Evaluation of *Metarhizium anisopliae* (Metsch.) Sorokin and *Beauveria bassiana* (Bals.) Vuill. as microbial insecticides for management of termites in agro forest eco-systems.

Thesis submitted for the Degree of Doctor of Philosophy in Science (Botany) of the University of North Bengal

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This is to certify that Mr. Rajeeb Kumar Ghosh has carried out his research work at Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal and at Research and Development Centre, Biotechnology Division, Goodricke Groups Ltd., Jalpaiguri under our joint supervision. His thesis entitled **" Evaluation of *Metarhizium anisopliae* (Metsch.) Sorokin and *Beauveria bassiana* (Bals.) Vuill. as microbial insecticides for management of termites in agro forest eco-systems"** is based on his original work and is being submitted for the award of Doctor of Philosophy (Science) degree in Botany in accordance with the rules and regulations of the University of North Bengal.

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CONTENTS

INTRODUCTION

Photosynthetic fixation of carbon dioxide in our biosphere yields approximately 136 x 10^{15}g of dry plant material annually, which represents Earth's most abundant form of biomass (Ljungdahl and Eriksson, 1985). Two major constituents of such biomass are cellulose and lignin, and hence this material is often referred to as lignocellulosic biomass, or simply lignocellulose. Most of the synthesis (about 2/3) occurs in terrestrial ecosystems where it is balanced, or nearly so, by the decomposition/ respiration side of the carbon cycle (Hobbie and Melillo, 1984). Decomposition of lignocellulose is carried out primarily by microorganisms, chiefly fungi and bacteria. However, augmenting the activities of microbes is an array of soil macro-invertebrates, whose effects may range from simple dispersion of plant material to actual dissimilation of the structural polymers of lignocellulose. Among the most abundant and important of these invertebrates are termites, which, with their associated microbial symbionts, dissimilate a significant proportion of the cellulose and hemicellulose components of the lignocellulosic plant material they ingest.

Termites cause serious economic damage to crops, forest trees, structural timbers in buildings and pastures in the tropics and the subtropics which unfortunately covers all the developing and underdeveloped countries such as Asia, Africa and Latin America (Sen-Sarma, 1995). Areas occupied by termites account for 68 percent earth's land area and 77 percent of the terrestrial net primary productivity (NPP). The world's termite population ($2.4x10^{17}$) processes material equivalent to 28 percent of the earth's annual NPP and an average of 37 percent of the NPP in areas where they occur. Out of two main groups of termites, viz, dry wood species and subterranean species, the latter are generally more harmful in this subcontinent. Around 200 species are found in India, of which *Odontotermes, Microtermes, Coptotermes, Heterotermes, Cryptotermes* and *Microcerotermes* are considered to be major pests in several parts of the country.

In order to find out the termite species available in and around the Jalpaiguri district, mainly Lataguri forest, Budhaganj forest and some other

areas like Danguajhar, Moriambasti, Dewniapara, Domohoni and Kadobari were surveyed. *Odontotermes obesus* (Plate 1C and Plate-2), *O. distans, O. horni* and *O. boveni* were collected from variable forest plant species. Extensive damage of wood due to the termite infestation by *Odontotermes obesus* were recorded (Plate-1A). Besides, infestation of *Microcerotermes sp.* was also recorded on tea plantations (Plate-1B).

Application of pesticide is one of the effective methods of pest control in forests, plantations and nurseries. However, the use of pesticides is not always advisable as this leads to the development of resistant strains of insect pests, damage caused to non-target biota, and the pollution of the environment. It is now generally agreed that insecticide application should be replaced by pest management strategies integrating silvicultural, biological, chemical and other methods to reduce the pest population below economic threshold levels. In this context, use of biocontrol agents, especially microbial pathogens, is safe in a forest ecosystem ; but, we require basic information on various pathogens associated with specific pests, their host range, and their efficacy as a control agent under both laboratory and field conditions.

Keeping in view the awareness about the environmental and contamination problems associated with the use of pesticides, it is now an opportune time to restructure the future termite control measures using the concept of integrated pest management (IPM). At present, there is a resurgence of interest in the use of entomogenous fungi for insect pest control. The muscardine fungi *Metarhizium anisopliae* (Metsch) Sorokin and *Beauveria bassiana* (Bals.) Vuill., are being evaluated worldwide for control of various pests (Hajek and Leger, 1994). As a result of the combined effects of high cost of application and environmental contamination, there has been a significant increase in the development of methods to replace the use of chemical insecticides. One such method is the development of the entomopathogenic fungi - *Beauveria bassiana* and *Metarhizium anisopliae* as bioinsecticides (Driver *et.al*, 2000, Magalhaes *et.al.*2000)

Termites are major pests of timber and timber products which live in warm, humid environments that are conducive to the development and spread of entomopathogenic fungi. Laboratory studies have shown that termite species are highly susceptible to entomopathogenic fungi from genera including the most commonly studied species, *M. anisopliae and B. bassiana* (Rath, 2000).

Field studies have shown mixed results. Direct application of fungi to nests has resulted in complete colony mortality, but studies where feeding sites or bait stations have been treated with fungus have yet to show similar success. The effectiveness of termite control in urban pest management, has yet to be reported in detail. *B. bassiana* is a widely distributed fungus of insect pest and is considered as commercial a bio-control agent (Niranjana, 2002). It has been effectively used against coffee berry borer (*Hypothenemus hampei)* in Colombia and many other coffee growing countries world wide. In search of alternatives to pesticides, mycoinsecticides, *B. bassiana* was identified to have potential in tea management by Hazarika *et. al* (2002). Use of the agent has drastically reduced the cost of production by managing the pests and minimizing the hazards caused by chemical pesticides.

The present investigation has been undertaken with a view to generate a database on microbial pathogens especially fungi associated with termites and to evaluate the efficacy of two fungal pathogens *Metarhizium anisopliae* (Metsch.) Sorokin and *Beauveria bassiana* (Bals.) Vuill., against available termite species (*Odontotermes sp.*) in laboratory as well as in field conditions with the following objectives :

1. Identification of strains of entomopathogenic fungi (*Metarhizium anisopliae* and *Beauveria bassiana*) with greater virulence.
2. Pathogenicity studies, standardization of conidial count of entomopathogenic fungi.
3. Determination of qualitative and quantitative effects of characterization of termite colonies (*Odontotermes obesus*,) foraging in terms of activity, population and territory using fungal bait blocks.
4. Development of mass production and formulation techniques in terms of dosage, time, shelf life, and storage stability of entomopathogenic fungi.
5. Serological detection of entomopathogenic fungi using immunoassays and immunofluorescence in the field.
6. Development of microbial insecticidal program and integration with other methods of management.

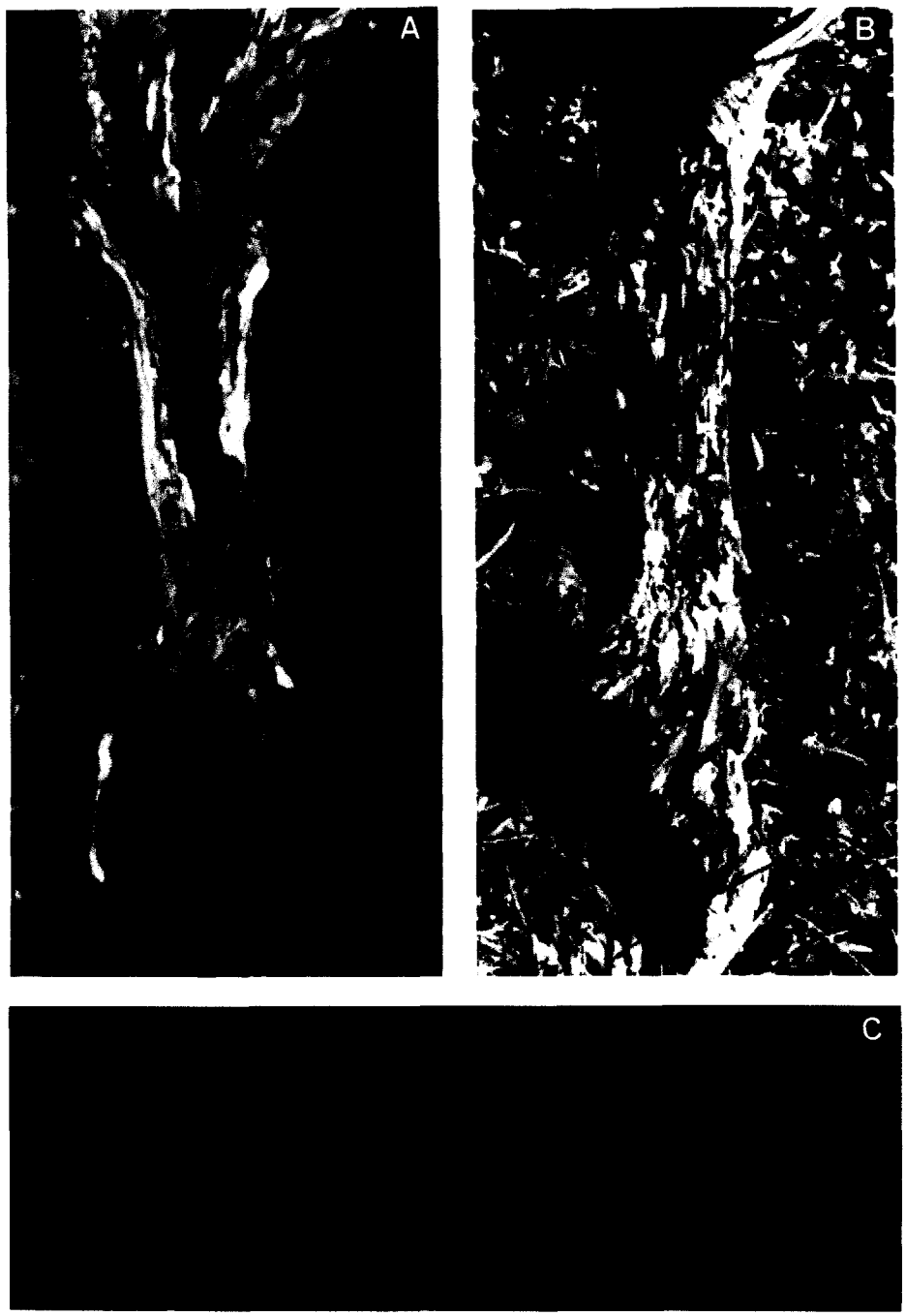


Plate 1 (A - C) : Termite infestation and damage of Teak (A) and Tea (B) plants; Underground termite nests (C).

REVIEW OF LITERATURE

Termites and their Microbial Symbionts

Termites belong to the order Isoptera, which includes over 2000 described species whose biology; behaviours, and nutritional ecology are remarkably diverse. Although frequently thought of as feeding on wood, their diet (depending on particular species) includes a variety of lignocellulosic plant material (either sound or in various stages of decay), as well as materials derived from it (e.g. animal dung), and soil rich inorganic matter (i.e. humus) as stated by Wood and Johnson (1986).

According to Wood and Sands((1978), roughly 2/3 of the Earth's land surface that lying between 45°N and S latitude is inhibited by one or more termite species. However, their populations are greatest in tropical and subtropical regions, where their numbers can exceed 6000 m^{-2} and their biomass densities (>50gm^{-2}) often surpass that of grazing mammalian herbivores (0.013-l7Sgm^{-2}) as stated by Collins and Wood (1984) and Lee and Wood (1971). It has been estimated that termites from all ecological regions (approximately $2x10^{17}$ individuals) together consume about $3\text{-}7x10^{15}$g of lignocellulose annually and mineralize a significant portion of it as stated by Collins and Wood (1984) and Khalil and Rasmussen (1990).

According to Krishna (1969) and (1970), termites are divided into two groups. The so-called lower termites (families Masto, Kalo, Hodo, Rhino and Seritermitidae) harbor in their alimentary tract a dense and diverse population of bacteria and cellulose digesting, flagellate protozoa. The latter represent unique genera and species found virtually nowhere else in nature as stated by Honigberg (1970). Higher termites (family Termitidae), comprising three fourths of all species, also harbor a dense and diverse array of gut bacteria, but they typically lack protozoa and have a more elaborate external and internal anatomy and social organization than do the lower termites.

The termite gut consists of the foregut (which includes the crop and muscular gizzard), the tubular midgut (which, as in other insects, is a key site for secretion of digestive enzymes and for absorption of soluble nutrients), and the relatively voluminous hindgut (which is also a major site for digestion and

for absorption of nutrients, and which may be further subdivided into various regions). Malpighian tubules, which transport urine and urinary metabolites for excretion, empty at or near the junction of the mid-and hindgut. Many higher termites also possess a so-called mixed segment, which is a portion of the gut consisting on one side of cuticle lined hidgut tissue as stated by Noirot and Noirot(1969).

Owing to the relatively small size of most termites, and microenvironmental conditions existing within the gut have been difficult to define precisely, especially with techniques that impose little or no invasiveness. Nevertheless, anoxic conditions become more apparent as one moves from the foregut to the hindguts typically have a relatively low redox potential (ranging from - 50 to 270mV in different species) and a pH around neutrality (6.2 to 7.6), but in soil-feeding termites some portions of the hindgut (i.e. the relatively large P1 region, immediately posterior to the mixed segment, but anterior to the enteric valve) may have a pH as high as 11 as indicated by Bignell (1984), Bignell *et. al.* (1980), Veivers *et. al.* (1980) and Veivers *et. al.*(1982).

The morphological diversity of the termite gut microbiota is remarkable and has been documented in recent years for both lower, Breznak and Pankratz (1977) and To *et. al.*(1980); and higher termites, Bignell, *et. al.* (1980), (I980a) and (1983), Czolij *et. al.* (1985). Although some bacteria colonize the fore-and midgut [some rather distinctive morphotypes are situated in the midgut between microvilli, Breznak, *et. al.* (1977); in the ectoperitrophic space Bignell *et. al.*(I980); and in the mixed segment, Bignell *et. al.* (1980a), Bignell *et. al.* (1983) and Kovoor (1968)], the bulk of the intestinal microbiota is found in the hindgut, especially in the paunch, the region immediately posterior to the enteric valve.

Gut microbes are important for termite vitality and much of the termites nutrition derives from the products of microbial metabolism. Acetate, propionate, and other organic acids produced during microbial fermentation of carbohydrate in the hindgut are important oxidizable energy sources for termite, Odelson and Breznak (1983), as well as carbon skeletons for biosynthesis, Blomquist *et. al.*(1979) and (1982), Gou *et. al.* (1991), Mauldin (1982) and

Presturich *et. al.* (1981).

Gut bacteria are important to nitrogen economy. They enhance the ability of termites to acquire new N through N_2 fixation, Bentley (1984) and Breznak (1984), and to conserve N by helping to recycle excretory N (uric acid) back to the termites for biosynthesis, Potrikus and Breznak (1981).

Spirochetes, a group of highly motile, spiral or undulate bacteria defined by distinctive ultrastructural features, Canale-Parola (1984), are a major component of the gut microbiota of all termites examined. Spirochetes rarely occur in nature in as great a density and morphological diversity as in the gut of termites, Breznak (1984) and Margulis and Hinkle (1992). The range in size from 0.2x3mm, Breznak (1982), to 1.0 x 100mm, Hollande and Gharagozlou (1967), with analogous variations in wavelength and amplitude of the spiral cell body. Comparative morphometry prompted Bermudes *et. al.* (1988) to create, or revive, generic and specific epithets for a number of distinctive spirochetes in the hindgut of termites.

