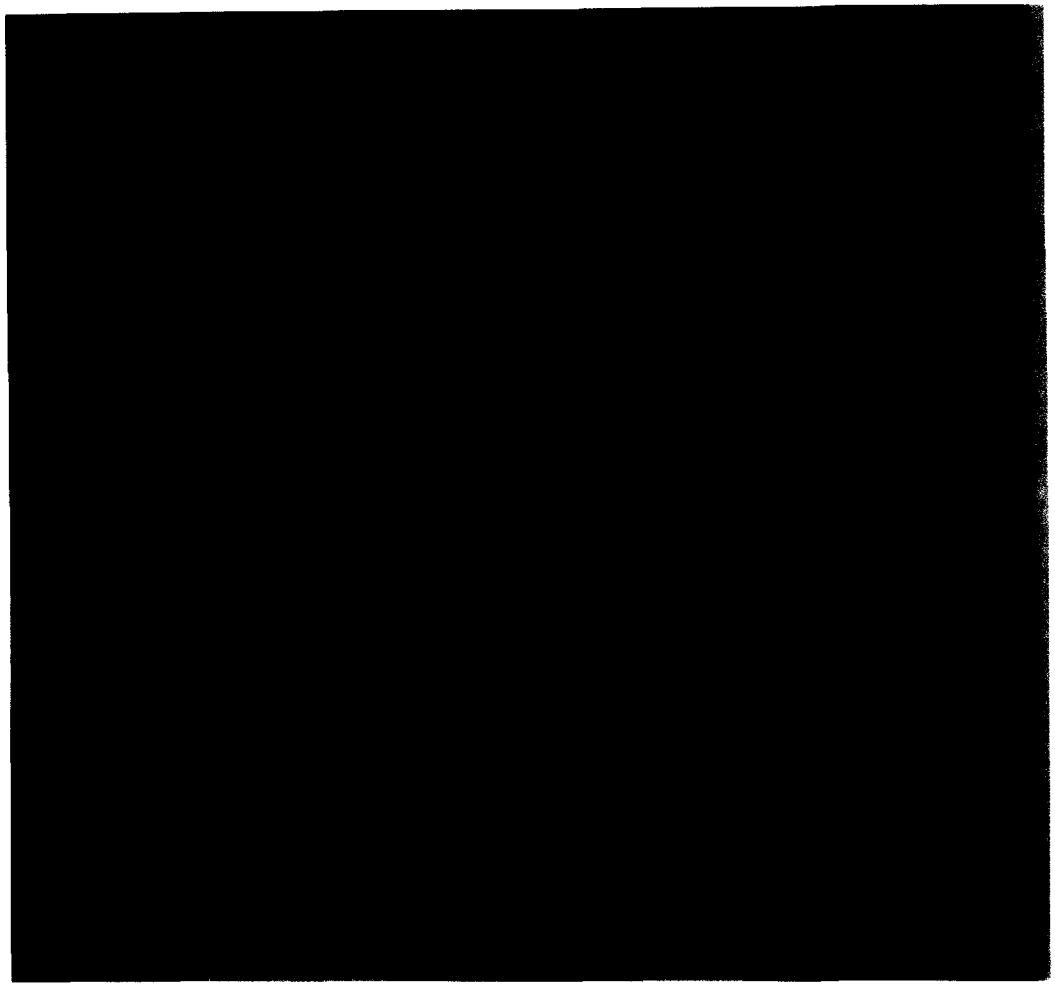

Plate 2 : *Odontotermes obesus* collected from the infested teak plant.

3.1 Source of Fungal cultures

Three isolates of *Beauveria bassiana*, designated as MTCC-984, MTCC-2028 and NCIM-1216 (Table-1) were originally isolated from infected *Ips typographus* (Bark Beetle) collected in India and one isolate of *B. bassiana*, designated as BBFF-135, was originally isolated from infected *Ips typographus* collected in Germany. One isolate of *Metarhizium anisopliae* (MTCC-892) and another isolate of (BBFF-140) were originally isolated from infected *Ips typographus*. The two isolates of *M. anisopliae* were collected in India and Germany respectively.

Fungi were grown in Yeast extract glucose agar (YGA) and Potato dextrose agar (PDA) media. These were incubated for 14-15d at 28-30°C to allow complete sporulation. All fungal cultures were maintained at room temperature (25-28°C) under weak fluorescent illumination (8 h/day). Every 3-4 weeks, cultures were subcultured by even distrubution of a conidial suspension over the surface of fresh plates.

Table 1 : Origin of Entomopathogenic fungal isolates.

Entomopathogenic fungal isolates	Strain No.	Host	Source
Metarhizium anisopliae (Metsch) Sorokin	BBFF-140	Bark Beetle, *Ips typographus*	Biologische Bundesanstalt Fur Landund forstwirtschaft Institut Fur biologischen ptlanzenschutz Darmstadt, Germany.
	MTCC-892	Bark Beetle, *Ips typographus*	Instituteof Microbial Technology, Chandigarh India.
Beauveria bassiana (Bals.) Vuill.	MTCC-984	Bark Beetle, *Ips typographus*	Institute of Microbial Technology, Chandigarh India.
	MTCC-202	Bark Beetle, *Ips typographus*	Institute of Microbial Technology, Chandigarh India.
	NCIM-1216	Bark Beetle, *Ips typographus*	National collection of Industrial Microorganisms. Biochemical SciencesDivision. National Chemical Laboratory. Pune. India
	BBFF-135	Bark Beetle, *Ips typographus*	Biologische Bundesanstalt Fur Land-und forstwirtschaft Institut Fur biologischen ptlanzenschutz Darmstadt,Germany.

3.2 Compatibility Tests for different fungal isolates.

In order to formulate a mycoinsectiside containing different isolates of entomogenous fungus, it is mandatory to find out compatibility between and among the chosen isolates. For this purpose all the isolates of *B. bassiana* and *M. anisopliae* were grown separately in petriplates containing Yeast Glucose Agar, media (Yeast extract - 5g, Glucose -10g, Agar 15g per liter distilled water). The cultures were allowed to grow for 20 days. Fresh YGA media was prepared and autoclaved at 15psi pressure for 15 minutes at 121°C. After sterilization, 15ml media was poured in each sterile petridishes, under laminar flow and allowed to cool down for solidification. From 20days old cultures, agar blocks were taken out with the help of sterile corkborer and placed asceptically on the freshly prepared YGA media on petri plates. Agar blocks of two separate isolates were placed at opposite ends on the media surface. Thus different combinations of isolates were undertaken to study their compatibility. For each test three replicates were kept.All the replicates were incubated for 10 days at 28 $\pm$ 2°C. After 10 days. observations on their growth were recorded on the basis of a grade system such as poor (+), medium (++), good (+++) and excellent (++++).

3.3 Preparation and standardization of spore suspensions.

The isolates of *B. bassiana* and *M. anisopliae* were allowed to grow and sporulate on yeast extract glucose agar (YGA) medium. Cultures were incubated at 28-30°C for 28 days. The spores were scraped off from the agar surface and suspended in 0.05% Tween 80 solution.

The spore suspension was then collected in a sterile test tube and shaken for two minutes to homogenize the spores. The suspension was filtered through a double layered mesh serially diluted and its conidial count determined microscopically by means of Neubauer haemocytometer.

3.4 Counting conidia by Light Transmission Measurements

Four isolate each of *B. bassiana* and two isolates of *M. anisopliae* were used for haemocytometer counts and transmission measurements from 28-day-old cultures. The experiment was repeated for 12 months after the initial counts and measurements. These dates will be referred to in the designation of the replications (0-6, 0-12, 6-12, 0-6, 0-12, 6-12 respectively for *M.anisopliae* and *B.bassiana*. The conidia were washed from petriplate in 0.05% Tween 80 solution. This suspension harvested from plates (referred to as suspension 0) was vigorously shaken and sieved (mesh diameter 0.2 mm). Serial dilutions (suspension 1 through 10) were prepared from this stock

suspension. The concentrations were halved with each step of the series.

Haemocytometer and transmission measurements

1. preparations of serial dilutions; conidia concentration halved at each step.
2. haemocytometer measurements (conidia/ml) on one dilution of each series ; the dilution with about 5 conidia per smallest square in the haemocytometer was chosen
3. Calculation of mean value (A) of conidia/ml per fungus per treatment
4. transmission measurements (%) on all serial dilutions
5. relation of numbers of conidia/ml (haemocytometer counts) with transmission (%) of the one dilution.
6. combining (1), (3), and (4) gives transmission and conidia values (conidia/ml%) for other dilutions

Evaluation

7. Calculation of ln-values (ln-A) for numbers of conidia/ml ; preparation of curves.
8. selection of 5 points per curve, nearest to 50% transmission and calculation of a regression line through the 5 points
9. transformation of each original haemocytometer value to a new ln-value on the 50% transmission line
10. calculation of actual numbers of conidia/ml from ln-values (from step 9)
11. comparison of 3 lines per fungus by comparison (t-test) of the transformed haemocytometer values at 50% transmission
12. preparation of one regression line per fungus based on 15 points of 3 line.