Spirochetes exist free in the gut fluid of all termites. In the lower mites they also occur within the cytoplasm, or they are attached to the surface of hindgut protozoa, Breznak, J.A. (1984) and participate in the motility of some of these protozoa, Cleveland and Grimstone (1964). Little else is know about termite gut spirochetes and their effect on termite vitality, as they have never been isolated and studied in pure culture. A reduced lifespan, Eutick *et. al.* (1978) and transient colonization of the gut by nonindigenous bacteria, *Serratia marcescens* was observed by Veivers *et. al.* (1982) when spirochetes were eliminated from *Nasutitermes exitiosus* by feeding the termites metronidazole, or by incubating them under pure oxygen. Such results are ambiguous, however, because elimination of spirochetes might have been accompanied by elimination of other, less morphologically conspicuous bacteria. In any case, there is no reason to believe that spirochetes are pathogenic to termites. They do not invade the gut epithelium, and termites harboring them appear vigorous and healthy.

According to Breznak and Brune (1994), higher termites in the subfamily Macrotermitinae have established an intriguing symbiotic, relationship with external basidiomycete fungi of the genus *Termitomyces*. The fungi are cultivated within the nest in convoluted, greyish-brown combs, consisting of plant material undergoing partial digestion by the fungal mycelium that permeates it, and on which the fungus develops round white nodules, or mycotetes, composed of masses of asexual spores (conidia).

The fungus combs are dynamic. New comb is formed by deposition of fresh termite feces containing finely comminuted (but only partially digested) plant material, which then becomes infiltrated with *Termitomyces* spp. Termites then feed upon older (i.e. more seasoned) parts of the comb, including the fungal nodules. Wood and Thoma (1989) have reviewed the biology of this fascinating association; however, a key question has always been the role of the fungus in termite nutrition. Evidence indicates that at least a partial digestion of plant polysaccharides and lignin by *Termitomyces* spp. can occur within the comb, Rohrmann and Rossman and Veivers *et. al.* (1991).

There is a widespread perception among biologists that cellulases are restricted to microbes. Although this restriction doesnot appear to be true, production by metazoans of cellulase components capable of depolymerizing polyglucan i.e. endo and exoglucanases) is relatively rare, Martin (1983) and (1987). A reasonably strong case can be made for symbiont-independent production of cellulases by termites, Slaytor (1993).

Lower Termites

Studies of axenic cultures of the protozoa (*Trichomitopsis termopsidis* and *Trichonympha sphaerica*), have shown the presence of CMC ase (i.e. an enzymatic activity capable of releasing reducing sugar from carboxy methylcellulose (CMC)], b-glucosidase, and CHA (cellulose hydrolyzing activity) in cell extracts, Odelson and Breznak (1985) and (1985a), Yamin (1978), (1980), and (1981), Yamin and Trager (1979).

According to Hogan *et. al.* (1985), O' Brien and Braznak (1984) and

Odelson and Braznak (1983), in termite hindgut fluid, acetate occurs at concentrations up to 80mM and can constitute over 90 mol% of the volatile fatty acid pool. It is taken up from the hindgut and oxidized to CO_2 and H_2O by termite tissues, a process that can support up to 100% of the respiratory requirement of some species.

Despite the critical role of hindgut protozoa in cellulose digestion by lower termites, evidence has also mounted that the termites themselves can produce cellulase components. Some workers suggested that some cellulase components' were produced by lower termites, Mishra (1980), Mishra and Sen Sarma (1987), Retief and Hewitt (1973), Yamaoka and Nagatani (1975) and Yokoe (1964).

Veivers *et. al.* (1982) demonstrated the presence of CMCase, CHA, and cellobiase in salivary glands, foregut, midgut, and hindgut of *Mastotermes darwiniensis.* Although most of the cellulase activity, Cookson (1992), was generally found in the hindgut and could be attributed largely to protozoa, the other enzyme activities were presumably of termite origin. Amylase and maltase activities were also found in salivary glands and midgut, indicating that *M. darwiniensis* should be able to utilize starch as a nutrient. Indeed, it was later discovered that *M. darwiniensis* could survive on a diet of starch, although this led to a loss of the large protozoa from the hindgut and, with them, the bulk of the hindgut cellulase activity, Veivers *et. al.* (1983). Such termites could no longer survive on a diet of wood, unless they were refaunted with cellulolytic protozoa. Presumably, when the termites were fed starch, a substrate more readily hydrolyzable than cellulose, the protozoa no longer had a competitive advantage in the hindgut and were displaced by bacteria, whose numbers increased. Curiously, starch and carbohydrates other than cellulose are also poor substrate for the *invitro* growth of the termite gut-protozoan *Trichomitopsis termopsidis*, Odelson and Breznak (1985) and Yamin (1978), despite the fact that such protozoa possess xylanase and amylase activities, Odelson and Breznak (1985). Even celluloses whose degree of polymerization is low will cause full or partial loss of the large cellulolytic protozoan *Pseudotrichnympha grassi* when fed to *Coptotermes formosanus*, with consequent decreased survivorship of

the host Yoshimura *et. al.* (1993).

A similar picture emerged in studies of *Coptotermes lacteus*, wherein a termite origin was attributed to enzymes that were found in regions anterior to the hindgut and that persisted following removal of protozoa, spirochetes, and other bacteria by treatment with oxygen or antibacterial drugs, McEwen *et. al.* (1980) and O'Brien *et. al.* (1979). Column chromatography of enzyme activities of putative termite origin (i.e. those from pooled extracts of salivary glands, foregut, and midgut) revealed a multiplicity of activities as stated by Hogan et. at (1988). Enzymes of apparent termite origin included an exoglucanase capable of releasing glucose from cellulose or CMC, two endoglucanases (each with activity on CMC, but not on cellulose), and at least one glucono-d-lactone-inhibitable b-glucosidase (=cellobiase). Enzymes from protozoan extracts included an exoglucanases with gluconolactone - inhibitable cellobiases.

According to Bignell and Anderson (1980) and Noirot and Noirot (1969), in higher termites, the anatomy of the gut is more elaborate and multicompartmentalized than that of lower termites, with some regions possessing cuticular spines protruding into the lumen. Such specializations may be sufficient for increasing the exposure time of lignocellulose food to hydrolytic enzymes, making the protozoa unnecessary.

Higher Termites

Kovoor (1968) and Potts and Hewitt (1973) were among the first to suggest that higher termites synthesize their own cellulases. They found CMCase and cellobiase activities in regions of the gut (midgut and midgut wall) that were virtually of microscopically observable bacteria. A partially purified CMCase behaved like a true endonuclease, but with some hydrolytic activity on xylan as well, Potts and Hewitt (1974) and (1974a), although the starting material used (extracts of whole termite abdomens) compromised the strength of the conclusion that the enzyme was truly of termite origin.

A stronger case for a termite origin ofcellulases has come from slaytors group, working with *Nasutitermes exitiosus* and *Nasutitermes walkeri*, Hogan

et. al. (1988), McEwen *et. al.* (1980), O'Brien *et. al.* (1979), and Schutz et. at (1986). They showed that (a) the bulk of the cellulase activity, including a CHA (referred to as exoglucanase, based on its ability to liberate glucose from microcrystalline cellulose), an endoglucanase, and a b-1, 4-glucosidase were present in the midgut, with a significant amount of endoglucanase and b-glucosidase associated with the midgut epithelium of *N. walkeri*; (b) the cellulase activity of *N. exitiosus* showed little or no change when gut bacterial populations were drastically reduced by starving the termites or feeding them tetracycline; and (c) attempts to reveal putatively occult, cell-bound cellulases from hindgut bacteria by sonication or by treatment with lysozyme ± EDTA were fertile. Importantly, the CHA activity of *N. walkeri* was significant in terms of the termite's respiratory activity, assuming that the CHA activity *in vitro* was comparable to that *in vivo*, and that oxidation of glucose (and/or a metabolite(s) derived from glucose) accounted for most of the oxygen consumption by this termite as stated by Breznak (1990).

The mechanism of cellulose digestion in the Macrotermitinae is not yet completely clear. The first evidence that ingestion of fungal comb contributed enzymes for cellulose digestion came from Martin and Martin's studies of *Macrotermes natalensis,* Martin and Martin (1978) and (1979). They found that most of the cellulase activity was located in the midgut and consisted of a C_1-cellulase activity (assayed as CHA), a C_x-cellulase activity (assayed as CMCase), and a b-glucosidase activity. The latter two were produced, in part, by the salivary glands and midgut. However, the C_1 -cellulase activity (which conferred upon midgut contents the ability to degrade crystalline cellulose) appeared to be derived only by ingestion of nodules present on the fungal comb. Evidence supporting this conclusion came from analysis of enzyme activities present in midguts of termites that were starved, or were fed normal or nodule-free comb material, Martin and Martin (1978), and from isoelectric focusing of enzyme activities present in midguts and in fungal nodules, Martin *et. al.* (1979).

The C_x(i.e. CMCase) components were presumably endoglucanases, because they had no activity on crystalline cellulose. However, it is not possible to tell whether the critical C_1-cellulase activities of fungal origin were endo-or

exoglucanases, because substrate-product relationships were not established. These studies led to the hypothesis that mycophagy (i.e. the ingestion of fungal tissue) may result in the acquisition of enzymes that permit an animal to increase efficiency of digestion, and perhaps to expand its range of natural substrates, Martin (1987). Abo-Khatwa (1978) reached a similar conclusion for *Macrotermes subhyalinus* on the basis of a somewhat limited study.

Rouland and co-workers achieved the first purification of cellulase components for *Macrotermes mulleri* and its associated *Termitomyces* sp. They were able to distinguish two b-glucosidases, one of termite origin and one from fungal nodules, Rouland *et. al.* (1986). After being characterized with respect to catalytic and physical properties, they were judged to be identical and of fungal origin, i.e.L_T=I_F Rouland *et. al.* (1988). I_F and I_T were both monomeric glycoproteins of M_r 34,000 KDa and showed respectable hydrolytic activity on microcrystalline cellulose, liberating reducing sugar at a rate roughly 5-10% of that observed with CMC as substrate. However, in contrast to results with *M. natalensis* the enzyme showing greatest activity on pure celluloses (enzyme II) was entirely of termite origin and appeared to be a cellobiohydrolase, Rouland *at. al.* (1988).

Enzyme II, a monomeric protein of M_r 52,000 KDa, also had relatively high activity on CMC, about 2/3 of that of component I. Synergism in CHA was observed between component I_F and enzyme II, and the synergism was further enhanced by the additional presence of the termite b-glucosidase, Rouland *et. al.* (1988a). This synergistic interaction between cellulase components may explain, in part, the enhanced survivorship of *M. mulleri* in the presence of its symbiotic *Termitomyces* sp., Rouland *et. al.* (1988b).

From the recent studies of Veivers *et. al.* (1991)*Macrotermes michaelseni* and *M. subhyalinus* another scenario for cellulose digestion has emerged. Cellulase activity in this species was greatest in the four worker castes (young and old, major and minor) and, again, most of the cellulase in the termites was found in the midgut, and in the fungus mainly in the nodules. Each cellulase complex comprised endoglucanase and b-glucosidase activities, both of which

were multicomponent as judged by column chromatography. When column chromatographic elution profiles of midgut and nodule endocellulases were compared, only about 90% of the endoglucanase activity in the midgut could be attributed to nodule enzymes. Thus, for *M. michaelseni* and *M. subhyalinus*, it seemed as though fungal enzymes were essentially irrelevant to cellulose hydrolysis in the midgut.

Cellulose digestion

Despite various reports on the isolation of cellulolytic bacteria from gut contents of lower or higher termites, there has never been a convincing demonstration that such bacteria are quantitatively significant to cellulose hydrolysis in situ. Much of the early literature on this issue has been reviewed previously. Breznak (1982) and (1984), O'Brien and Slaytor (1982). Current reports include one of the isolation of cellulolytic actinomycetes (*Streptomyces* spp. and *Micromonospora* spp.) from wood feeding, soil-feeding and fungus-cultivating termites, Pusti and Belli (1985) and on isolation of the cellulolylic *Clostridim termitidis* from *Nasutitermes lujae*, Hethener *et. al.* (1992).

Most of the energy available to termites from cellulose digestion appears to come from oxidation of the acetate derived from cellulose, Odelson and Breznak (1983). Although higher and lower termites have all the enzymes necessary for converting glucose to pyruvate, and for oxidizing acetate to CO_2 and H_2O via the tricarboxylic acid (TCA) cycle, they appear to lack pyruvate hydrogenase or any other enzyme capable of converting pyruvate to acetyl CoA or acetate to feed into the TCA cycle, as said by O'Brien and Breznak (1984). Thus, production of acetate in the hindgut, from glucose or other intermediates of lignocellulose degradation, may be a major, critical role of many of the microbes harbored there. The bacteria capable of fermenting glucose and / or cellobiose to acetate are present in guts of higher and lower termites as refered by Eutick *et. al.* (1978) and Schutz *et. al.* (1986). The bacteria are capable of forming acetate from the reduction of CO_2 and removal of gut bacteria from higher or lower termites usually results in death of the termites within a relatively short time as indicated by Eutick *et. al.* (1978) and O'Brien *et. al.* (1979). If the

mentioned hypothesis is true, one has to wonder why both higher and lower termites have apparently turned the process of acetate formation over to their gut microflora. At this moment there is still no clear answer. However, considering that termites thrive on diets relatively poor in nitrogen, and that many bacteria can use inorganic N or even N_2 as sole N source, one might speculate that this represents an evolutionary trade-off in order to maintain a population of microbes capable of upgrading poor sources of N to nutritious ones.

Hemicelluloses hydrolysis

Xylanase activity has been demonstrated: (a) in the hindgut of lower termites, Mishra (1980), where it is produced, at least in part, by hindgut protozoa, Odelson and Breznak (1985); (b) in the gut of soil-feeding termites, Rouland *et. al.* (1989); and (c) in the gut contents and fungal nodules of fungus-cultivating termites, Martin and Martin (1978) and Rouland *et. al.* (1988). According to Potts and Hewitt (1974a), partial purification of a cellulase from the higher termite *Trinervitermes trinervoides* revealed that it was also capable of hydrolyzing xylan. However, the most detailed information on xylanases so far has come from a single report by Rouland *et. al.* (1988d). These investigators purified two xylanases : one from workers of *M. mulleri* (X), and one from nodules of its symbiotic *Termitomyces* (X_F). Both were glycoproteins and proved to be identical with respect to physical and catalytic properties, including molecular weight (28.5KDa), pH optimum (5.2), and substrate specificities. Hence, they were judged to be the same enzyme, whose origin was the fungus and which was acquired by the termites through ingestion of nodules. Given the quantitative significance of xylan in lignocellulosis plant material, and its high digestibility by termites, the xylanases constitute an enzyme system sorely in need of further study.

Lignin Degradation

Studies on lignin degradation in termites are scarce. Early estimates according to Breznak (1982), based on chemical analysis of lignin in food versus feces, range from virtually no degradation to astonishingly high values (83%).

These discrepancies are not surprising, given the problems and potential pitfalls of accurate determination of the lignin content of sound wood, Lai and Sarkanen (1971), let alone of fecal materials. Approaches using radiolabeled lignin preparations are better suited to address the question of lignin digestibility in termites. The release of $^{14}CO_2$ as a final respiration product is a sure indication of complete mineralization of those subunits canying the lable.

Entomopathogenic fungus

Hallsworth and Magan (1999) evaluated the effects of temperature (5-50°C), water availability (0.998-0.88 water activity, aw), and aw X temperature interactions (15-45°C) on growth of three entomogenous fungi, *Beauveria bassiana, Metarhizium anisopliae*, and *Paecilomyces farinosus*, on a sabouraud dextrose-based medium modified with the ionic solute KCl, the non-ionic solute glycerol, and an inert solute, polyethylene glycol (PEG) 600. The temperature ranges for growth of *B. bassiana*, *M. anisoplae* and *P. farinosus* were 5-30, 5-40, and 5-30°C and optimum growth temperatures were 25, 40 and 20°C, respectively. All three species grew over a similar aw range (0.90-0.998) at optimum temperatures for growth. However, there were significant interspecies variations in growth rates on media modified with each of the three aw-modifying solutes. Growth aw optima ranged between 0.99 and 0.97 on KCl- glycerol, and PEG 600-modified media for *M.anisopliae* and *P. farinosus*. *B. bassiana* grew optimally at 0.998 aw, regardless of aw. Comprehensive two-dimensional profiles of aw x temperature relations for growth of these three species were constructed for the first time. There results were discussed in relation to the environmental limits that determine efficacy of entomogenous fungi as biocontrol agents in nature.