3.4.1 Haemocytometer counts

Conidia were counted under the microscope (40x) in a haemocytometer cell with an improved Neugebauer ruling. The suspension of the series nearest to 5 conidia per smallest square of the grid (0.0025 mm^2) was selected and counted (step 2). This relatively low number was selected because counts were more variable at higher concentrations where more conidia were found on the boundaries between cells. For the observation, 270 blocks of 16 smallest squares (0.04 mm^2), and 90 blocks for the 2nd and 3rd observation were counted. With this data, the numbers of conidia in suspensions (0) and their standard deviations were calculated (step 3).

3.4.2 Turbidity measurements

The turbidities of all suspensions of the series were determined in a Erma AE-IIM colorimeter using the cuvets (Step 4). A Tween 80 solution (0.05%) was used as a standard. A wavelength of 620 nm was chosen for all measurements because at longer wavelengths (500-600 nm) variation is minimized. After placing a sample of solution in a cuvet of the colorimeter, the transmission value was recorded after 10-15 seconds.

3.4.3 Evaluation

The numbers of conidia/ml, calculated from the haemocytometer counts can be related to a transmission value (step 5). Other transmission values were measured on the other suspensions of the serial dilutions (step 6). The actual number of conidia (A) was transformed to x=lnA because our series of suspensions were prepared with a constant dilution factor (step 7). The ln of the mean value of haemocytometer counts determined the placement of each curve on the x-axis.

A simple mathematical relationship to straighten the transmission curves was not found ; non-linear deviations persisted either at the toe or at the shoulder of the curves. This asymmetry in the curves is mainly due to increased levels of secondary and tertiary scattering of light at increasing concentrations of particles - thus the number of conidia in dense concentrations are consistently underestimated. Therefore, only the more symmetrical and relatively linear central parts of the curves were used for analysis.

Of each line, the 5 points nearest to 50% transmission were used and regression lines calculated (step 8). In the measurements, the only source of variation was the haemocytometer counts ; variations in transmission measurements in the spectrophotometer were negligible.

Dilutions with about 5 conidia per smallest cell in the haemocytometer were counted. Between replicates these dilutions differed slightly and thus the mean haemocytometer counts could not be statistically compared. Therefore, all haemocytometer counts (lnA) were individually adjusted to numbers of conidia around a mean at Y=50% (step 9). This adjustment was made by individual transformation of all lnA values along the regression lines (step 9). Adjusted A values were calculated (step 10). Differences between these means (A) were tested on significance (t-test, with the level of significance at P=5% ; (step 11). Also, regression analysis was done on all 15 data points of each fungus (step 12). Confidence limits (95%) of the regression lines were calculated based on the standard deviations of the transformed haemocytometer counts.

3.5 Termite survey, collection and maintenance.

A survey had been conducted in Jalpaiguri district, India and the collected termite specimens were sent to the different agencies such as, Zoological Survey of India, Calcutta and Jodhpur, Forest Research Institute, Dehradun, and International Institute of Entomology. London, U.K., for identification (Table-2).

Termites were collected from termite infested trees (plate-2) with the help of a fine soft brush. They were maintained in the plastic tubs (40 cm diameter) containing soil amended with dried wooden sticks of *Glyricidia sepium, Bambusa sp* and dried leaves of guatemala grass, *Lagerstroemia lanceolata, Tectona grandis* and sawdust. The tubs containing termites were kept at 25-26°C and 80-90% moisture level. Acclimatised termite workers were taken for bioassays.

Table 2 : Survey of termite species in and around Jalpaiguri district.

Location	Termite species*	Host plant
Budhaganj forest	*Odontotermes obesus* *Odontotermes distans* *Odontotermes horni* *Odontotermes boveni*	Teak *(Tectona grandis)*, Sisoo*(Dalbergiasisoo)* Zarul *(Lagerstroemia lanceolata)*, Kum tree, Sidha,Baheri,Chap *(Michelia champaca)*, Chulli, Nagiza,Ghugura, Sal *(Shorea robusta)*, Tetul *(Tamarindus indica*. Odhara
Domohoni more	*Odontotermes obesus* *Odontotermes distans*	Sisoo *(Dalbergia sisoo)*, Sal *(Shorea robusta)*, Teak *(Tectona grandis)*. Gamari, Coke Sal
Lataguri forest	*Odontotermes obesus* *Odontotermes horni* *Odontotermes boveni*	Teak *(Tectona grandis)*, Sisoo *(Dalbergia sisoo)*, *Odontotermes distans* Zarul *(Lagerstroemia lanceolata)*,kum tree, Sidha, Baheri, Chap *(Michelia champaca)*, Chulli, Nagiza, Ghugura, Tetul, Sal *(Shorea robusta)*
Kadobari	*Odontotermes distans*	Coke Sal, Teak *(Tectona grandis)*. Zarul *(Lagerstroemia lanceolata)*, Sisoo *(Dalbergia sisoo)*, Mahogani *(Swietenia mahagoni)*
Danguajhar	*Odontotermes horni* *Odontotermes obesus*	Teak *(Tectona grandis)*, Sisoo *(Dalbergia Odontotermes distans sisoo)*,Gamari, Zarul*(Lagerstroemia lanceolata)*,Chap *(Michelia champaca)*, Kadam *(Anthocephalus indicus)*
Moriambasti	*Odontotermes distans*	*Eucalyptus* sp.. Sisoo *(Dalbergia sisoo)*, Teak *Odontotermes obesus (Tectonagrandis)*, Sal *(Shorea robusta)*. Akashmoni *(Acacia obegrandis)*, Sal *(Shorea robusta)*. Akashmoni *(Acacia moniliformis)*
Dewniapara	*Odontotermes obesus*	Zarul *(Lagerstroemia lanceolata)*. Bamboo *(Bambusa* sp.)

* family : Termitidae

3.6 Bioassay methods for contact/oral toxicity of entomogenous fungus.

3.6.1 To individual termite (Bioassay-I)

Termite workers of *O. obesus* were placed individually on cellulose filter paper disks (No. 1, Whatman paper) in the wells (3.5 x 3.5 x 3.5 cm) of a ice-cube preparation plate (26.5 x 10 x 3.5 cm). 0.5 ml of unformulated dilutions of spore suspension of isolates of *B. bassiana* (MTCC-2028, NCIM-1216, MTCC-984 and BBFF-135) and *M. anisoplia* (BBFF-140 and MTCC-892) at the concentrations of 10^4 - 10^7 conidia/ml was applied by pipet to the top of each well. 0.5ml water was applied to each controls.

Each test contained 20 termite workers per treatment (each individual in a separate well), the test was replicated five times. The well plates were incubated under constant conditions, temperature 25-27°C and ~80-95% RH. The termite mortality was recorded daily for seven days. The mortality data were analyzed by probit analysis of dosage mortality response (LC_{50}) and time mortality response (LT_{50}) after correcting for control mortality by Abbott's formula. Data were subjected to analysis of variance (ANOVA) and were compared between treatments based on critical difference at 0.05 probability level.

3.6.2 To group of termites (Bioassay-II)

In this test, a filter-paper disk (4cm diameter) was placed in a disposable cup (4.5 x 6.5 x 5.0 cm) and saturated with 1ml of unformulated dilutions of spore suspension of isolates of *B. bassiana* and *M. anisopliae* at the concentrations 10^4-10^7 conidia/ml. 1ml water was applied to each controls. A group of 20 termite workers of *O. obesus* was placed on the moist paper in the cup. Five replicates of each treatment were included in the test. The cups were incubated as previously described and termite mortality recorded as mentioned earlier. Mortality data were analyzed and reported as described above.

3.6.3 To groups of termites in soil application (Bioassay-III)

40g of acetone washed and autoclaved soil was taken and saturated with sterile water and placed in disposable cup (4.5 x 6.5 x 5.0 cm). 5g of saw dust was placed on top of the soil and each cup was treated with 1ml of unformulated dilutions of spore suspension of the respective fungal isolates. 1ml water was used for each control. A group of 20 termite workers was placed on the soil surface, and the cups were incubated as previously described. The test included 9 replicates of each treatment. Mortality data were recorded and analysed as described above.

3.7 Bioassays of Pathogenicity toward *Odontotermus obesus*

Two bioassays were conducted with each isolate of *B. bassiana* (135 and 984). In each bioassay workers of *O. obesus* were inoculated with each concentration of

fungal spores described below. Control termites were treated with water.

Groups of 30 termites were placed in plastic cups (2x2") containing 40g soil and 5g saw dust. Spore suspension of 1 ml was used for each treatment. Different spore concentrations of *B. bassiana* such as 10^6 to 10^4 spore/ml for isolate BBFF 135 and 10^6 to 10^4 spore/ml for isolate MTCC 984 were applied in each treatment. Five replicates were kept per treatment. Spore concentrations were prepared from fungus cultured for different periods of time (10, 14, 20, 28 and 34 d respectively).

Biossays were performed under asceptic laboratory conditions with temperature, humidity and photoperiod ranged from 25 ± 2°C, 80-90% RH, and 12:12 (L:D)h. Termites were checked daily for mortality. Dead insects were placed in petri dishes containing a disk of damp sterile filter paper to allow fungal sporulation. Termite mortality, time to death, and the number of cadavers showing visible symptoms of sporulation were recorded. Datas were subjected to statistical analysis using ANOVA and after analysis of variance, the means were separated by Tukey's multiple range test.

3.8 Mass production of different fungal isolates and their formulation.

Four isolates of *B. bassiana* (984, 2028, 135 and 1216) and *M. anisopliae* (892 and 140) were selected for mass production and formulation.

3.8.1 Multiplication on solid / grain medium

In this method, dry Bajra (*Pennisetum typhoides)* grains crushed in an electric mixer were used. Crushed Bajra grains were autoclaved at 15 Psi pressure for 15 minutes at 121°C in 1000 ml capacity Erlenmeyer flasks. Sterilized Bajra grains (crushed) were taken in plastic tub (36 cm diameter, 12.5 cm depth) predisinfected with 0.1% formaldehyde solution and UV light exposure. Two sets, one containing 400g and other containing 200g crushed Bajra were taken in predisinfected tubs. The level of moisture was maintained at 60-80% per tub. Each tub was inoculated with 50 ml homogenous spore suspension ($1x10^8$ conidia/ml) of a particular isolate. The tubs were covered with sterile black muslin cloth and tied with rubber band. Experiment was performed with three replicates for each isolates. The tubs were incubated at 28 ± 1°C for 30 and 20 days for 400 g and 200g set respectively.

Spores of each isolate were suspended in 3 litres of 0.05% Tween 80 solution in distilled water for collection of spores from the substrate (Bajra). The above suspension was filtered by using cheese cloth for the separation of spores from the solid mass debris. The spore suspension obtained from the above was subjected for conidial count using Neugebauer haemocytometer. The spore concentration of different isolates were tested for its bioefficacy against termite workers.

3.8.2 Multiplication on liquid/broth medium

3.8.2.1 Preparation of inoculum

After 20 days of suitable growth and sporulation on yeast extract glucose agar (YGA) medium, the fungal spores of *Metarhizium* and *Beauveria* spp. were collected on a piece of sterile butter paper and suspended in sterile distilled water with 0.05 percent tween 80 under aseptic conditions. Then, the suspension was shaken on mechanical shaker for 15 min, spore concentration ml^{-1} (10^7-10^8) was determined and used for the inoculation.

3.8.2.2 Preparation of medium and inoculation

Two medium namely Yeast Glucose (yeast extract-5g/lit and Glucose -10g/lit) and Molasses Yeast (Molasses - 1% and Yeast - 0.3%) broth medium were used for the mass culture. The broth mediums were adjusted to pH 6.0 and autoclaved at 15 Psi pressure for 15 minutes at 121°C in 1000 ml capacity Erlenmeyer flasks. Each flask containing 200ml of media (broth) was inoculated with 4ml of spore suspension (10^7-10^8 conidia/ml).

The inoculated media were poured in predisinfested (0.1% formaldehyde solution and UV exposure) plastic tubs (5 litres). Each tub was asigned for each isolate. The plastic tubs were covered with sterile black muslin cloth and tied with rubber band. The tubs were incubated at 28 ± 1°C for 15 days.

3.8.3 A pilot scale mass production

After standardization of liquid culture technique for the isolates of *B. bassiana* and *M. anisopliae*, a pilot scale production of the above was initiated by using 200 litres capacity synthetic tanks. Initially the synthetic tanks were surface sterilized by using 0.1% formaldehyde solution. The tanks were filled with sterile water (autoclaved at pressure 15 psi for 15 minutes at 121°C). Water was boiled inside the tanks with the help of immersion heater. The media ingredients (Molasses 1% and yeast extract 0.3%) were sterilized in an autoclave (pressure 15 psi, temperature 121°C for 15 minutes) and transferred to the synthetic tanks and mixed throughly with a sterile glass rod. The media in the tanks were cooled and 1% of spore suspension (10^7-10^8 conidia/ml) of isolates was used to inoculate the mass culture tanks separately. Inoculated tanks were incubated at 28 ± 1°C for 15-20 days. The maturity of the cultures were determined on the basis of mat formation and sporulation. After maturation the cultures were homogenised with the help of an electric stirrer. Conidial counts were recorded with a Neugebauer haemocytometer. The conidial suspensions obtained from the above procedure were tested against termite workers of *O. obesus* to confirm the efficacy.

3.8.4 Formulation of fungal pathogens

3.8.4.1 Harvesting of fungal biomass.

After 15-20 days of inoculation, the fungal biomass of *M. anisopliae* and *B. bassiana* isolates produced on molasses yeast broth was collected along with the spent medium and properly blended in an electric mixer for 1-2 min to get a homogenous slurry. Then, it was strained through muslin cloth to remove debris under aseptic conditions. In case of grain substrate, the spore mass along with grain carrier was taken out from tubs after 20-30 days of incubation and air dried under laminar flow for 48-72h. The spore count per ml or per gram was determined with a Neugebauer haemocytometer.

3.8.4.2 Mixing of bioagent into carrier

Fuller's earth was sterilized in autoclave at 20 psi pressure for 20 minutes twice. After cooling, the fungal slurry of known spore strength was mixed in the fuller's earth to obtain the formulation of desired strength. Carboxy methyl cellulose (5g/kg) was added as a sticker. Then, this mixture was dried under laminar flow for 2-3 days under aseptic conditions, seived through mesh screen and packed in sterilized polypropylene bags. Similarly, grain-spore powder mixed in sterilized fuller's earth and carboxy methyl cellulose were packed in the same manner. These formulations are designated as dust formulation.

For the preparation of liquid formulation, homogenous fungal slurry of known spore strength was mixed throughly with sterilized 1% soap solution (Hogla) aseptically. Hogla soap solution act as surfactant and sticker. Both the formulations were used in various field experiments against the termites.

3.8.5 Test on substrate variation and carbon source.

Six substrates like Bajra (*Pennisetum typhoides*), Ricebran, Wheat bran, Maize (*Zea mays*), Wheat and Ground nut were selected for the growth of the isolates of *B. bassiana* and *M. anisopliae*. The grain substrates were partially crushed in an electric mixer. 20g of each crushed substrates were taken in an Erlenmeyer flask (100 ml). Six replicates for each substrates were taken. Distilled water (25 ml) was added to each of the flasks to maintain the moisture at desired level. Out of six replicates for each substrates, three were supplemented with 2% dextrose (400 mg). All the flasks were then sterilized in an autoclave at 15 psi pressure for 20 minutes at 121°C. After sterilization, the flasks were shaken vigorously to destroy the lumps of grain formed due to high temperature and pressure. From the cultures of each isolates of *B. bassiana* and *M. anisopliae* (grown on YGA media), Conidial suspension ($2 \pm 1 \times 10^7$ conidia/ml) was prepared and inoculated on the different substrates (with and without dextrose) aseptically under laminar flow. All the flasks were then incubated at 28 ± 2°C for 28 days.

It was necessary to shake all the flasks two or three times a weak on an electrically operated shaker to ensure a homogenous development of the fungus on the substrates. After 28 days, the conidia were harvested by suspending them in 50 ml of 0.05% Tween 80 solution. The suspension was filtered through cheese cloth and the number of conidia determined with a haemocytometer.

Bioassay tests were conducted under laboratory conditions in polythene cups (4.5 x 6.5 x 5.0 cm) containing 40 g soil and 5 g of *Glyricidia* powder. Moisture content of the soil was maintained at 80%. 20 termite workers (*O. obesus).* were released in each cups and 1 ml of conidial suspension (1 x 10^7 conidia/ml) was applied per cup. Three replicates were kept per treatment and mortality data of the termites were recorded daily for a period of 7 days.