De-Croos and Bidochka (1999) assessed the effects of temperature (8°C, I5°C and 22°C) on germination, growth rate and conidia production in thirty-two isolates of the entomopathogenic fungus *Metarhizium anisopliae*. Most isolates were obtained from various locations in Ontario, Canada. Ten out of thirty-two (31-3%) isolates were deemed cold-active because of their ability to grow at 8°C. Growth rates in all isolates increased as the incubation temperature was

increased. There were significant differences in growth rates and conidia production among isolates. They observed that conidia production among isolates. They observed that conidia production had no relationship with a growth rate. With respect to the geographical origin, there was no general relationship between latitude and growth rates. However, they found that all the cold-active isolates were isolated from the more northern sites and no isolate originating below 43.5 degree latitude showed cold activity. Cold-active germination and growth of this biocontrol fungus have implications for strain selection and application in Canadian insect control efforts.

Jeffs *et. al.* (1999) studied the surface properties of aerial conidia from 24 strains of entomopathogenic fungi and compared using the salt-mediated aggregation and sedimentation (SAS) assay, electron microscopy, FITC-labelled lectins, and spore dimensions. Spores with rugose surfaces were hydrophobic, where as hydrophilic spores had smooth surfaces. Correlation analysis found no link between spore dimensions and either hydrophobicity or surface carbohydrates. However, they observed a strong positive correlation between spore hydrophobicity and surface carbohydrates .The three spore types of *Beauveria bassiana* were all shown to possess discrete surface hydrophobicities which were also strongly linked to surface carbohydrate profiles. Various chemical treatments had pronounced effects on spore surface properties, with sodium dodecyl sulfate (SDS) and formic acid reducing both lectin binding and surface hydrophobicity. When formic acid-protein extracts were separated and analysed using SDS-PAGE, only the hydrophobic spores had low molecular weight hydrophobin-like peptides that were unglycosylated and contained disulfide bonds. The strains with hydrophilic aerial conidia had much lower levels formic acid extractable protein per spore dry weight compared to their more hydrophobic counterparts.

The differentiation of a Brazilian isolate of *Metarhizium flavoviride*, a promising candidate for the biocontrol of grasshoppers, was investigated by Xavier *et. al.* (1999). Conidia were spread onto solid medium (1% yeast extract, 2.8% agar, 96.2% distilled water), incubated at 28°C and observed during 26h.

Germination initiated as conidia size increased from 5.3 (± 0.6) x 3.1(± 0.3) mum (Oh incubation). Germ tubes started to appear after 10h incubation showing a high degree of multipolarity. Twenty six hours after inoculation, hyphal differentiation and anastomosis among hyphae from adjacent conidia were recorded. Appressoria were formed only from conidia incubated in liquid medium containing minimum concentration of yeast extract (0.06% w/v). Appressoria were firmly adhered to the bottom plastic dishes.

Strain of hydrocarbon-degrading microorganisms (bacteria and fungi) were isolated from an agricultural soil in France by Chaineau *et. al.* (1999). In a field, a portion was treated with Oily cuttings resulting from the drilling of an onshore well. The cuttings which were spread at the rate of 600g HC m-2 contained 10% of fuel oil hydrocarbon (HC). Another part the field was left untreated. Three months after HC spreading, HC adapted bacteria and fungi were isolated at different soil depths in the two plots and identified. The biodegradation potential of the isolated strains was monitored by measuring the degradation rate of total HC, saturated hydrocarbons, aromatic hydrocarbons and resins of the fuel. Bacteria of the genera *Pseudomonas*, *Brevundimonas*, *Sphingomonas, Acinetobacter, Rhodococcus, Arthrobacter, Corynebacterium* and fungi belonging to *Aspergillus, Penicillium, Beauveria, Acremonium, Cladosporium, Fusarium* and *Trichoderma* were indentified. The most active strains in the assimilation of saturates and aromatics were *Arthrobacter* sp., *Sphingomonas spiritivorum, Acinetobacter baumanii, Beauveria alba,* and *Penicillum simplicissimum.* The biodegradation potential of the hydrocarbon utilizing microorganisms isolated from polluted and unpolluted soils were similar according to them. They indicated that, in laboratory pure cultures, saturated HC were more degraded than aromatic HC, where as resins were resistant to microbial attack. They concluded that on an average, individual bacterial strains were more active than fungi in HC biodegradation.

Smits *et. al.* (1999) say that *Metarhizium flavoviride*, a fungal pathogen of grasshoppers and locusts, appears to be an effective, nop-chemical insecticide (mycoinsecticide) for control of grass hoppers and locusts. They

conducted a study during June and July, 1997. and examined the pathogenic potential of this entomopathogenic fungus to non-target avian species that encounter infected insect prey items or contaminated food sources. Ring-necked pheasant (*Phasianus colchicus*) chicks were exposed to one of three diets, (Spore-coated feed, infected insects, or untreated feed), either from 4 to 9 days of age, or, from 35 to 40 days of age. Necropsies were conducted on birds 10 days and 46 days old, respectively. They observed that neither consumption of infected insects, nor of spore-coated feed, resulted in pathological changes, or significant changes in weight growth rate, behaviour, or mortality rate. Histological examination of organs indicated either no changes related to treatment, or normal tissue responses to antigenic challenge.

Kmitowar *et. al.* (2000) performed laboratory experiments and assessed on effect of plant extracts from stingin nettle (*Urtica dioica* L.) wormwood (*Artemisia absinthium* L.) and field horsetail (*Equisetum arvense* L.) on the colony growth, sporification, germination and pathogenicity level of the four species of entomopathogenic fungi *Beauveria bassiana* (Bals.) Vuill., *Paecilomyces farinosus* (Dicks) Brown et. Smith, *P. fumosoroseus* (Wize) Brown et. Smith and *Metarhizium anisopliae* (Metsch.) Sorok. The pathogenicity was tested on *Galleria mellonella* (L.) larvae. A reaction to extract added to the medium was species - specific. The smallest changes of studied parameters under the influence of the extracts were observed in *P. fumosorseus*, and the largest in *M. anisopliae*. Studied extracts, those from nettle and wormwood in particular, influenced colony growth and germination of the spores while a horsetail extract influenced spore production from the unit area.

Wagner and Lewis (2000) used light and electron microscopy to describe the mode of penetration by the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin into corn, *Zea mays* L. After inoculation with a foliar spray of conidia, germinating hyphae grew randomly across the leaf surface. Often a germtube formed from a conidium and elongated only a short distance before terminating its growth. Not all developing hyphae on the leaf surface penetrated the cuticle. However, when penetration did occur the penetration sites were ramdomly located, indicating that *B. bassiana* doesnot require specific

topographic signals at an appropriate entry site as do some phytopathogenic fungi. Long hyphal structures were observed to follow the leaf apoplast in any direction from the point of penetration. A few hyphae were observed within xylem elements. This is because bundles are interconnected throughout the corn plant, this may explain how *B. bassiana* travels within the plant and ultimately provides overall insecticidal protection. Virulency bioassays demonstrate that *B. bassiana* does not lose virulence toward the European cornborer, *Ostrinia nubilalis* (Hubner), once it colonizes corn. This endophytic relationship between an entomopathogenic fungus and a plant suggests possibilities for biological control, including the use of indigenous fungal inocula as insecticides.

A novel Chitinase was detected by Kang *et. al.* (1999) in extracellular culture fluids of the entomopathogenic fungus *Metarhizium anisopliae* (ATCC 20500) grown in liquid medium containing Chitin as a sole carbon source. They purified Chitinase to near homogeneity from culture broth of *M. anisopliae* by DEAE-sephadex, CM-Sepharose CL-6B ion-exchange chromatography, and gel filtration with Superose 12 HR. The molecular mass of the enzyme determined by SDS-polyacrylamide gel electrophoresis was approximately 60 KDa and the optimum pH of the enzyme was 5.0. This molecular mass is different from values of 33, 43.5, and 45 KDa for endochitinases and 110 KDa for an exochitinase (N-acetylglucosaminidase) from *M. anisopliae* ME-1 published previously. In addition they found that N-terminal sequences of 60-DKa Chitinase are different from those of 43.4 and 45-KDa endochitinases. The purified enzyme showed high chitinolytic activity against colloidal, crystalline chitin of crab shells as well as against p-nitrophenyl-beta-D-N-acetylglucosamide, pnitrophenyl-beta-D-N, N'-diacetylchitobiose, and p-nitrophenyl-N, N'-N"-triacetylchitotriose, indicating that tins enzyme has both endo-and exochitinase activity.

The biohydroxylation of a series of amides and related amino, Keto and hydrocarbon substrates by the fungal biocatalyst Beauveria bassiana ATCC 7159 has been examined by Holland *et. al.* (1999). The product distributions, together with data obtained from selective inhibition experiments using Cyt. P-450 inhibitors isosafrole, l-amino-benzotriazole and phenylacetylene, suggest

that *B.bassiana* contains a range of hydroxylase enzymes with different substrate specificities. They presented a paradigm for the interpretation of the results of microbial hydroxylation and for the application of existing active site models for *B.bassiana*.

Beauveria sp. BTMF S10 isolated from marine sediment produced extracellular L-glutaminase as observed by Keerthi *et. al.* (1999). Maximal L-glutaminase yield (46.8 U/ml) was obtained in a medium supplemented with 1% (w/v) yeast extract and sorbitol, 9% (w/v) sodium chloride and 0.2% (w/v) methionine, initial pH 9.0 and at 27 degree C after 108h. This enzyme was inducible and growth-associated as stated by Keerthi *et. al.* (1999).

St. Leger *et. al.* (1999) stated that ambient pH regulates the expression of virulence of *Metarhizium anisopliae*, but it was unknown if *M. anisophae* can regulate ambient pH. Mutants of *M anisopliae* altered in production of oxalic acid were evaluated for the interrelationship of ambient pH, buffering capacity added to media, growth, and generation of extracellular proteases and ammonia. Wild-type and acid-overproducing mutants (Acid(+)) grew almost as well at pH 8 as at pH6, but acid, non producing (Acid (-)) mutants showed limited growth at pH 8, indicating that acid production is linked to the ability to grow at higher pH. Production of ammonia by *M.anisopliae* was strongly stimulated by low levels of aminoacids in the medium when cells were derepressed for nitrogen and carbon. Likewise, although *Aspergilus fumigatus* and *Neurospora crassa* produced some ammonia in minimal media, addition of low levels of amino acids enhanced production. Ammonia production by *A .fumigatus*, *N. crassa* and *M.anisopliae* increased the pH of the medium and allowed production of subtilisin proteases, whose activities are observed only at basic pH. In contrast, protease production by the Acid (+) mutants of *M.anisopliae* was greatly reduced because of the acidification of the medium. This suggests that alkalinization by ammonia production is adaptive by facilitating the utilization of proteinaceous nutrients. Collectively, the data imply that ammonia may have (functions related to regulation of the microenvironment and that it represents a previously unconsidered virulence factor in diverse fungi with the potential to harm tissues and disturb the hosts immune system.

Epoxide hydrolase activity was produced during the exponential and stationary growth phases of the fungus *Beauveri bassiana* ATCC 7159 as observed byMoussou *et. al.* (2000). They said that it was completely cell-associated. After cell disruption epoxide hydrolase activity was recovered in both the cell debris (EH "A") and the soluble fraction (EH "B"), but not in the membrane fraction. Activity assays of these fractions with two different substrates indicated that this substrate specificity, as well as the corresponding E value and, to a lesser extent,their regioselectivity, were different. Also, they could observe that the absolute configuration of the residual epoxide was opposite. This indicates that these two epoxide hydrolase activities are substantially different and are, therefore interestingly complementary biocatalysts for the preparation of the corresponding epoxides and or vicinal diols in nearly enantiopure form.

Mass production, Formulation and Application

Aerial conidia of *Metarhizium anisopliae* (flavoviride) var. acridum strain IMI 330189 were used by Cherry *et. al.* (1999) for the inundative biological control of grasshoppers and locusts in sub-Saharan Africa. Those aerial conidia were produced in a purpose-built facility at the International Institute for Tropical Agriculture in Benin using a standard, two-stage mass production system. The yields average 31. lg of dry conidia powder /kg of rice substrate, the production capacity was 300-350 kg of conidial year and the production costs were estimated at US dollar sign 21/100g (their recommended dose for I ha). The production process parameters varied within narrow limits established during optimization, but the yield was characterized by a high level of variation over time. The incubation period and temperature were identified as key factors, although they accounted for less than 40% of the yield variation. The variation in conidial viability and contamination were correlated with several parameters, but none could adequately explain that variation. They said that the handling time, a principal limiting factor, could be reduced by increasing the substrate quantity unit of production. They concluded that an awareness of these factors presented the opportunity to fine tune production, although the options for increasing or improving production efficiency were limited within the constraints

of the system.

Four fungicides were used for controlling foliar diseases of potato (*Solanum tuberosum*) and they were evaluated under field and laboratory conditions for their effects on the colorado potato beetle, *Leptinotarsa decemlineata* (CPB) by Jaros *et. al.* (1999). They investigated the direct effect of time between fungicide and *B. bassiana* application. Effects of fungicide on conidial survival in soil and on foliage were examined by them in the field. Jaros *et. al.* (1999) observed significantly more larval mortality when larvae were sprayed with *B. bassiana* than with water control. Fungicide had no significant effect on larval mortality in the field. In the laboratory, survival of larvae was significantly lower among larvae fed fungicide-treated foliage. They observed that *B. bassiana*-induced mortality in the laboratory was only when larvae were fed foliage treated with copper hydroxide or water. Larvae fed mancozeb or chlorothalonil-treated foliage experienced high mortality regardless of *B. bassiana* treatment. They said that, while there was no significant effect of fungicide on *B.bassiana* sporulation on cadavers in the field, a pattern emerged that indicated higher proportions of cadavers producing conidia in plots sprayed with water or copper hydroxide than in plots sprayed with chlorothalonil or mancozeb. They concluded that survival of *B. bassiana* conidia in the soil and on foliage was significantly greater in plots treated with copper hydroxide or water than in plots treated with macozeb or chlorothalonil. According to them fungicides such as copper hydroxide may be less deleterious to the fungus than mancozeb and chlorothalonil.

The entomopathogenic fungus *Metarhizium anisopliae* was formulated into a bait for assessment against *Musca domestica* (housefly) by Renn *et. al.* (1999). They tested the efficacy of the formulated bait by placing either one, two or four baits on the floor of a $10m^3$ polythene cubicle and releasing 100 female and 50 male *M.domestica.* They observed that one, two or four baits had equal effects and, between 95.2 and 100% of flies were killed after 10 days. In a second experiment, the durations of visits by single or pairs of flies to the baits and subsequent pick-up of conidia were recorded by them. Single flies

remained in the baits for tip to 33min and picked up a mean of 38235 + 8291 conidia. When pairs of flies were exposed, the individuals picked up a mean of 95879 + 23838 conidia, which was significantly more ($P<0.05$) than the single flies, although the mean duration of the visits was not significantly different ($P>0.05$). However they observed that the numbers ofconidia picked up by the flies didnot correlate with the duration of the visit. Lastly they exposed 25 female and 25male flies in 0.027m^3 arenas, to I0 *M.anisopliae* - killed house flies, showing sporulating hyphae. After 8 days, they noticed that 97.3% of female flies and 100% of male houseflies had succumbed to infection with *M.anisopliae*.