3.8.6 Conidial production of *B. bassiana* in different substrates at different combinations of water and vegetable oil.

In order to determine the most fabourable conditions for growth and sporulation of entomogenous fungus, *B. bassiana* (isolate 2028) was selected for the test. 7 food substrates like bajra, maize, wheat, wheat bran, ground nut, barley, and rice bran were taken and the grain substrates were partially crushed in an electric mixer. 50 g of each crushed substrates were taken in an Erlenmeyer flask (250 ml). The moisture content of each substrates were maintained at 50%, 60%, 70%, 80% and 90% respectively. Sunflower oil was added to each substrate at 0, 2, 4, 8, and 16% respectively. Then all the flasks were autoclaved at 15 psi pressure for 20 minutes. After sterilization, the flasks were shaken vigorously to destroy the lumps of grain formed due to high temperature and pressure. The flasks were cooled and inoculated asceptically with 1 ml conidial suspension (1 x 10^7 conidia/ml). All the flasks were incubated at 27 $\pm$ 2°C for 28 days in dark.The flasks were shaken two or three times a week on an electrically operated shaker to ensure a homogenous development of the fungus on the substrates. After 28 days, the conidia were harvested from each flask by suspending them in 50 ml of 0.05% Tween 80 solution. The suspensions were filtered through cheese cloth and the number of conidia determined with a haemocytometer.

3.9 Food preference and deterrence test for termites

Food preference test was conducted with different substrates like maize, bajra, *Glyricidia* powder, rice bran, filter paper, and wheat bran with termites (*O. obesus)* following two different methods named as glass trough test and four arm glass chamber test.

3.9.1 Glass trough test

Food preference test was conducted in glass trough measuring 10 cm high and 20 cm diameter with different substrates as wheat bran, filter paper, maize, bajra, *Glyricidia* powder, rice bran. 2g of each substrates were placed on the periphery of the base of glass trough at equal distance and moistened. 100 termite workers (*O. obesus)* were released at the centre of the trough. The number of termites in contact with each substrate were recorded at every 10 minutes of interval. Readings were recorded at every 10 minutes of interval. Readings were recorded for 1h and 40 minutes (10 counts). The total number of termites in contact with each substrate was summed up.

Deterrence test of different isolates of *B. bassiana* and *M. anisopliae* were performed in a glass trough with the most preferred substrate obtained from the above food preference test. For this, bajra (2g) was mixed with 1 ml of spore suspension of each chosen isolates. Then all are separately placed in a same glass trough opposite to each other at the periphery. Untreated control was also kept. 100 termite workers of *O. obesus* were released at the centre of the glass trough. The test was replicated thrice. Number of termites in contact with the substrate containing the isolate was recorded at every 15 minutes of interval for 1h (4 counts). The total number of termites in contact with each substrate was summed up over 1 h period.

3.9.2 Four arm glass chamber test

Both preference and deterrence tests were performed in a four arm glass chamber which contain 4 arms each measuring 20 cm length and 2.5 cm diameter. Arms are connected to a centrally located flat bottom round glass chamber (6 cm diameter). The central chamber was filled with soil and arms were provided with strips of paper for each movement of the termite workers. From the open ends of the arm 2g of different substrates such as bajra, filter paper, maize, *Glyricidia* powder, wheat bran and rice bran were placed as food source and moistened. The open ends were plugged with cotton pads.

100 termite workers (*O. obesus)* were introduced in the central chamber and counts were recorded every 10 minutes interval for 1h (6 counts). The total number of termites in contact with each substrate was summed up over 1 h period. The experiment was repeated three times.

Deterrence test of isolates was performed by using suitable substrate, observed from the above test. 2g substrate (Bajra) was mixed with 1ml spore suspension of isolates of *B. bassiana* and *M. anisopliae*. Number of termites introduced, counting, replication of test and so on were followed as described above.

3.10 Soil factors on the pathogenicity of the fungus

Surface samples (top soil) of the acid (pH=4.8) and alkaline (pH=7.3) types were taken from Bodaganj and Jaldapara respectively. The soil samples were sifted to a uniform texture and then sterilized by autoclaving at 15 psi for one hour. The samples were used to test the effect of different soil factors on the pathogenicity of different isolates of *M. anisopliae* and *B. bassiana* which were found to be the most promising pathogen against termites. Prior to any treatment, representative sample was taken from each for moisture determination by gravimetric method.

Sixteen treatment combinations were prepared using two soil pH (4.8 and 7.3), four moisture levels (15, 30, 60 and 90% wt/wt) and sterility conditions (autoclaved and unautoclaved). Uniform amount of spores at ca. 1 $x10^7$ conidia/40g of solid were added to both autoclaved and unautoclaved soil, 40g of which was provided for each plastic disposable cup. Sterile distilled water was then added to give 15, 30, 60 and 90% (wt/wt) moisture levels of the soil. Eighty termite workers at 20 nos./cup were used per treatment per isolate. The plastic cups containing the workers were provided with a piece of *Glyricidia sepium* wood piece to serve as food for workers. Daily mortality was noted for seven days.

3.11 Formulation and its application.

3.11.1 Preparation of formulations with entomogenous fungus and their pathogenicity test

Different formulations were prepared with entomogenous fungus, *B. bassiana* and *M. anisopliae*. Formulations are in form of dust, wettable powder and liquid.

3.11.1.1 Fungal dust formulation

The fungal biomass of *M. anisopliae* and *B. bassiana* isolates were harvested from the grain substrate (crushed *Pennisetum typhoides)* after 15-20 days of inoculation. The fungal biomass were air dried under laminar flow for 48-72 h. Talc, China clay, and clay were sterilized in autoclave separately at 20 psi pressure for 1h. twice. After cooling, the fungal biomass of known spore strength was mixed in talc (1:10), china clay (1:10), and clay (1:10) respectively. Carboxy methyl cellulose (5g/kg) was added as a sticker to each of the above mixtures. Then, these mixtures were dried under laminar flow for 2-3 days under asceptic conditions, seived through mesh screen and packed in sterilized polypropylene bags.

3.11.1.2 Wettable powder formulation

As in the fungal dust formulation, the fungal biomass of different isolates of *M. anisopliae* and *B. bassiana* were harvested from the crushed grain substrate of *Pennisetum typhoides* after 15-20 days of inoculation. Then the harvested biomass

methyl cellulose (5g/kg) was added to each of the above mixtures. Then, the mixtures were dried under laminar flow for 2-3 days under aseptic conditions and packed.

3.11.1.3 Liquid formulation

After 15-20 days of inoculation, the fungal biomass of *M. anisopliae* and *B. bassiana* isolates produced on molasses yeast broth was collected along with the spent medium and properly blended in an electric mixer for 1-2 min to get a homogenous slurry. Then, it was strained through muslin cloth to remove debris under asceptic conditions. The homogenous fungal slurry of each isolates were mixed with Tween 80 solution (0.05%), Lavolin (0.01%) and detergent Hogla (0.2%). Further, certain adjuvants like sunflower oil (0.2%), coconut oil (0.2), palm oil (0.2%), mustard oil (0.2%), soyabean oil (0.2%), crude sugar (20%), molasses (20%) and U.V. protectants (Robin blue and Ranipal, 1%) were also added to increase the potentiality and stability of the liquid formulated products.

Pathogenicity tests were conducted by using the aforementioned different formulations against workers of *O. obesus*. The trials were conducted in disposable cups (4.5 x 6.5 x 5.0 cm) with 40 gm of acetone washed and autoclaved soil saturated with sterile distilled water 5gm of autoclaved saw dust was placed on top of the soil and treated with 1ml of the respective formulations comprising a conidial strength of 1×10^7 conidia/ ml or gm. A group of twenty termite workers of *O. obesus* were released per cup per formulation and replicated for three times. A set of control was also kept.

3.11.2 Field application of liquid formulation.

3.11.2.1 Experimental area

The study was conducted in 2.5 hectare young tea area in the tea garden of Goodricke Group Ltd. at Danguajhar, Jalpaiguri. The main tea plants were TV-25 clones (3 years, 105 x 75 x 75 cm planting style, 15,000 plants/ha). The tea plants were severely infested with live wood termites. 2.5 hectare area was divided into 5 equal blocks (I-V) measuring 0.5 ha each. Each block was further subdivided into 5 plots (A, B, C, D & E) measuring 0.1 ha. The number of plants per plot ranged from 1480-1500. Randomised block design method was followed which is appended below:

I. Chemical treatment (Chlorpyriphos-700ml in 400 litres of water/ha.

II. Half dose of chemical (Chlorpyriphos - 350 ml) plus Metabass 8 litres (liquid formulated mycoinsecticide) in 400 litres of water/ha.