Lopez *et. al.* (1999) used six plant waste substrates Palm leave (*Phoenix dactyhfera, Phoenix canariensis, Washingtonia filifera* and *Chamaerops humilis*), *Phoenix dactylifera,* seed and almond mesocarp, to produce entomopathogenic (*Verticillium lecanii, Paecilomycesfarinosus, Metarhizium anisopliae,Beauveria bassiana)* and mycoparasitic *(Trichoderma harzianum* and *Gliocladium virens*) fungi.

Uninoculated plant waste substrates had very little microbial colonization. In inoculated substrates, germination conidia of *T. harzianum* were found on *P. canariensis* leaves. Widenings of hyphal apex, resembling appressoria, were found for *T. harzinum* and *B. bassiana* on palm leaves. Hyphae of *G. virens* produced adhesives on the surface of palm leaves. The development of antagonistic fungi on plant waste substrates depended on the combination fungus-substrate tested. Of the entomopathogens *P. farinosus* did not grow on almond mesocam. This fungus grew well on both *P. dactylifera* seed and *P canariensis* leave *P. farinosus* grew extensively on the edges of the leaf fragments and then started colonizing the leaf surface. *V lecanii* colonized and sporulated on almond mesocarp. Of all the plant waste substrates tested *M. anisopliae* only did grow on almond mesocarp. *C. humilis* leaves were excellent substrates for the growth and sporulation of both *V. lecanii* and *B. bassiana. B. bassiana* grew best on *P. daetilyfera* seed. The mycoparasitic fungi were, in general terms, faster and better colonizers of plant waste substrates than the entomopathogens. *T. harzianum* and *G. virens* sporulated well on *P. dactilyfera*

seed *C. humilis* leaves also supported abundant growth of *T. harzianum*. *P canariensis* leaves were also readily colonized by the fungus. So their results show that plant waste has potential as substrate for production and perhaps formulation of important biocontrol fungi.

Sharma *et. al.* (1999) selected molasses yeast broth as synthetic medium for mass production for all the three pathogens which produced 8 $x10^7$, I x 10^9 and 2 x 10^9 conidia ml^{-1} in the slurry of *M. anisopliae*. *B. bassiana* and *B. brongniartii,* respectively. Amongst grain media, crushed maize grains for *M. anisopliae* and whole cowpea grain for *Beauveria* spp. were employed for mass multiplication using 2kg high density polypropylene bags each containing hundred grams of grains moistened with 60ml of distilled water. *M. anisopliae, B. bassiana* and *B. brongniartii* and their respective temperature yielded a grain spore dust of 2 x 10^9, 1.5 x 10^9 and 1.8 x 10^9 conidia g^{-1} dry grain weight. Fungal slurry and dried grain spore mass, after blending in electric mixture for 30 sec was incorporated in sterilized talc powder (carrier) in the proportion of 1:2 to 1:5 depending on density of spore in order to achieve 4 – 5 x 10^8 conidia g^{-1} in formulation.

They observed that *Maladerainsanabilis* was more susceptible as compared to *H. consanguimea* as it took less time to cause mortality, when exposed to the different doses of the tested pathogens by soil inoculation method. *M. anisopliae* and *B. brongniartii* showed high virulence against both the target insects with LT_{50} of 7.95-16.20 and 9.93-13.98 days, respectlvely with third instar larvae exposed to their highest doses of inoculum. *B. bassiana* was found to be weak pathogen against both insects.

According to *Gillespie et. al.* (2000) topical application of *Metarhizium anisopliae* var. acridum to the desert locust *Schistocerca gregaria* in changes in the biochemistry and antimicrobial defenses of the haemolymph. *M. anisopliae* var acridum colonized the host haemolymph from day two post application. The haemocytes did not attach to, phagocytose or nodulate elements of the fungus. However, the presence of the fungus appeared to stimulate haemocyte aggregation over the first few days of mycosis though the

number of aggregates declined subsequently. The total hemocyte count increased two days after application, indicating an overall stimulation of the immune system, but declined to a value below that for uninoculated controls by day four. The differential haemocyte count showed that the initial increase in total haemocyte count was primarily due to a larger number of coagulocytes. After day two consistent declines in cell number were observed for all haemocyte classes in mycosed insects. The activity of the enzyme, phenoloxidase, decreased during the course of infection. However, the converse was true for prophenoloxidase. Lysozyme levels were significantly smaller in infected than control locusts. They observed a significant correlation between lysozyme and PO activities when data from mycosed and control insects were combined. The total protein content of the haemolymph decreased during the course of infection.

Hong *et. al.* (2000) harvested conidia of the entomopathogenic *Metarhizium flavoviride,* 8,12, or 15 d after inoculation at 25°C and then (as conidiated rice) dried rapidly (10-12% r.h. and 17-20 degree for 17h to about 15-22% moisture content) or slowly (50-60 % rh. and 27 degree for 5 d to about 27-32% moisture content initially). The subsequent survival of these conidia in air-dry storage at 50 degree with 8.1% moisture content was then assessed. Conidia longevity (assessed by the duration of storage until conidia viability was reduced to 50%, P^{50}) was maximal when conidia were harvested 10d after inoculation, and was much greater following slow rather then rapid drying. The substantial beneficial effect of slow desiccation to subsequent conidia survival is consistent with that detected in other propagules in anhydrous biology, and is also of considerable practical utility for the biological control of insects by entomopathogenic fungi.

The efficacy of mycoinsecticide formulated vegetable oil was tested in Brazil against the grasshopper *Rhammatocerus schistocercoides* by Magalhaes *et. al.* (2000) A set of experiments was conducted in the Chapada dos Parecis region (Mato Grosso state), a permanent zone of out breaks for this pest. Experiments were performed in zones of natural vegetation, against

grasshopper bands in the third nymphal instar. Three nymphal bands were treated with a mycoinsecticide formulation based on conidia of the entomopathogenic fungus *Metarhizium anisopliae* Var. acridum (=*M. flavoviride*), strain CG 423. Three non-treated bands were used as control. The application was made with the aid of a hand held ULV sprayer adjusted to deliver 2l of the formulation ha^{-1}, containing 1 x 10^{13} conidia. Treatments were limited to the surface of the grasshopper bands and this immediate borders(5-10m), The efficacy of the mycoinsecticide was evaluated through band survival after treatment (grasshopper numbers, surface, density, behaviour and daily movement of the band), allowing the insects to move freely in their natural environment. They regularly surveyed and maintained the insects in the laboratory, allowing estimates of the infection rate. Field and laboratory studies showed a clear effect of the product 10 days after treatment. At 14 days post-spraying, mortality caused by the mycoinsecticide in the field was approximately 88%.

Inyang *et. al.* (2000) investigated the effect of simulated rain on the persistence of oil and water formulations of conidia of the entomogenous fungus *Metarhizium anisopliae* when applied to oilseed rape foliage, using third instar larvae of the mustard beetle (*Phaedon cochleariae*) as the target host. Rain significantly ($p < 0.01$) reduced the susceptibility of the beetle larvae to *M. anisopliae* but the amount of inoculum removed was influenced by the formulation. Larvae exposed to plants treated with conidia formulated in aqueous Tween, shellsol T, or sunflower oil/Shell sol T resulted in 55,82.5 and 72.5%, mortality, respectively. The mortality for these respective formulations was reduced by 42,57 and 51% if the plants were exposed for I h to simulated rain. Laboratory and field studies showed that more inoculum collected beneath plants sprayed with conidia formulated in shellsol T or aqueous Tween than in the more viscous sunflower/ shellsol T mixture. Mortality studies on leaves taken from field plots suggested that conidia on leaf surfaces could be replenished by repeated application. The number of conidia isolated from field plots was greater where inoculum was applied bi-weekly than once weekly.

Vanninen *et. al.* (2000) studied in 1988-1991 the persistence and

penetration into soil of surface-applied informulated conidia of two isolates of *Metarhizium anisopliae* and one of *Beauveria bassiana* at sites with clay, peat and two kinds of sand as their soil types and at depths of from 0 to 20 cm under conditions characterised by permanent snow cover and frozen soil in the winter time. At 0-5 cm depth, *M. anisopliae* persisted throughout the experiment at all sites clay being most and peat the least favourable soil for persistence. Clay and one of the sandy soil were the least and peat the most conducive soil to penetration of *M. anisopliae* from the surface to deeper soil layers and persistence therein. Differences in persistence were evident between the two *M anisopliae* is lates in the sandy and peat sites, but not in clay site. Three years post- application there were still enough infections propagules of *M anisopliae* in soil of all sites to infect over 80% of the *Tenebrio molitor* larvae used as baits in samples taken the cylinders of all soils. All the augmented propagules of *B. bassianc* disappeared during the first winter after application in clay and one of the two sandy sites, but some persistence one year post-inoculation was evident at 0-5 depth in one of the sand soils and at 0-5 and 0.-JO cm. depths in peat.

Booth *et. al.* (2000) demonstrated that a dried mycelium formulation of an indigenous strain of *Metarhizium anisopliae* has good potential as an effective biopesticide against the black vine weevil (BVW), *Otiorhynchus sulcatus* (F.), and the cranberry girdler (CG), *Chrysoteuchia topiaria* (Zeller). The formulation was produced at moderate sized batches scales (approx. 100g product per batch) using inexpensive and easily obtainable equipment . It was observed that the levels of conidiation were consistent among production batches and storage intervals with the product remaining viable for long time periods at 4°C in one case for more than a year. Levels of contamination by yeasts and other benign fungi were also consistent. Fewer BVW and CG were found in small plots treated with the dried mycelium formulation than in untreated plots, but differences were not always significant. A waxmoth *(Galleria mellonella)* bait viable in the soil over long time periods (>7 months). Percentage of *M. anisopliae* infected waxmoth larvae was signficantly affected by rate.

According to Nankinga and Moore (2000) one of the major constraints

for banana production in Uganda is the banana weevil, *Cosmopolites sordidus* (Germar), (Coleoptera : Curculionidae). Investigations were carried out to evaluate the efficacy of maize, soil-based and oil formulations of an indigenous isolate of *Beauveria bassiana* for the control of the banana weevil. Weekly trapping of weevils over a 9 month monitoring period showed significant reduction in unmarked and marked weevil population in *B. bassiana treated plots.* Application of maize formulation at 2×10^{15} conidia ha^{-1} proved most effective, reducing the weevil populations by 63-72% withing 8 weeks after a single application. They observed that the soil based formulation at 2×10^{14} conidia ha^{-1} was intermediate while the oil formulation at 6×10^{15} conidia ha^{-1} was least effective. Trapping efficiency declined in *B. bassiana* treated and untreated banana plots but was greatest in the latter.

Reduction in grasshopper population density between treated and untreated plots was significantly different, but did not convince farmers, as full grown L5 larvae had emerged as adults and began migrating. However, cadavers collected from cags containing insects from treated plots allowed farmers to follow how the fungus sporulates and contaminates healthy grasshoppers. They observed that farmer were interested in the product but were reluctant to buy a slow-acting insecticide.

Gurusubramanian et. al. (1999) evaluated the pathogenic effects of *Beauveria bassiana* (Bals) *Vuil. (Deuteromycotina : Hyphomycetes)* against workers of the termite *Odontotermes obesus (Rambur)* (Isoptera : Termitidae). Termite mortality ranged from 41-91% in treatments, compared with 19-21% in controls. Both lethality and lethal time varied significantly with conidial concentration and age of culture. Mycelial growth was recorded on the leg joint, abdomen, antenna, thorax and head regions of treated cadavers after 24h. Also observed in treatments were body surface shrinkage, colour change, and hardened and brittle appendages.

Laboratory evaluation of the pathogenicity of three isolates of the entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuillemin on the American cockroach (*Periplaneta americana*) were performed by Mohan *et. al.* (1999)

where they treated the insects in three different ways, i.e. by direct contact with spore mass, a spore-wheat flour mixture and a spray of an aqueous spore suspension. They observed a mortality of 100% in the first treatment, 57-100% in the second treatment and 17- 7500 in the third treatment. These results suggest that *B. bassiana* spore formulations in food baits can be developed for cockroaches. They concluded that mycopesticides can be ideal for the biocontrol of cockroaches because the habitat of these insects promotes initial fungal infection and its subsequent spread.

Rica and Cogburn (1999) evaluated a *Beauveria bassiana* isolate from the rice water weevil, *Lissorhoptrus oryzophilus* his Kuschel (Coleoptera: Curculionidae), as a conidial powder against 3 colepteran pests of stored grain: the rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae); the lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera : Bostrichidae); and the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). They assessed the bioactivity of *B. bassiana* isolate 22292A on 3 types of food substrates (red flour beetle media, medium grain brown rice, and long grain rough rice) at 27 degree C and 60% RH. They observed that adult mortality was 80-100% at the higher dosage levels for all insects tested on all media types at 21d after treatment. Emergence of adult progeny on brown and rough rice was reduced by 83-99% at the 2 highest dosage level. They concluded that the abrasive nature of rice hulls on the rough rice may contribute to the accelerated mortality seen on this medium.

Sharma *et. al.* (1999) selected molasses yeast broth as a synthetic medium for mass production for all the three pathogens which produced 8 x 10^8, 1x10^9 and 2x10^9 conidia ml^{-1} in the slurry of *M. anisopliae, B. bassiana* and *B. brongniartii,*, respectively. Amongst grain media, crushed maize grain for *M. anisopliae* and whole cowpea grain for *Beauveria* spp. were employed for mass multiplication using 2kg high density polypropylene bags each containing hundred grams of grains moistened with 60m1 of distilled water. *M. anisopliae*, *B. bassiana* and *B. brongniartii* at their respective temperature yielded a grain spore dust of 2x10^9, 1.5x10^9 and 1.8 x 10^9 conidia g^{-1} dry grain

weight. Fungal slurry and dried grain spore mass, after blending in electric mixer for 30 sec was incorporated in sterilized talc powder (carrier) in the proportion of 1:2 to 1:5 depending on density of spores in order to achieve 4-5x10^8 conidia g^{-1} in formulation. *Maladera insanabilis* was more susceptible as compared to *H. consanguinea* as it took less time to cause mortality, when exposed to the different doses of the tested pathogens by soil inoculation method. *M anisopiiae* and *B. brongniaritti* showed high virulence against both the target insects with LT_{50} of 7.95-16.20 and 9.93-13.98 days, respectively when third instar larvae exposed to their highest does of inoculum. *B. bassiana* found to be weak pathogen against both insects.

Zurek and Keddie (2000) tested a new isolate of the entomopathogenic fungus, A *Beauveria bassiana* by laboratory bioassays for potential use as a microbial control agent of the early (third) and last (seventh) instars of the satin moth, *Leucoma salicis* L. Results demonstrate that this fungus is very effective against last instars of this lepidopteran pest. Although *B. bassiana* was cultured successfully from internal contents of all cadavers examined, vegetative hyphae emerged from intact cadavers of *L. salicis* infected as early (third) instar caterpillars only.

The susceptibility of *Megalurothrips sjostedti* to *Metarhizium anisopliae* when reared on susceptible, tolerant, and moderately resistant varieties of cowpea at different constant temperatures was evaluated in the laboratory by Ekesi *et. al.* (2000). Insects were exposed either to direct spray of the conidia or to fungus-treated floral tissues. Mortality was significantly higher on the moderately resistant variety at all temperatures compared to the susceptible and tolerant varieties. Corresponding, lethal time and lethal concentration values were significantly shorter and lower, respectively on the moderately resistant variety compared to the other varieties, thus indicating that the two control methods are compatible as part of an integrated pest management strategy. Thrips raised on the tolerant variety incurred an exceptionally low level of mortality when the inoculum was sprayed directly on the insects or when the insects were exposed to fungus-treated floral tissues. Observations on the effects of

airborne volatiles and crude extracts of this variety revealed in inhibitory effect on fungal germination, colony forming units and growth. This suggests the existence of anti-fungal substances in the tolerant variety.

The pathogenicity of *Vespula vulgaris* wasp workers and larvae to a range of fungi was determined by Harris *et. al.*, (2000). All fungi were isolated in New Zealand and included islates from Vespula, known generalist insect pathogens, and isolates generally nonpathogenic to insects. Workers and larvae were highly susceptible to pathogenic isolates at high spore concentrations (>$1.75x10^5$ cfu / individual). Eight isolates, two of *Metarhizinum anisophiae*, five of *Veauveria bassiana*, and one of *Aspergillus flavus* were pathogenic while a single isolate of M. flavouiride var. *novazealandicum*, *Cladosporium* so. and *Paecilomyces* sp. were not. The transfer of spores between workers, and between workers and larvae, was also investigated using several different application methods. Transfer of spores occurred between treated and untreated individuals, and for some of the application methods sufficient spores were transferred to cause mortality of the nontreated individuals. These findings are related to the potential of fungi for the control of wasps.

Beauveria bassiana (Balsamo) Vuillemin is a fungus with broad spectrum insecticidal activity as stated by Martin *et. al.* (2000). As a biological control agent used against Colorado potato bettles (*Leptinotarsa decemlineata*), this fungus has been attributed to formulation problems, UV sensitivity, and humidity. They observed that in a multisite test, *B. bassiana* controlled Colorado potato beetle larvae in both Poland and the Czech Republic, but not in Maryland. Control was measured by reduction in populations of beetle larvae. One of the major differences among these sites was temperature. In Poland, the mean temperature ranged from 5°C to 23°C, in the Czech Republilc the average temperature ranged from 6.7°C to 18.7°C; and in Maryand, temperatures at time of applicatin exceeded 45°C at canopy level. This led them to examine *B. bassiana* growth *in vitro*. While *B. bassiana* grew in the laboratory from 16 to 30°C, the *B. bassiana* from a formulated product (Mycotrol TM, Mycotech, Butte, MT) did not germinate at temperatures above 37°C. Germination and

subsequent development of this entomopathogenic fungi are critical factors in the infection and control of the Colorado potato bettle. As a consequence of the inabiity to germinate at high temperatures, *B. bassiana* would not be expected to effectively control pest insects in climates with hot summers. This fungus, however, may be for insect control in early spring or in cool temperature climates during the growing season.

Edgington *et. al.* (2000) considered *Beauveria bassiana* to the one of the few natural enemies available for use against the coffee berry borer. In an attempt to enhance to efficany of this pathoge, a range of concentrations of 22 substances was tested in simple laboratory tests using natural sunlight or a UV light source. Unprotected *B. bassiana* spores were almost copletely inactivated by exposure to 60mim of direct sunlight or 20s of UV ight of 302nm wavelength. Seven of the 22 substances tested were egg albumen an skimmed milk powder which could extend the persistence of *B.bassiana* spores by a factor of almost three. A mixture of 3% (w/v) albumen and 4% (w/v) milk powder gave the highest degree of spore protection per unit cost. Young coffee plants sprayed with this mixture did not suffer any significant phytotoxic effects. A field trial, involving two applications of spores with or without the milk and albumen mixture, failed to show that improved spore persistence resulted in increased coffee berry control. Very low levels of pest infestation was observed in field plots together with unusual, unfavourable weather condiditons may have accounted for this unexpected result.

The laboratory studies of Rath (2000) have shown that termit species are highly susceptible to entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana.* According to his obseervations there appears to be very little host specificity among fungal isolates with many isolates being highly virulent to many species of termites. The grooming and other social intractions between termites are seen to have the potentional to spread the fungus through the colony, allowing for colony control by the treatment of remote feeding sites. However, factors such as avoidance of the fungus conidia by the termites, the removal and burial of fungus-killed termites, together with defensive secretions and

inhibitory components in termites, together with defensive secretions and inhibitory components in termite frass and the possibility of humoral resistance may limit the spread of the disease in the colony. Field studies have shown mixed resuts. Direct application of fungus to nests has resulted in complete colony mortality, but studies where feeding is ites or bait stations have been treated with fungus have yet to show similar success. The effectiveness of termite control in urban pest management, particularly in structural timber and dwelings, has yet to be reported in details, as indicated by Rath (2000). Such studies require the examination of the complex relationships between does, speed of kill, virulence. horizontal transmission and ultimate colony death, combined with aboidance and recognition factors, and survival of the fungi under field conditions.

The susceptibility of immature stages of the legume flower thrips, *Megalurothrips sjostediti,* to the entomopathogenic fungus *Metarhizium anisopliae,* was investigate under laboratory conditions by Ekesi *et. al.* (2000). The adult stage was found to be more susceptible to infection than the larval and pupal stages. Mortality at all stages was does-dependent, with the highest concentration of $1x10^8$ conidia ml^{-1} producing the highest mortality (256, 46 an 100% for larvae,l plupae and aduts, respectively) at 8 days post- inoculation. At the same concentration, daily pollen consumption was significantly reduced 2 days after treatment in infected adults but more slowly in infected larvae. Fecundity, egg fertility and longevity in adults surviving infection as larvae were significantly reduced compared to the control.

Noma and Strickler (1999) evaluated the entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillemin, in alfalfa seed plots for control of lygus bug (primarily *Lygus hesperus* knight) in comparison with conventional chemical insecticides and a water control. *B. bassiana* applications reduced lygus bug mymphs relative to the water control during one of three prebloom (June) trials but not during any bloom (July) trials. A single applications of conventional insecticides reduced ygus bug populations more frequently and to a greater extent compared to three applications of *B. bassiana.* The noticed that infection rates were relatively high in treated plotsdespite lower spray

coverage than expected. Reduced *B. bassiana* infection during the bloom trial compared to the prebloom trial has been attributed to reduced spray penetration within alfalfa as the canopy closed. Faster nymphal growth rates relative to growth of *B. bassiana* hyphae at field temperatures could also explain seasonal differences in the effectiveness of *B. bassiana*.

The impact of relative humidity (RH) on the infective potential of the isolate BbINRA 297 of *Beauveria bassiana* (Bals) Vuillemin (Deuteromycotina Hyphomycetes) against first instar nymphs of *Rhodnius prolixus* Stal.l (Hemiptera Reldluviidae) was determined by Luz and Fargues (1999). Fungus-treated insects were exposed to RHs ranged from 75 to 100% at 25°C. Results clearly showed a threshold of humidity at ca. 96% for high and rapid mortality. After initial exposure to increasing periods of 97% (4, 8, 16, 24, 36 and 48h) and subsequent transfer to constant lower RHs (43, 53, 75, and 86%) at a constant 25°C, an incubation of atleast 48h at 97% RH was necessary to kill all insects. On changing RHs of 97/75% and different regimes of temperature (15/ 28°C, 20/25°C and 25/35°C), at least 72h of initial exposure to favourable moisture condition (97% RH), significantly affected infection for up to a 3-day delay within the various temperature humidity regimes tested.

The efficacy of the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuilemin was tested as a control agent by Mulock and Chandler (2000) for adult western corn rootworm, *Diabrotica virgifera* virgifera LeConte, in walk in fied cages. Suspensions of *B. bassiana* conidia were applied to corn plants in cages into which laboratory-reared beetles had been released. Beetles were collected at 3 and 5 days post- application and evaluated in the laboratory for mortality. Mortality was 10l.29 and 50%, at rate equivalents of $7x10^{12}$, $2x10^{13}$ conidia / ha, respectively. They observed no significant difference in mortality of beetles collected at 3 days compared with 5 days post-application. Mortality due to *B. bassiana* was 24% when beetles were released into field cages 24h post-application ($5x10^{13}$ conidia/ha) compared with 50% when beetles were present during the application. Beetle mortality declined significantly with increasing time from application. Beetle mortality declined

significantly with increasing time from application in feeding assays carried out with leaf samples removed from plants at 0, 12, 24 and 72h post application. Mortality of beetles collected from treated plants within cages and maintained is the laboratory was bound to overestimate the population decline by approx. 10% when compared with beetle estimates from treated plants within field cages.

Wraight *et. al.* (2000) assessed the microbial control potential of *Beauveria bassiana* against Bemisia white flies. Laboratory assays demonstrated the capacity of the pathogen to infect *Bemisia argentifolii* nymphs on excised hibiscus leaves incubated at relative humidities as low as 25% at 23±2 degree C (ca. 35% infection by *B. bassiana* resulted from applications of 0.6–1.4x10^3 conidia / mm^2 of leaf surface). In small-scale field trials using portable air-assist sprayers, applications at a high rate of 5x10^{13} conidia in 180 liters water / ha produced conidial densities of ca. 1–2.5x10^3 conidia / mm^2 on the lower surfaces of cucurbit leaves. Multiple applications of four isolates of *B. bassiana* made at this rate at 4 to 5 day intervals provided >90% control of large (third and fourth instar) nymphs on cucumbers and cantaloupe melons. The same rate applied at 7 days intervals also provide > 90% control in zucchini squash, and a one-fourth rate (1.25x10^{13} conidia/ha) applied at 4 to 5 day intervals reduced numbers of large nymphs by >85% in cantaloupe melons. In contrast to the high efficacy of the fungal applications against nymphs, effects against adult whiteflies were minimal. The results indicated that *B. bassiana* have strong potential for microbial control of nymphal whiteflies infecting cucurbit crops.

In insects, fungal disease are common and widespread and often decimate insect populations in spectacular epizootics. Entomogenous fungi infect insects by breaching the host cuticle; they are the principal pathogens among sucking insects because these host cannot ingest other pathogens that infect through the gut wall.

The use of newly available biochemical and molecular markers has

facilitated other studies on species structure and geographical spread. For example, in recent allozyme studies of St. Leger *et. al.* (1992a), the level of genetic distance observed between cluster groups of *Beauveria bassiana* and Metarhizium anisopliae indicated that each represents a species aggregate, components of which isplay overlapping genetic variability, with some isolates currently assigned to other rarer species in the same genus. Except for isolates of *Metarhizium anisopliae* var. *majus*, most *Metarhizium* isolates are homozygous at each locus in a manner consistent with hapoidy. In spite of the maintenance of high diversity in *B. bassiana* and *M. anisopliae*, the majority of isolates are contained in a few geographically widespread genotypic classes.

According to Kulye *et. al.* (2002), fungal agents act on insect pest by contact and the cuticle degrading enzymes (CDEs), mainly chitinase, protease, and lipase contribute significantly in the process of entomo-pathogenesis. The control of *Helicoverpa armigera* infested on chickpea crop was studied using insect pathogens such as *Metarhizium*, *Beauveria*, and *Nomuraea* as well as with CDE complex produced by another fungus *Myrothecium verrucaria.* The percent efficacy of *Metarhizium anisopliae* and the chemical control agent, endosulfan was highest (70%). *Beauveria* showed percent efficacy in the range 45-62%.

Niranjana (2002) made and attempt to use an entomopathogenic fungus *Beauveria bassiana* in managing Coffee Berry Borer (CBB). Soil, infested berries, leaf materials, coffee twigs, and gleanings were collected from different coffee plantations and screened for presence of infected and dead CBB. These were plated on PDA and the expressed fungi were isolated and identified. *B. bassiana* isolates were grown on YPD media and conidial suspension (10^6 conidia / ml) was prepared. The borers were dipped in the suspension and maintained at ambient temperature and humidity. Mortality was recorded daily and the dead insects were transferred to sterile petriplates with moist blotters and incubated at 25 ± 2^0C for 5 days. Only those insects, which were colonised by *B. bassiana* and showed sporulation, were considered for calculation of percent mortality. The tested isolates of *B. bassiana* obtained from naturally

dead CBB showed pathogenicity towards CBB with varied mortality rates. Isolate Bb2 recorded high mortality 0f 93%, while isolates Bb4 and Bb1 stood next to Bb2 with 64.25% and 59.25% borer mortality rates respectively. *B. bassiana* was mass multiplied in Roux bottles and the culture was vacuum dried at – 40°C which was then suspended in sterile distilled water containing 0.02% Tween 20. The conidial concentration was adjusted to $1x10^6$ conidia/ml using a haemocytometer and the same was used as mycopesticide. Large-scale experiments were conducted at Green Hill estate of Virajpet from 1995-2002. Mycopesticide was applied and evaluation was done after 24 days of spraying. Percent insect mortality was calculated. The mean insect mortality after spraying with *B. bassiana* calculated from five different plots was 75.5% against zero mortality rates in unsprayed plots. In *robusta* insect infestation was ranging from 1.46-9.65%. The mean insect mortality after 1.33% and robusta 1.76% insect infestation.

MATERIALS AND METHODS

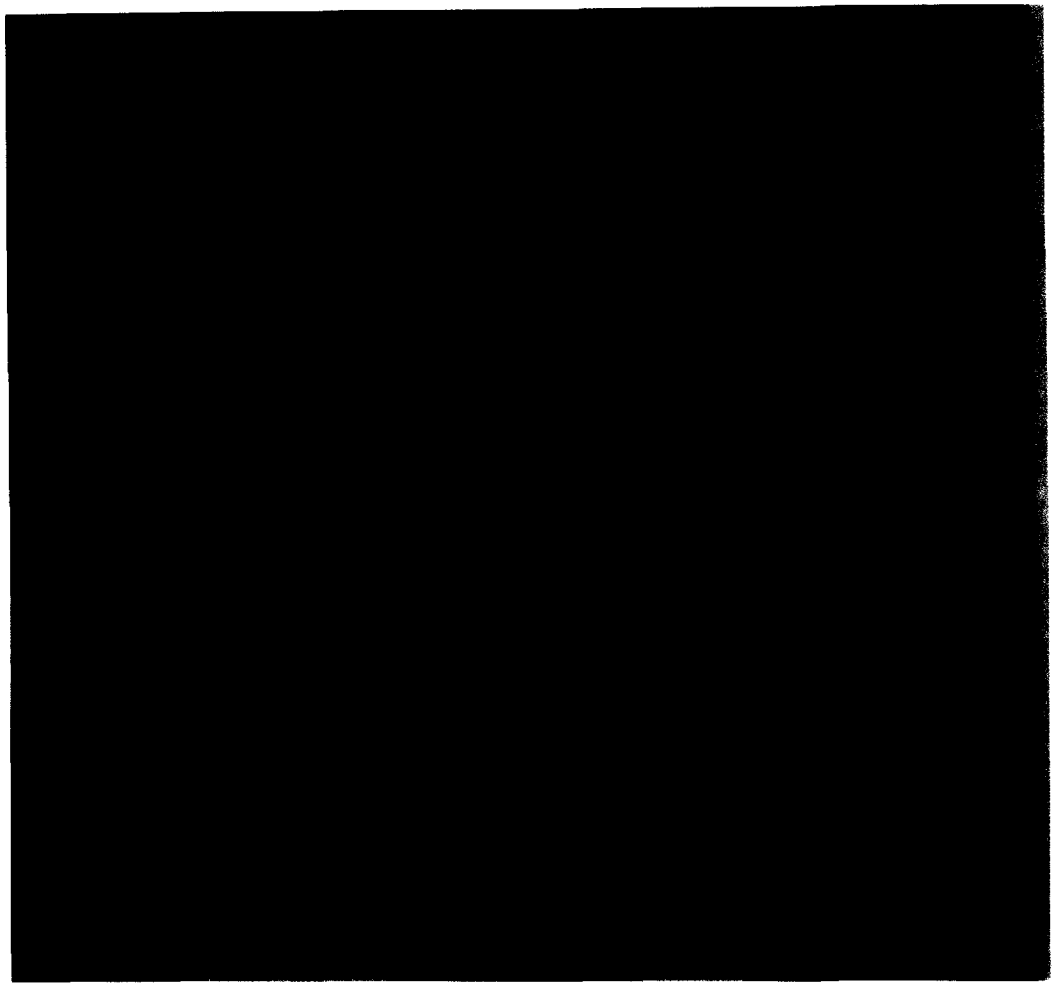


Plate 2 : *Odontotermes obesus* collected from the infested teak plant.