III. Quarter dose of chemical (Chlorpyriphos-175ml) plus Metabass 8 litres in 400 litres of water/ha.

IV. Metabass (8 litres) in 400 litres of water/ha.

were air dried under laminar flow for 48-72 h. Fuller's earth, Arrowroot powder and Dedenol were double sterilized in autoclave separately at 20 psi pressure for 1h. After cooling the fungal biomass of known spore concentration (conidia/ml) was mixed in Fuller's earth (1:10). Arrowroot powder (1:10) and Dedenol (0.01%) respectively. Carboxy V. Untreated control.

Block

0.5 ha

0.1 ha

I	IV	III	II	V	
1487	1499	1485	1500	1500	Plot - A
V	I	I	III	IV	Plot - B
1490	1491	1483	1486	1480	
III	V	IV	II	I	Plot - C
1489	1495	1492	1489	1486	
II	IV	III	I	V	Plot - D
1492	1483	1495	1500	1498	
V	I	II	IV	III	Plot - E
1490	1500	1500	1495	1495	

2.5 ha

Number mentioned within the box are the number of plants.

3.11.2.2 Field Trials

Five treatments namely, I) Chemical (chlorpyriphos-700 ml in 400 litres of water/ ha), II) Half dose of Chemical (Chlorpyriphos - 350 ml) plus formulated mycopesticide (8 litres) in 400 litres of water/ha, III) Quarter dose of Chemical (Chlorpyriphos-175 ml) plus formulated mycopesticide (8 litres) in 400 litres of water/ha, IV) Formulated mycopesticide (8 litres) in 400 litres of water/ha and V) Untreated control were reckoned with for field trials. The chosen chemical, formulated mycopesticide (Metabass) and their Combinations were sprayed on the respective plots as mentioned in the Randomised block design (RBD) method at an interval of 15 days. The spraying was done with the sprayer fitted with a nozzle which enabled a flow rate of approximately 60 ml min^{-1}. The spraying was done only in the evening hours. The temperature and relative humidity at the beginning (18:00) and end (18:55) of application were 26.5-25.5°C and 76-79%, respectively.

Before the first spraying, pretreatment data were recorded and cultural operations like forking of the collar region around 10cm, removal of earthern runs, dead snags and hollowed out portions were followed. Control plots were also subjected to all operations except spraying. The data regarding percentage of infestation ratio was ascertained following the formula as mentioned below :

$$\text{Percentage of infestation ratio} = \frac{\text{No. of plants infested with termites}}{\text{No. of plants in the plot}} \times 100$$

Population size of termite workers was observed in the respective plots for 50 bushes at random per plot and recorded on a weekly basis for a period of 8 weeks by disturbing the earthern runs on the bush frames for a total number of 250 plants/treatment.

3.12 Bait application for population suppression of subterranean Termites

3.12.1 Characterization of subterranean termite colonies' Foraging : Activity, Population, and Territory.

The two field colonies of *Odontotermes obesus* and one colony of *O. distans* were selected for testing. Wooden-stake surveys were done in areas of known termite activity. Survey stakes (*Glyricidia sepium*, length 30 cm and diameter 3 cm) were driven into soil adjacent to teak and shade trees (*Albizia spp* and *Lagerstroemia speciosa*) known to be infested with termites and were examined monthly. Infested stakes were replaced by underground monitoring stations that included wooden blocks surrended by earthern collars measuring 12 cm diameter by 14 cm high. The blocks of monitoring stations were composed of 10 wooden boards (four boards [each 2.0 by 6.0 by 12.5 cm] encircling six boards [0.5 by 6.0 by 12.5 cm]) nailed together. The six thinner boards were separated by wooden applicator sticks (2mm diameter) used as spacers to maximize the surface area available to foraging termites. Wooden blocks were dried at 80°C for 48h and weighed (±0.1g) before placement in monitoring stations. Termites readily entered wooden blocks in the stations were separated from debris. Wooden block remnants were rinsed under running water, oven dried, and weighed as described above. Weight loss of a wooden block was determined monthly to assess foraging activity of the subterranean termite colony being tested. Termite activity was measured 0.5-1 year before the introduction of formulated bait tubes.

After the establishment of five or more monitoring stations for each colony, a triple mark recapture procedure was used to estimate the foraging territory and the population. Worker termites collected from a station with high activity (>500 termites) were fed on filter disks (Whatman No. 1, 5.5 cm) stained with 0.05% (wt/wt) Nile blue A (Su and Scheffrahn 1991) for 3 days before being released back to the same station. This blue marker remained visible in termites 6 months after staining. Termites were collected at monitoring stations 1 week after the release of staining termites. Termites

collected from stations containing marked termites from the first release were counted and again stained and released to their respective stations. The mark-release-recapture cycle was repeated three times for each colony. The numbers of marked and unmarked workers were recorded for each cycle. A weighted mean model was used to estimate the foraging populations (N) and associated standard errors (SE)

$$N = (\Sigma \text{ mini}) \quad [(\Sigma \text{ mi}) + 1]$$

$$SE = N\sqrt{[\Sigma mi+i)]+[2/(\Sigma mi+1)^2]+[6/(\Sigma mi+1)^3]}$$

Where for each i^{th} cycle, ni is the number of marked individuals among captured termites, and Mi is the total number of marked individuals up to the i^{th} cycle. For each termite collection, mean body weight of termite workers was determined by weighing five groups of 10 individuals each. Numbers of collected workers were determined by the total weight of collected workers and the mean worker weight. The foraging territory of each colony, defined as the area encompassed by interconnected stations, was determined by the presence of marked termites.

3.12.2 Bait Tube

Sisoo (*Dalbergia sisoo*) saw dust, *Glyricidia sepium* dust, rice bran were equally mixed at a ratio of 1:1:1. The fungal mycelial mat was collected from the culture medium (Molasses Yeast broth) and blended in an electric mixer. After air drying under laminar flow for 48-72h, carboxymethyl cellulose was added to the blended fungal mass (5g/kg) and packed. For the preparation of bait, the above fungal formulation 10% (wt/wt) was added to the mixture of saw dust and rice bran (1:1:1:) along with 0.1% coconut oil. The above ingredients were thoroughly mixed and filled inside the bait tubes (a plastic tube, 2.6 cm inside diameter by 15 cm high, one end rounded, the other end capped). Four rows of four holes, 0.4 mm were predrilled into the walls of the tube.

3.12.3 Baiting Procedure

Four stakes (*Glyricidia sepium* : height 30 cm and 3 cm diameter) were driven 20-25 cm into the soil evenly surrounding each active monitoring station at 30 cm radii and were examined monthly. Once infested by termites, each wooden stack was gently pulled out of the soil, leaving an intact hole into which a bait tube was inserted. The infested stake was split into two pieces by cutting and gently tapped to dislodge termites into a funnel inserted in the recruiters' chamber of a bait tube. The bait-tube lid was attached and covered with soil. Bait tubes were examined monthly. Bait tubes were examined monthly and replaced with new tubes when termite activity was observed, those without termite activity were replaced with survey stakes. At the time of each monthly inspection, newly infested survey stakes also were replaced with bait tubes. Bait tubes were retrieved monthly from the field regardless of the bait consumption. Bait matrix in each tube was removed, separated from soil, and reweighed to determined

the amount of bait matrix consumed by termites.

3.13 Different application procedures with formulated mycoinsecticide.

The efficacy of formulated mycoinsecticide was tested against the termites (*O. obesus)*. A set of experiments was conducted in the Danguajhar region, a permanent zone of outbreaks for termite infestation. Experiments were performed in zones of natural vegetation. 400m^2 experimental area was chosen and divided into four sections 100m^2 each. Each section was planted with 100 saplings of sissoo (*Dalbergia sissoo*), Lebbeck (*Albizia lebbeck*), Champac (*Michelia champaca*) and Queen crape myrtle (*Lagerstroemia speciosa)* respectively.

Four different types of application procedures were followed which are described below :

3.13.1 Planting Pit application

25 pits were dug in a row on each of the four sections. 100g formulated fungal dust (1x10^7 conidia/gm) was sprinkled in each pit and mixed properly with the soil. 25 saplings of *Dalbergia sissoo* were planted in the pits of section I. Similarly 25 saplings each of *Albizia lebbek, Michelia champaca* and *Lagerstroemia speciosa* were planted in the pits of section II, III and IV respectively.

3.13.2 Container application

Saplings raised on polythene container were selected for this experiment. 100 ml liquid formulation (1x10^7 conidia/ml) was added on the container of each sapling. As mentioned above, 25 saplings per species were planted in a row on each section.

3.13.3 Post-Planting application

25 saplings per species were planted on each section is a row. 100 ml liquid formulation (1x10^7 conidia/ml) was added on the soil around the base of each sapling.