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.

EXPERIMENTAL

4.1 Evaluation of mycelial growth and sporulation of fungal cultures

Mycelial growth rate of four different strains of *Beauveria bassiana* and two strains of *Metarhizium anisopliae* were observed initially in four different culture media such as Yeast extratct glucose agar (YGA); Potato dextrose agar (PDA), Potato carrot agar (PCA) and Beef extract agar (BA). Maximum growth and sporulation were observed in PDA and YGA media (Table-3 & Plate - 3) while in BA media mycelial growth was found to be very slow even after 20 days of incubation. On the basis of this result YGA and PDA were selected for spore production for further experiments. Standardization of conidial concentration were done with 28 days old cultures when maximum spore production was noticed. All the isolates of *B.bassiana* and *M.anisopliae* were incubated for 28 days and their conidial concentrations were determined from the serial dilutions of the stock suspension . *B.bassiana* isolate 2028 registered the highest conidial concentration (4.56 x 10^6 conidia/ml) at 10^{-1} dilution while *M.anisopliae* isolate 140 registered lowest conidial concentration (2.3x10^6 conidia/ml)at same dilution (Table-4).

4.2 Compatibility tests for different fungal isolates

Among the chosen isolates intra and inter-compatibility tests were perfomed. The results of compatibility tests for isolates of *B.bassiana* and *M. anisapliae* are given in Table 5. Excellent growth was observed among the isolates of *B.bassiana* ; Bb-2028 and Bb-1216 ; Bb-1216 and Bb-984 ; and between *B.bassiana* (135) and *M.anisopliae* (140). The growth was at medium rate in the combinations of (a) Bb-2028 and Bb-135; (b) Bb-1216 and Bb-135 ; (c) Bb-984 and Bb-135. Good form of growth was obtained in the combinations of Bb-2028 and Bb-984; Bb-2028 and Ma-892; Bb-1216 and Ma-892; Bb-1216 and Ma-140; Bb-984 and Ma-892; Bb-135 and Ma-892; and Ma-892 and Ma-140 (Table-5). In general the fungi secreting metabolites during their growth may inhibit the growth of the other fungi. In this study no inhibition zone was found between and among the isolates of *B.bassina* and *M.anisopliae*. In other words no antibiosis effect was observed and all the chosen isolates seem to be compatible with each other.

Table 3: Growth of different strains of *B. bassiana* and *M. anisopliae* in different culture media

Fungi (strain)	[a]Average mycelial growth in different media (cm)			
	YGA	PDA	PCA	BA
Beauveria bassiana (135)	5.2	7.7	3.9	2.5
Beauveria bassiana (984)	5.4	6.8	2.5	1.8
Beauveria bassiana (2028)	7.2	7.1	3.2	1.3
Beauveria bassiana (1216)	7.0	7.8	2.8	1.5
Metarhizium anisopliae (140)	7.2	8.2	4.2	1.2
Metarhizium anisopliae (892)	7.3	8.1	4.4	2.5

Incubation period : 20 days
Temperature : 28 ± 2°C

[a]Average of five replicates

YGA = Yeast extract- Glucose- Agar
PDA = Potato- Dextrose- Agar
PCA = Potato- Carrot- Agar
BA = Beef Extract- Agar

Table 4: Conidial concentrations (conidia/ml) for different serial dilutions of strains of *B. bassiana* and *M. anisopliae*

Strains	'O' Suspension	Serial dilutions			
		10^{-1}	10^{-2}	10^{-3}	10^{-4}
Bb-2028	5.2×10^7 (12)	4.6×10^6 (13)	9.9×10^5 (15)	2.7×10^5 (14)	5.4×10^4 (12)
Bb-1216	2.9×10^7 (10)	3.6×10^6 (11)	8.9×10^5 (11)	2.4×10^5 (11)	5.9×10^4 (11)
Bb-984	4.0×10^7 (14)	3.8×10^6 (14)	8.7×10^5 (16)	2.5×10^5 (16)	8.4×10^4 (14)
Bb-135	3.0×10^7 (8)	3.3×10^6 (8)	7.3×10^5 (7)	2.7×10^5 (7)	6.8×10^4 (7)
Ma-892	3.0×10^7 (18)	3.3×10^6 (18)	7.2×10^5 (22)	2.7×10^5 (20)	6.1×10^4 (18)
Ma-140	2.9×10^7 (18)	2.3×10^6 (18)	3.2×10^5 (18)	1.2×10^5 (18)	3.6×10^4 (18)

* Figures in the parentheses are the number of replications.

Bb - *Beauveria bassiana* ; Ma - *Metarhizium anisopliae*

Conidial concentrations were determined by haemocytometer measurements on each dilutions.

Table 5: Intra- and Inter- compatibility test among the chosen isolates of *B.bassiana* and *M. anisopliae*

Combinations	Growth performance *
1 and 2	++++
1 and 3	+++
1 and 4	++
1 and 5	+++
1 and 6	++
2 and 3	++++
2 and 4	++
2 and 5	+++
2 and 6	+++
3 and 4	++
3 and 5	+++
3 and 6	++
4 and 5	+++
4 and 6	++++
5 and 6	+++

1= Bb - 2028; 2=Bb - 1216;
3= Bb - 984; 4=Bb - 135;
5= Ma - 892; 6=Ma- 140.

* After 10 days observation with three replicates.

++ = Medium; +++ = Good; ++++ = Excellent

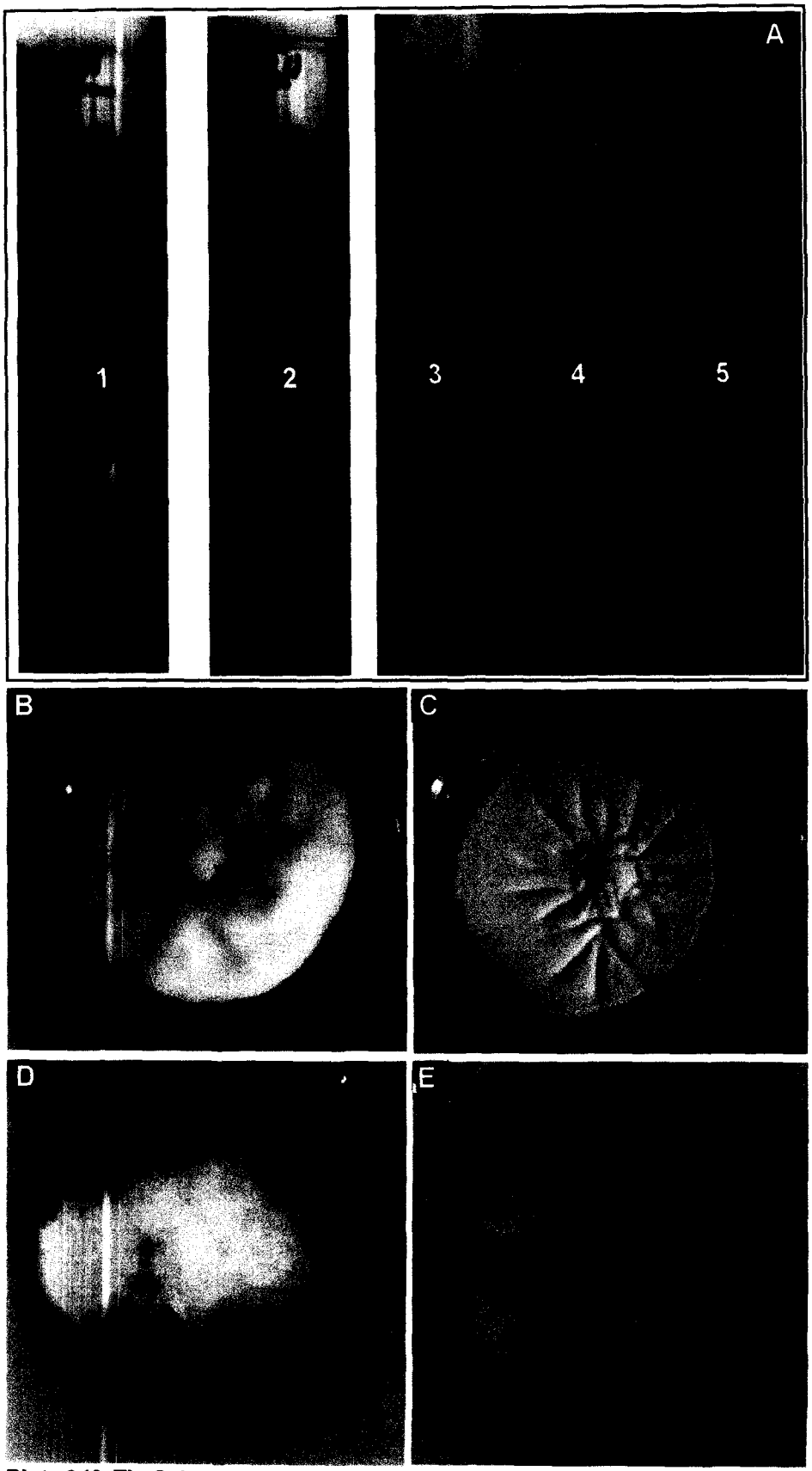


Plate 3 (A-E) : Cultures of *Metarhizium anisopliae* (A1-892, A2-140) and *Beauveria bassiana* (A3-2023, A4-1216, A5-984). Mycelial growth of *M.anisopliae* isolate - 892 (B&D) *B.bassiana* isolate-2028 (C&E) in YDA media (B&C) and in maize meal media (D&E).

4.3 Determination of conidial concentration of fungal isolates using transmission measurements.

Four isolates each of *B. bassiana* and two isolates of *M. anisopliae* were used for haemocytometer counts and transmission measurements from 28 days old cultures for a period of 12 months. Bright field microsopic observations of the conidia were done (Plate - 4). For each of the six isolates of *M. anisopliae* and *B. bassiana*, 3 lines, representing the measurements on the different chosen periods (0 - 6, 0-12 and 6-12 months) are given in Figures 1-3. Regression lines for *M. anisopliae* isolates 140 and 892 based on 15 selected data points at different periods nearest to 50% transmission, are given in figure 4 Regression lines for *B.bassiana* isolates 1216 and 2028 are given in figure 5 while that of isolates 135 and 984 are given in figure 6. The results of the statistical analysis are shown in table 6. The regression relationship between the numbers of conidia in suspension (X) and percentage transmission (Y) at different periods of 0-6, 0-12 and 6-12 months for different isolates of *M.anisopliae* and *B.bassiana* are as follows :

Isolates	
Ma 140	X = 1.95 + (- 0.004)Y Y = 87.68 + (- 18.5)X
Ma 892	X = 1.96 + (- 0.0006)Y Y = 80.74 + (- 14.5)X
Bb 1216	X = 2.00 + (- 0.001)Y Y = 87.05 + (- 16.75)X
Bb 2028	X = 4.87 + (- 0.057)Y Y = 646.27 + (-303.68)X
Bb 135	X = 1.99 + (- 0.001)Y Y = 166.53 + (-55)X
Bb 984	X = 1.98 + (- 0.0008)Y Y = 64.7 + (- 4)X

Table 6: Results of t-test on the 3 different replications for each isolates of *M.anisopliae* and *B.bassiana*

Fungus	Isolate	*Replicate	T-value	d.f.	P=0.05
Beauveria bassiana	984	0-6 , 0-12	0.03	49	2.010
		0-6 , 6-12	0.91	43	2.017
		0-12 , 6-12	0.96	48	2.011
	2028	0-6 , 0-12	0.75	53	2.035
		0-6 , 6-12	1.39	45	2.014
		0-12 , 6-12	0.58	52	2.007
	135	0-6 , 0-12	1.73	23	2.064
		0-6 , 6-12	1.42	31	2.040
		0-12 , 6-12	0.89	36	2.028
	1216	0-6 , 0-12	1.61	35	2.030
		0-6 , 6-12	1.70	22	2.074
		0-12 , 6-12	3.09	29	2.045
Metarhizium anisopliae	892	0-6 , 0-12	0.18	41	2.020
		0-6 , 6-12	1.35	31	2.040
		0-12 , 6-12	1.37	36	2.028
	140	0-6 , 0-12	0.33	31	2.040
		0-6 , 6-12	1.29	30	2.042
		0-12 , 6-12	1.97	33	2.035

* 0-6, 0-12 and 6-12 denotes *B. bassiana* and *M. anisopliae* culture used for first 6 months, 12 months and from 6 to 12 months respectively.

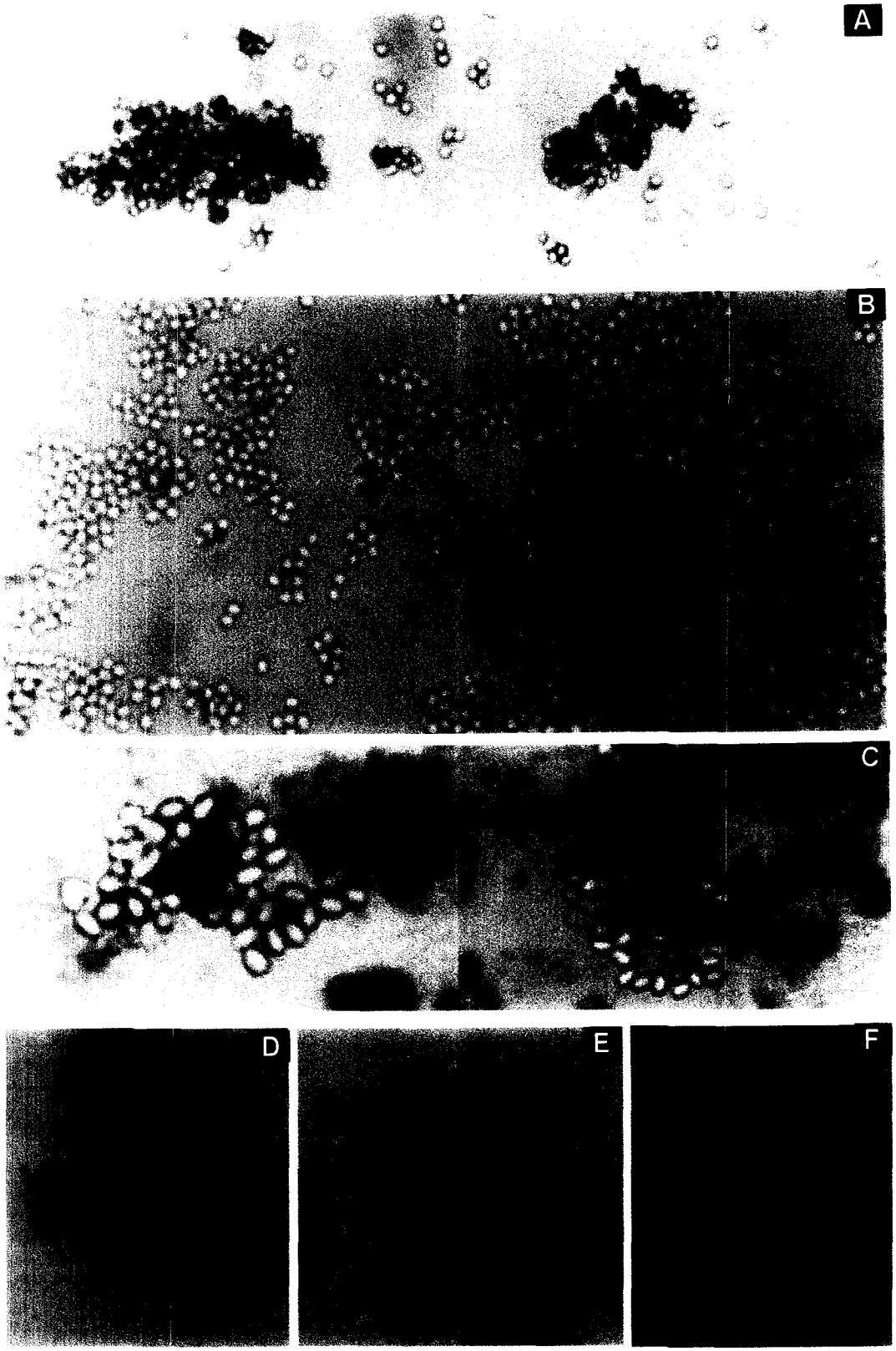
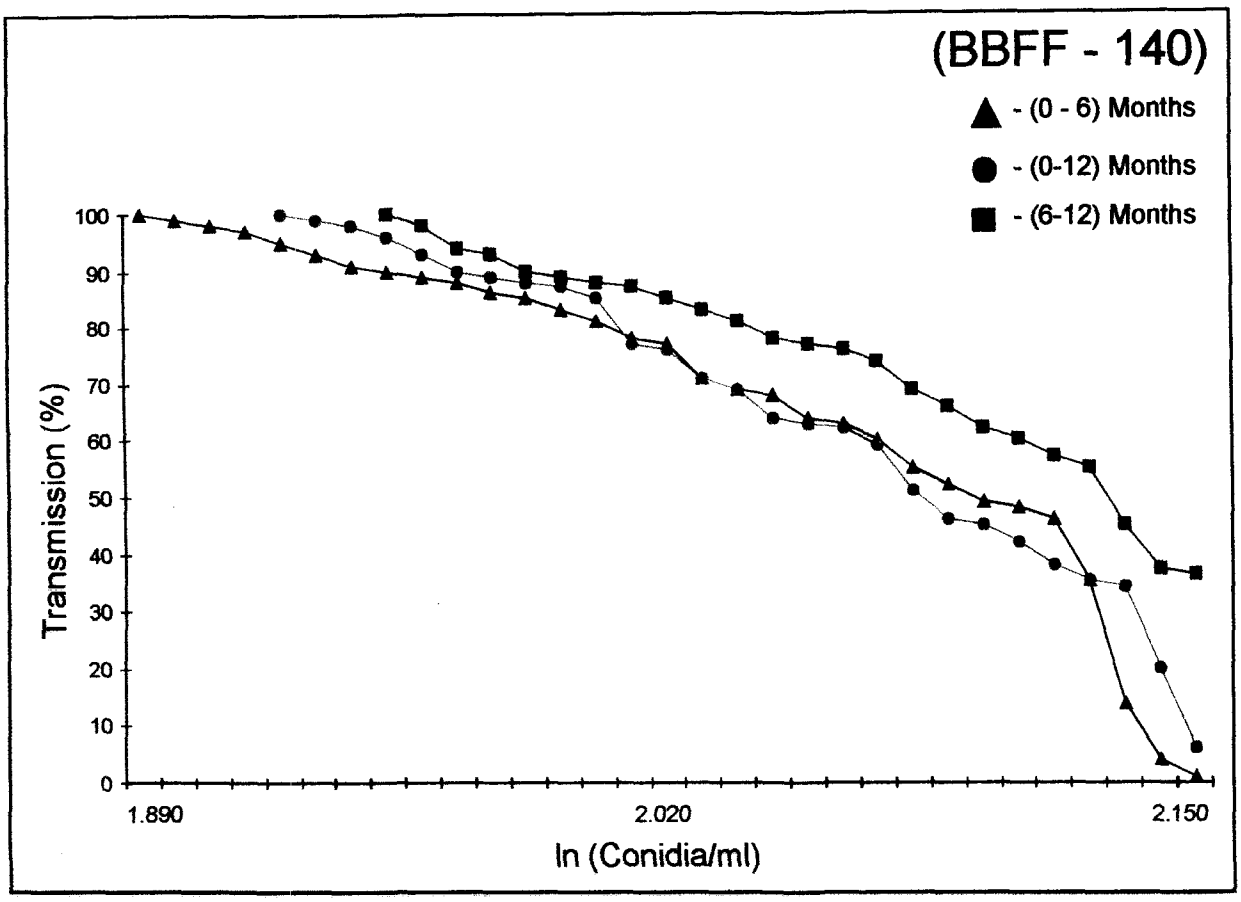


Plate 4 (A-F): Conidia of *Metarhizium anisopliae* isolates 892 (A&F), 140 (B) and *Beauveria bassiana* isolates 2028 (C), 1216 (D) & 984 (E)



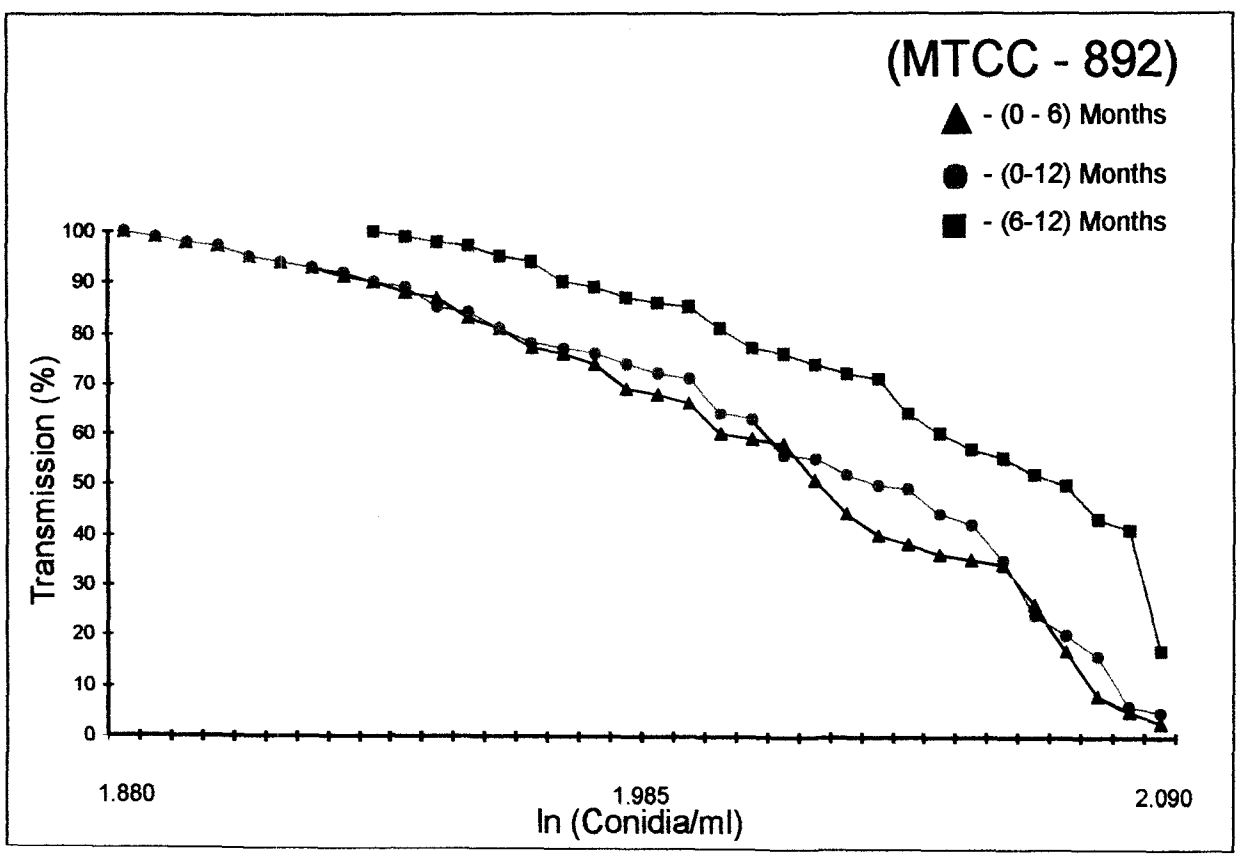
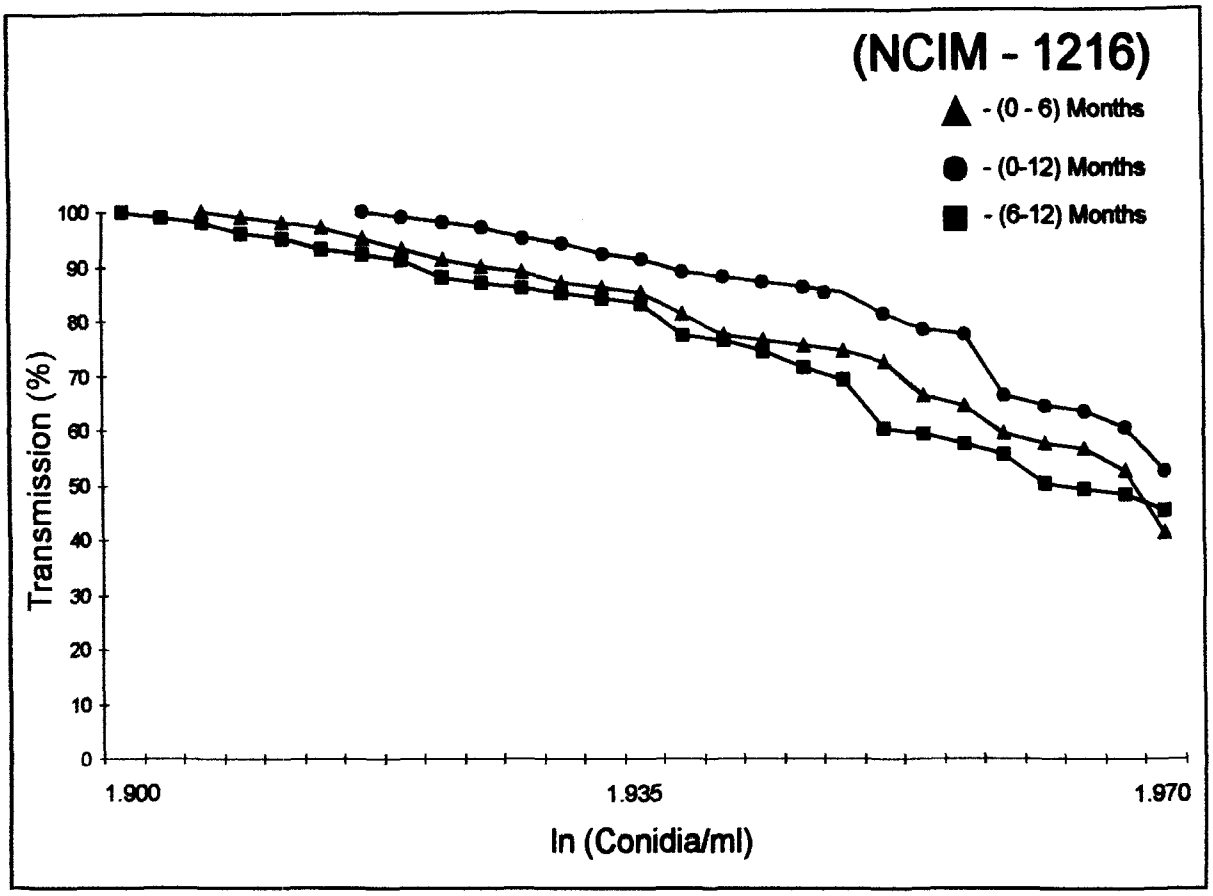


Figure 1. Transmission (%) and their corresponding conidial count for isolates of *Metarhizium anisopliae* cultured in different periods.



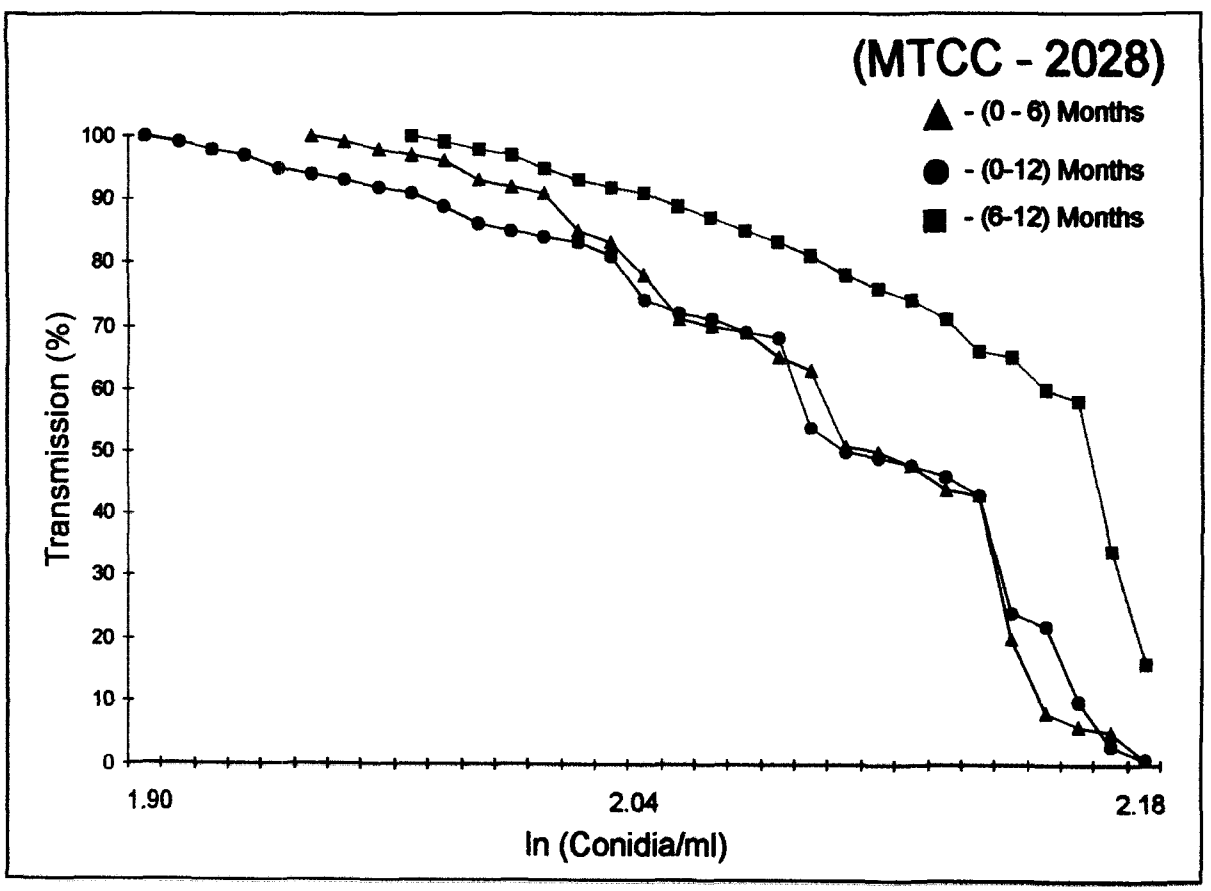
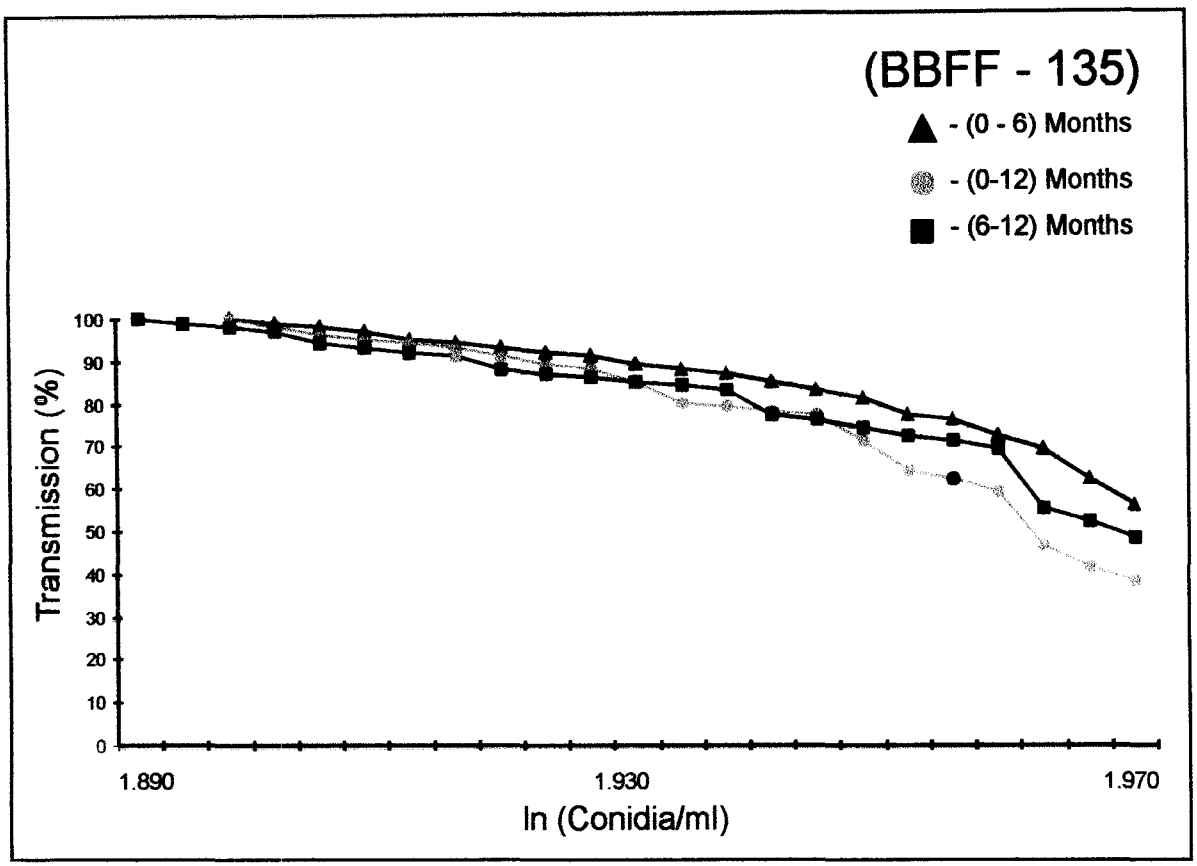