3.13.4 Root dip treatment

Before transfering saplings from polythene container into the pits, the roots were immersed in liquid formulated mycoinsectiside (1x10^7 conidia/ml) for 15 minutes. Then 25 saplings per species were planted on each section as mentioned above. A separate section for 100 (25 each species) untreated saplings were kept as control.

The saplings were watered regularly. Growth and mortality (due to termite infestation) of the saplings were recorded on monthly basis for a period of 12 months.

3.14 Storage life and pathogenicity of the fungus

3.14.1 Storage life

Storage life was measured for each liquid formulated strain by comparing the number of conidia sporulated after storage at temperature 4°C and three intervals (6,

12 and 18 months). After storage treatments, formulated fungal strains were cultured on each of three replicate YGA (Yeast extract glucose and agar) plates for 21 days at 28°C. Each plate was drenched with 10 ml of 0.05% Tween 80 suspension and shaken gently by hand for 2 min. The resulting conidial suspension was pipetted into a small vial containing three glass beads, shaken vigorously for 2 min to help to separate aggregated conidia, then diluted in 90 ml of Tween 80 suspension and placed on an automatic stirrer. Conidia in three subsamples from each suspension were counted using a haemocytometer.

3.14.2 Pathogenicity

The conidial suspensions of four isolates of *B. bassiana* and two isolates of *M. anisopliae* obtained from the aforementioned fungal formulation stored for 0,6,12 and 18 months period at 4°C in a freezer were subjected to pathogenicity test against workers of *O. obesus* separately and also in different combinations. Pathogenicity test was carried out in disposable plastic cups (4.5 x 6.5 x 5.0 cm) with 40 gm of acetone washed and autoclaved soil saturated with sterile distilled water 5 gm of autoclaved saw dust was placed on top of the soil and treated with 1ml of the respective conidial suspension at a concentration of 1 x 10^7 conidium/ml. A group of twenty termite workers were released per cup per treatment and replicated for three times. A set of control (Untreated) was also maintained. Pathogenic efficacy of the formulated isolates stored at different storage intervals was calculated as percentage cumulative mortality caused by mycosis, corrected according to Abott's formula, leading to the same results but based on the number of dead instead of living individuals :

$$\% \text{ efficacy} = \frac{\% \text{ mycosis} - \% \text{ mycosis in control}}{100 - \% \text{ mycosis in control}}$$

3.15 Preparation of antigen

Fungal antigen

Mycelia - Mycelial antigen was prepared following the method of Chakraborty & Saha (1994). Initially the fungal mycelium (4mm disc) were transferred to 250 ml Ehrlenmeyer flask each containing 50 ml of sterilized liquid yeast extract glucose medium (g/l distilled water, glucose, 10; yeast extract, 5) and incubated for 10 days at 28 ± 1°C. For extraction of soluble antigens, mycelial mats were harvested, washed with 0.2% NaCl and rewashed with sterile distilled water. Washed mycelia (50 g fresh wt.), were homogenized with 0.05 M Sodium phosphate buffer (pH-7.2) supplemented with 10 mM sodium metabisulphate and 0.5 mM magnesium chloride and 0.85 NaCl in mortar and pestile in the presence of sea sand. Cell homogenates were kept overnight at 4°C and then centrifuged (15000g) for 30 min at 4°C. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring and kept overnight at 4°C. After this period the mixture was centrifuged (15000 rpm) for 30 min at 4°C, the precipitate was dissolved in 10ml 0.05 M sodium phosphate buffer (pH 7.2).

The preparation was dialysed for 72h through cellulose tubing (Sigma Chemical Co, USA) against 1L of 0.005 M sodium phosphate (pH 7.2) with ten changes. Then the dialysed material was stored at - 20°C and used as antigen for the preparation of antiserum and other experiments.

3.16 Purification of mycelial antigen

Saturated ammonium sulphate fractionation

Freshly harvested mycelium (150g) of *B. bassiana* and *M. anisopliae* was crushed in a mortar with pestle at 4°C using seasand and homogenized with 150 ml of 0.1 (M) Tris HCl buffer (pH-7.0). The slurry was strained through muslin cloth and the filtrate was centrifuged at 15,000 rpm for 30 min. Finally into the supernatant finely ground ammonium sulphate crystals were added slowly with constant stirring at 4°C. Finally saturations of ammonium sulphate of 20, 40, 60, 80 and 100% were obtained. In each case, stirring was done for 6h and then it was kept overnight at 4°C for precipitation. After centrifugation at 15000 rpm for 1h the precipitates were dissolved in 2-3 ml of 0.1M Tris-HCl buffer pH 7.0, for each saturation level. Dissolved precipitates were then dialysed by using cellulose dialysing tubing (Sigma Co., U.S.A) against 0.01 M Tris-HCl buffer, pH-7.0 at 4°C for 72 h with 6 hourly changes.

3.17 Antisera production

3.17.1 Rabbits and their maintenance

For the production of antisera against different fungal antigens, New Zealand white, male rabbits were used. Before immunization, the body weights of rabbits were recorded and were observed for at least one week inside the cages. They were regularly fed with 500 g green grass each time in the morning and evening. Every alternate day they were also given 50-75g of gram seeds soaked in water. Besides this, they were given saline water after each bleeding for three consecutive days. Cage were cleaned everyday in the morning for better hygeinic conditions.

3.17.2 Immunization

Antisera were raised in separate rabbits against antigen preparation of mycelia of *M. anisopliae* isolate 892 and *B. bassiana* isolate 2028 sera collected before immunization were used as controls. After preimmunization bleeding, immunogen (1ml) emulsified with an equal volume of Freund's complete adjuvant (Difco) followed by incomplete adjuvant were injected intramascularly at weekly intervals, upto 18 weeks.

3.17.3 Bleeding

Blood was collected from the marginal carvein puncture 3 days after seventh week of first immunization and subsequently seven times more every fortnight. During bleeding, rabbits were placed on their backs on a wooden board after taking them out from the cage. The board was fixed at a 60° angle. The neck of the rabbit was held tight

in the triangular gap at the edge of the board, and the body was fixed in such a way that the rabbits could not move during bleeding. The hairs were removed from the upper side of the ear with the help of a razor and disinfected with rectified spirit. Then the ear vein was irritated by xylene and an incision was made with the help of a sharp sterile blade and blood samples (2-5 ml) were collected in a sterile graduated glass tube. After collection, all precautionary measures were taken to stop the flow of the blood from the puncture. For clotting, the blood samples were kept at 30°C for 1hr and then the clot was loosened with a sterile needle and the antiserum was clarified by centrifugation at 2000 rpm for 10 min. Finally, blood samples were distributed in 1ml vials and stored at - 20°C, until used for experiments.

3.18 Immunodiffusion tests

3.18.1 Preparation of agar slides

The glass slides (5cm x 5cm) were degreased successively in 90% (v/v) ethanol, ethanol : di-ethylether (1:1 v/v) and ether, then dried in hot air oven and sterilized inside the petridish each containing one slide. A conical flask containing Tris - barbiturate buffer (pH 8.6) was placed in a boiling water bath, when the buffer was hot, 0.9% agarose was mixed to it & boiled for the next 15 min. The flask was repeatedly shaken thoroughly in order to prepare absolutely clear molten agarose which was mixed with 0.1% (w/v) sodium azide (a bacteriostatic agent). The molten agarose was poured in glass slides (5ml / slide) and kept 15 min for solidification. After that 7 wells were cut out with a sterilized cork borer (4 mm dia.) at a distance of 5 mm from the central well.

3.18.2 Diffusion

Agar gel double diffusion test was performed following the method of Ouchterlony (1967). The antigens and undiluted antisera (50 µl/well) were pipetted directly into the appropriate wells and diffusion was allowed to continue in a moist chamber for 48-72h at 25°C. Precipitation reaction was observed in the agar gel only in cases where common antigens were present.

3.18.3 Washing, staining and drying of slides

After immunodiffusion, the slides were initially washed with sterile distilled water and then with aqueous NaCl solution (0.9%) NaCl and 0.1% NaN_2) for 72 h with 6 hourly changes to remove unreacted antigen and antibody widely dispersed in the agarose. Then slides were stained with 0.5% amido black (0.5g amido black, 5g $HgCl_2$, 5ml glacial acetic acid, 95ml distilled water) for 10 min. at room temperature. After staining, slides were washed thrice in distaining solution [2% (v/v) acetic acid] for 5h to remove excess stain. Finally, all slides were washed with distilled water and dried in hot air oven for 3 h at 50°C.

3.19 Purification of IgG.

3.19.1 Precipitation

IgG was purified as described by Clausen (1988). The polyspecific / polyclonal crude antiserum (2ml) was first diluted with two volume of distilled water and an equal volume of 4M Ammonium Sulphate. The pH was adjusted to 6.8 and the mixture was stirred for 16hrs at 22°C for 1hr. Then the precipitate was dissolved in 2ml of 0.02M Sodium Phosphate buffer, pH 8.0.

3.19.2 Column preparation

Eight gram of DEAE cellulose (Sigma Co. USA) was suspended in distilled water for overnight. The water was poured off and the gel was suspended in 0.005 M Phosphate buffer, pH 8.0 and the buffer washing was repeated for 5 times. The gel was then suspended in 0.02M Phosphate buffer, pH 8.0 and was applied to a column 2.6 cms in diameter 30 cm high and allowed to settled for 2hr. After the column material had settled, 25 ml of buffer (0.02M Sodium Phosphate buffer, pH 8.0) washing was given to the gel material.

3.19.3 Fraction collection

All the top of the column, 2 ml of Ammonium Sulphate precipitate was applied and the dilution was performed at a constant pH and a molarity continuously changing from 0.02M to 0.03M. The initial elution buffer was 0.02M Sodium Phosphate buffer pH 8.0 (I). The final elution buffer was 0.3M Sodium Phosphate buffer pH 8.0 (2). The buffer was applied in a flask on which one rubber connection from its bottom was supplying the column. Another connection above the surface of buffer (1) was connected to another flask with buffer (2). The buffer (2) had also connection to the open air. During the draining of buffer (1) to column, buffer (2) was sucked into buffer (1) thereby prducing a continuous rinse in molarity. Ultimately, 40x5 ml franctions were collected and the optical density values were recorded by means of UV Spectrophotometer at 280 nm. The fractions showing >2 reading were stored as purified IgG.

3.20 Protein estimation

Proteins were extracted from the mycelia of *B. bassiana* and *M. anisopliae*. Soluble proteins were estimated following the method as described by Lowry et.al. (1951). To 1ml of protein sample 5 ml of alkaline reagent (0.5 ml of 1% $CuSO_4$ and 0.5 ml of 2% Potassium sodium tartarate, dissolved in 50ml of 2% Na_2CO_3 in 0.1 N NaOH) was added. This was incubated for 15 min at room temperature and then 0.5 ml of Folin-Ciocalteau's reagent (diluted 1:1 with distilled water) was added and again incubated for 15 min for colour development following which optical density (OD) was measured at 750 nm. Using bovine serum albumin (BSA) as standard, the protein concentrations were computed.

3.21 Enzyme linked immunosorbent assay (ELISA)

The following buffers were prepared following the method as described by Chakraborty *et al*, 1995, with modifications.

1. Antigen coating buffer : Carbonate Bicarbonate buffer 0.05M pH-9.6

Stocks

A. Sodium Carbonate - 5.2995g in 1000 ml Dist water.

B. Sodium bicarbonate - 4.2g in 1000 ml Dist water

160ml of stock A was mixed with 360ml of stock B and pH was adjusted 9.6

2. Phosphate Buffer Saline : 0.15 M PBS pH-7.2

Stocks

A. Sodium dihydrogen phosphate - 23.40g in 1000ml Dist water

B. Di-Sodium hydrogen phosphate - 21.2940 in 1000ml Dist water 280 ml of stock A was mixed with 720 ml of stock B and the pH was adjusted to 7.2.

Then 0.8% NaCl and 0.02% KCl was added to the solution.

3. 0.15M Phosphate buffer Saline - Tween (0.15 M PBS - Tween, pH 7.2).

To 0.15M PBS 0.05% Tween 20 was added and the pH was adjusted to 7.2.

4. Blocking reagent (Tris buffer saline, pH 8.0)

0.05M Tris, 0.135M NaCl, 0.0027M KCl

Tris - 0.657g

NaCl - 0.81g

KCl - 0.223g

Distilled water was added to make up the volume to 100ml. Then pH was adjusted to 8.0 and 0.05% Tween 20 and 1% bovine albumin (BSA) were added.

5. Antisera dilution buffer (0.15M PBS - Tween, pH 7.2).

In 0.15M PBS - Tween, pH 7.2, 0.2% BSA, 0.02% Polyvinylpyrrolidone. 10,000 (PVPP 10,000) and 0.03% Sodium azide (NaN2) was added.

6. Substrate

p-Nitrophenyl phosphate (Himedia) 1mg/ml dissolved in 100ml of di ethanolamine (1.0% w/v, 3mM NaN_2) pH 9.8.

7. 3N NaOH solution was used to stop the reaction.

This ELISA was performed following the method as described by Chakraborty

et al,1995 with modifications. Fungal antigens were diluted with coating buffer and the antigens were loaded (200ul/well) in 8 welled ELISA strips (Costar EIA/RIA, strip plate USA), arranged in 12 rows in a (casette) ELISA plate. After loading, the plate was incubated at 25°C for 4 hrs. Then the plate was washed 4 times under running tap water and twice with PBS - Tween and each time shaken to dry. Subsequently, 200µl of blocking reagent was added to each well for blocking the unbound sites and the plate was incubated at 25°C for 1hr. After incubation, the plate was washed as mentioned earlier. Purified polyspecific IgG was diluted in antisera dilution buffer and loaded (200µl/well) to each well and incubated at 4°C overnight. After a further washing, antirabbit IgG goat antiserum labelled with Alkaline Phosphates diluted 10,000 times in PBS, was added to each well (100µl/well) and incubated at 37°C for 2 hrs. The plate was washed, dried and loaded with 100µl of p-Nitrophenyl Phosphate substrate in each well and kept in dark for 60 mins. Colour development was stopped by adding 50µl /well of 3N NaOH solution and the absorbance was determined in an ELISA Reader (LISA-5 Trans Asia model) at 405nm. Absorbance values in wells not coated with antigens were considered as blanks.

3.22 SDS - PAGE analysis of total soluble protein

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed for the detailed analysis of protein profile following the method of Laemmli (1970).

3.22.1 Preparation of stock solutions

The following stock solutions were prepared :

A) Acrylamide and N'N' - Methelene bis acrylamide.

A stock solution containing 29% Acrylamide and 1% bis acrylamide was prepared in warm water. As both of them are slowly deaminated to acrylic and bis acrylic acid by alkali and light, the pH of the solution was kept below pH - 7.0 and the stock solution was filtered through whatman No. 1 filter paper and was kept in brown bottle stored at 4°C and used within one month.

B) Sodium Dodecyl Sulphate (SDS)

A 10% stock solution of SDS was prepared in warm water and stored at room temperature.

C) Tris buffer

a) 1.5 Tris buffer was prepared for resolving gel. The pH of the Tris was adjusted to 8.8 with conc. HCl and stored at 4°C for use.

b) 1.0 M Tris buffer was prepared for use in the stacking and loading buffer. The pH of this Tris was adjusted to pH 6.8 with concentrated HCl and stored at 4°C.

D) Ammonium Persulphate (APS)

Fresh 10% APS solution was prepared with distilled water each time before use.

E) Tris - Glycine electrophoresis buffer

This running buffer contains of 25mM Tris base, 250mM glycine (pH-8.3) and 0.1% SDS. A solution can be made by dissolving 3.02g Trisbase. 18.8g glycine and 10ml of 10% SDS in 1L of distilled water.

F) SDS loading buffer

This buffer 50mM Tris Cl (pH- 6.8). 10mM â Mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol. A 1x solution was made by dissolving 0.5 ml of 1M tris buffer (pH 6.8), 0.5ml of 14.4M âMeraptoethanol, 2ml of 10% SDS, 10mg bromophenol blue, 1ml glycerol in 6.8 ml of Distilled water.

3.22.2 Preparation of Gel

Slab gel was prepared for the analysis of protein patterns by SDS-PAGE i.e. mini gel (8cm x 10cm). For slab gel preparation, two glass plates were thoroughly cleared with dehydrated alcohol to remove any traces of grease and then dried. Then 1.5mm thick spacers were placed between the glass plates at the three sides, and the three sides of glass plates were sealed with high vaccum grease and clipped thoroughly to prevent any leakage of the gel solution during pouring. Resolving and stacking gels were prepared by mixing compounds in the following order by pasture pipette leaving sufficient space for any unpolymerized acrylamide. Stacking gel solution was poured over the resolving gel and comb was inserted immediately and overlayered with water. The gel was kept for 30 minutes. After polymerization of the stacking gel, the comb was removed and washed thoroughly. The gel was then finally mounted in the electrophoresis apparatus. Tris-glycine running buffer was added sufficiently in both upper and lower reservoir. Any bubble, trapped at the bottom of the gel, was removed very carefully with a bent syringe.

3.22.3 Sample preparation

Sample (34µl) was prepared by mixing the sample protein with 1xSDS gel loading buffer (16µl) in cyclomixture. All the samples were floated in boiling water bath for 3 mins, to denature the protein sample. The samples were immediately loaded in a pre-determined order into the bottom of the wells with a microtiter syringe. Along with the samples, protein markers consisting of a mixture of six proteins ranging in molecular weight from high to low molecular wt. (Phosphorylase b-97, 400; Bovine serum Albumin - 68000; Ovalbumin - 43000; Carbonic Anhydrase-29000; Soyabean trypsin inhibitor - 20,000; Lysozyme - 14300 daltons was treated as the other samples and loaded in separate well.

Composition of solution for 10% resolving gel :

Name of the Compound	Minigel (7.5ml)
1. Distilled water	2.85 ml
2. 30% acrylamide mix	2.55 ml
3. 1.5M Tris (pH 8.8)	1.95 ml
4. 10% SDS	0.075 ml
5. 10% APS	0.075 ml
6. TEMED	0.003 ml

Composition of solutions for 5% stacking gel

Name of the Compound	Minigel (7.5ml)
1. Distilled water	2.1 ml
2. 30% acrylamide mix	0.5 ml
3. 1M Tris (pH 6.8)	0.38 ml
4. 10% SDS	0.03 ml
5. 10% APS	0.03 ml
6. TEMED	0.003 ml

3.22.4 Electrophoresis

Electrophresis was performed at constant 17 mA current for a period of 3 hrs in case of mini gel until the dye front reached the bottom of the gel.

3.22.5 Fixing and Staining

After electrophoresis, the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally fixed in glacial acetic acid : methanol : water (10:20:70) for overnight.

The staining solution was prepared by dissolving 250 mg of Coomassie brilliant blue (Sigma R 250) in 45 ml methanol. After the stain was completely dissolved, 45 ml of water and 10 ml of glacial acetic acid were added. The prepared stain was filtered through Whatman no 1 filter paper.

The gel was removed from fixer and stained in this staining solution for 4 hrs at 37°C with constant shaking at a very low speed. After staining, the gel was finally destained in destaining solution containing methanol, water and acetic acid (4.5:4.5:1) at 37°C with constant shaking until back ground became clear.

3.23 Fluorescence antibody staining and microscopy

Indirect fluorescence staining of fungal mycelia and spores were done using FITC labelled goat antirabbit IgG following the method of Chakraborty and Saha (1994).

3.23.1 Mycelia

Fungal mycelia of *B. bassiana* and *M. anisopliae* were grown in liquid potato dextrose medium as described earlier. After four days of inoculation young mycelia were taken out from the flask and kept in eppendrof tube. After washing with PBS (phosphate buffer saline), pH7.2, mycelia were treated with normal sera or antisera diluted (1:125) with PBS, pH-7.2 and incubated for 30 min. at 27°C. Then mycelia were washed thrice with PBS - Tween (pH7.2) as mentioned above and treated with goat antirabbit IgG (conjugated with fluorescein isothiocyanate (Sigma) diluted 1:40 with PBS (pH 7.2) and incubated in dark for 30 min at 27°C. After incubation, mycelia were washed thrice in PBS (pH 7.2) and mounted in 10% glycerol. A cover slip was placed on mycelia and sealed. Then slides were observed under Leica microscope, equipped with I-3 UV-fluorescence filter and photographed in a wild MPS camera on 400 ASA konica film (Leitz).

3.23.2 Spore

Fungal spore of *B. bassiana* and *M. anisopliae* were collected from 15 day-old culture and a suspension of this was prepared with PBS, pH 7.2. Conidial suspensions were taken in micro-centrifuge tubes and centrifuged at 3000 r.p.m. for 10 min and the PBS supernatant was discarded. Then 200 µl of diluted (in PBS, pH 7.2) (1:125) was added into the microcentrifuge tube and incubated for 2h at 27°C. After incubation, tubes were centrifuged at 3000 r.p.m. for 10 min and the supernatant was discarded. Then the spores were rewashed 3 times with PBS - Tween pH - 7.2 by centrifugation as before and 200 µl of goat antirabbit IgG conjugated FITC (diluted 1:40 in PBS) was added and the tubes were incubated in dark at 26°C for 1h. After the dark incubation excess FITC-antisera was removed by repeated washing with PBS -Tween pH 7.2 and the spores were mounted on glycerol jelly and observed under Leica microscope, equipped with I-3 UV-fluorescence filter. Photographs were taken in a wild MPS camera on 400 ASA konica film (Leitz).

3.24 Dot - Blot

Dot - blot was performed following the method suggested by Lange et al.(1989) Following buffers were used for dot-blot :

i) Carbonate - bicarbonate buffer (0.05M, pH-9.6 coating buffer).

ii) Tris buffer saline (10mM, pH-7.4) with 0.9% NaCl and 0.05% Tween 20 for washing

iii) Blocking buffer - 10% Casein hydrolysate in 0.05M Tris, 0.5 NaCl, 0.5% Tween - 20, pH-10.3.

Nitrocellulose membrane (Millipore, H5 SMO 5255, 7cm x 10cm, pore size 0.45µm, Millipore Corporation, Bedford) was first cut carefully into the required size

and placed inside the template. 2µl of coating buffer (carbonate - bicarbonate buffer) was loaded in each well of the template over the NCM and kept for 25 mins. to dry. Following this 2µl of test samples (antigen samples) were loaded into the template wells over the NCM and kept for 3 hrs at room temperature. Template was removed and blocking of the NCM was done with 10% non-fat dry milk (casein) prepared in TBS for 30 mins. Polyclonal antibody (IgG-E, Vexans 1:40 was added directly in the blocking solution and further incubated at 4°C for overnight. The membrane was then washed several times in TBS-Tween (pH-7.4). Enzymatic reactions were done by treating the NCM membrane with Alkaline phosphatase conjugate (1:7500) for 2 hrs at 37°C. This was followed by washing for 25 mins. in TBS - Tween. Substrate (66 µl Nitro Blue Tetrazolium chloride + 33 µl 5 - Bromo - 4-chloro -3 Indolyl phosphate Di sodium salt in 10 ml of Tris buffer saline (pH 7.4). Finally, reaction was stopped by floating the NCM in deionized water.

3.25 Western blotting

Blot transfer was done in three steps, following the method as described by White et.al. (1994) with modification.

3.25.1 Extraction of soluble proteins

Soluble proteins were extracted from the mycelia of the entomogenous fungus *B. bassiana* and *M. anisopliae* and protein content was estimated.

3.25.2 SDS-PAGE

Analysis of total soluble protein was performed as described previously.

3.25.3 Transfer process

Preparation of transfer buffer : (Towbin) 25mM Tris, 192mM glycine in 20% Reagent grade Methanol, pH 8.3.

(Tris - 3.03g, Glycine - 14.4g, 200ml Methanol - volume make upto 1 litre)

SDS gel electrophoresis was carried out in a mini gel unit. Following gel run, it was transferred to Towbin buffer and equilibrated for 1hr. The transfer unit was attached to a power pack. The presoaked filter paper was placed on the platinum anode and air bubbles were rolled out with a glass rod over the pre-wetted membrane, followed by the gel and finally on top again another presoaked filter paper was placed. The cathode was placed on the sandwich and pressed. The unit was run for 45 mins. at 15 volts constant voltage. After the run the membrane was dried for 1hr and proceeded for immunological probing.

3.25.4 Immunoblotting

Blocking was done by 5% non fat dried milk and 0.02% sodium azide in 0.15M PBS, pH 7.2 with 0.02% Tween - 20 in a heat sealable plastic bag kept for 1 hr with occasional shaking. Antibody was added (1:40) to the blocking solution and incubated in plastic bag at 4°C overnight. All the processes were done by occasional shaking. The nitrocellulose membrane was washed properly in 200ml of 150 mM NaCl, 50mM Tris HCl, pH 7.5 to remove azide and phosphate from filter before enzyme coupled reactions. Enzyme was added (1:10,000 in alkaline phosphatase buffer) and kept for 1hr at room temperature.

The membrane was washed in 150mM NaCl, 50mM Tris HCl, pH 7.5 and substrate was added (66µl NBT(Nitro Blue Tetrazolium Chloride) + 33µl BCIP(5 Bromo - 4 - Chloro - 3 Indolyl Phosphate) + 10 ml of Alkaline phosphatase buffer). The reaction was monitored carefully and when bands were observed of the desired intensity the filter was transferred to a try of 200µl of 0.5 M EDTA, pH 8.0 in 50 ml of 0.15M PBS.