Figure 2. Transmission (%) and their corresponding conidial count for isolates of *Beauveria bassiana* cultured in different periods.



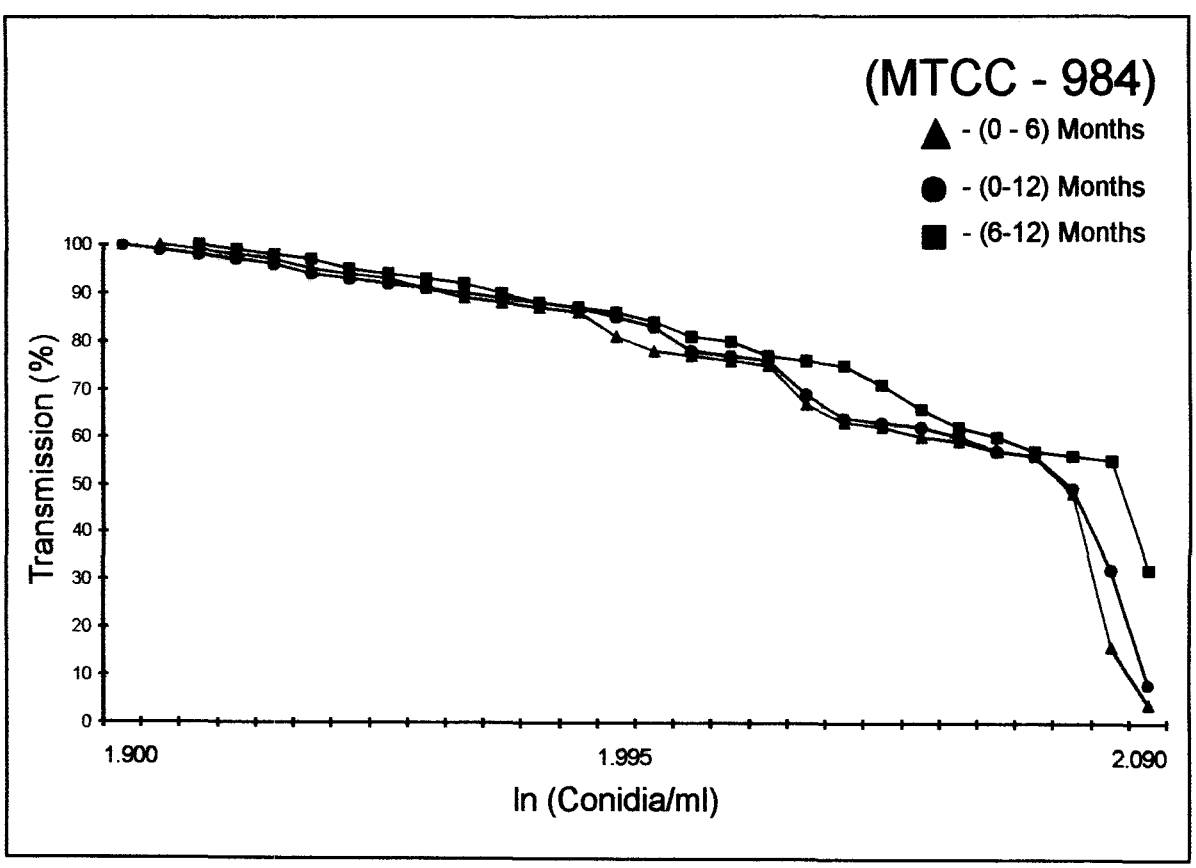
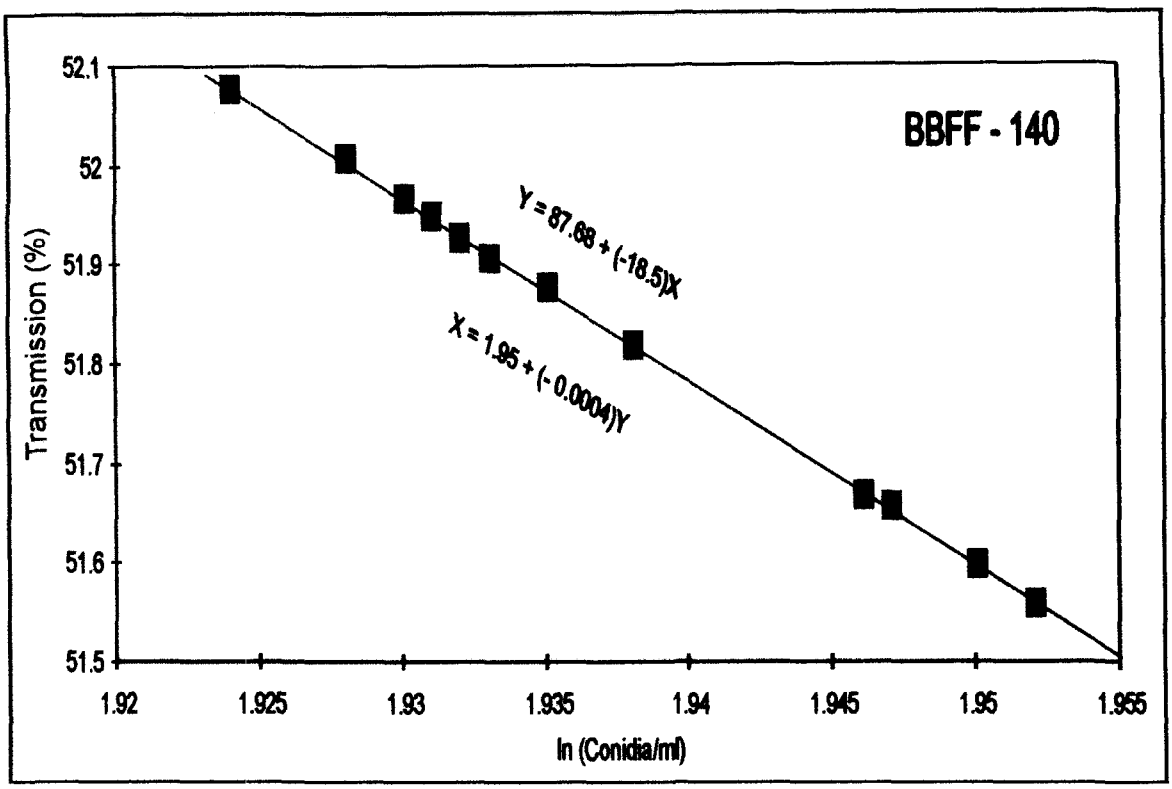


Figure 3. Transmission (%) and their corresponding conidial count for isolates of *Beauveria bassiana* cultured in different periods.



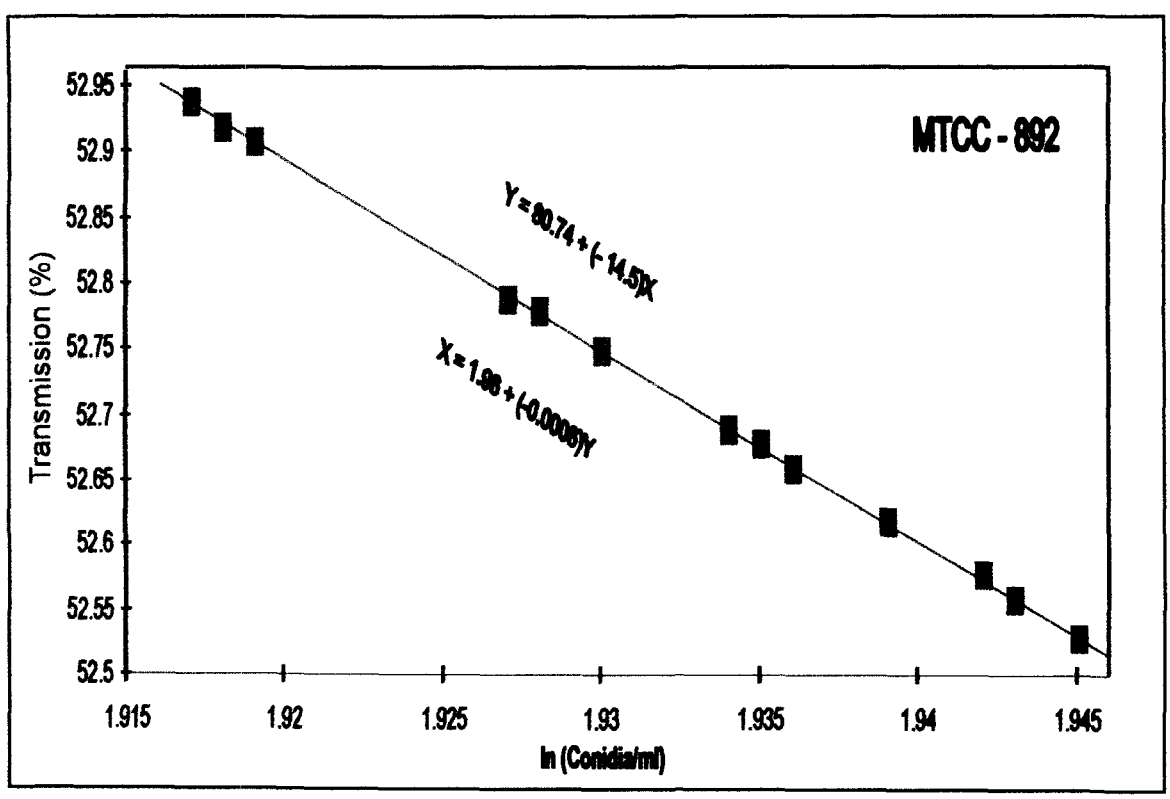
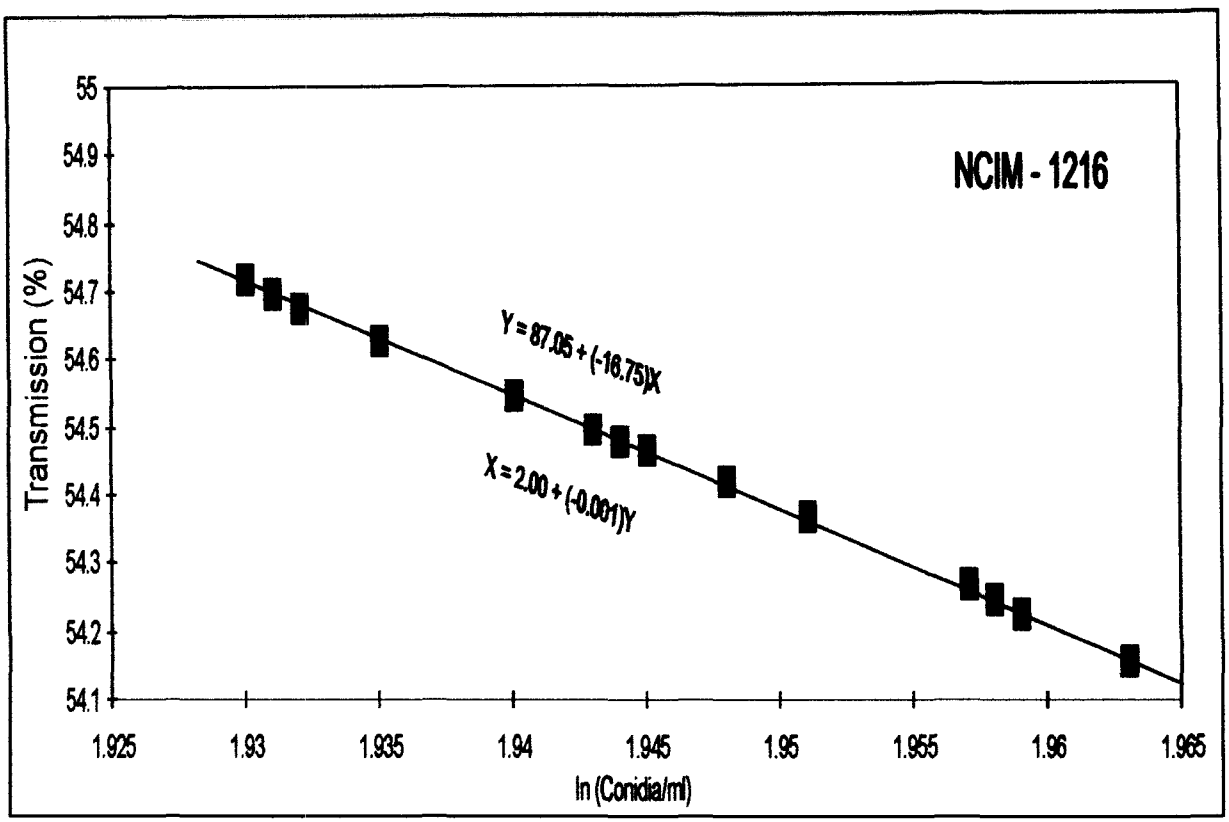


Figure 4. Regression line for *Metarhizium anisopliae* isolates for the relationship between transmission (%,Y) and numbers of conidia in suspension (X)(ln-value, based on 15 selected data points at different periods of 0-6, 0-12 and 6-12 months nearest to 50% transmission).



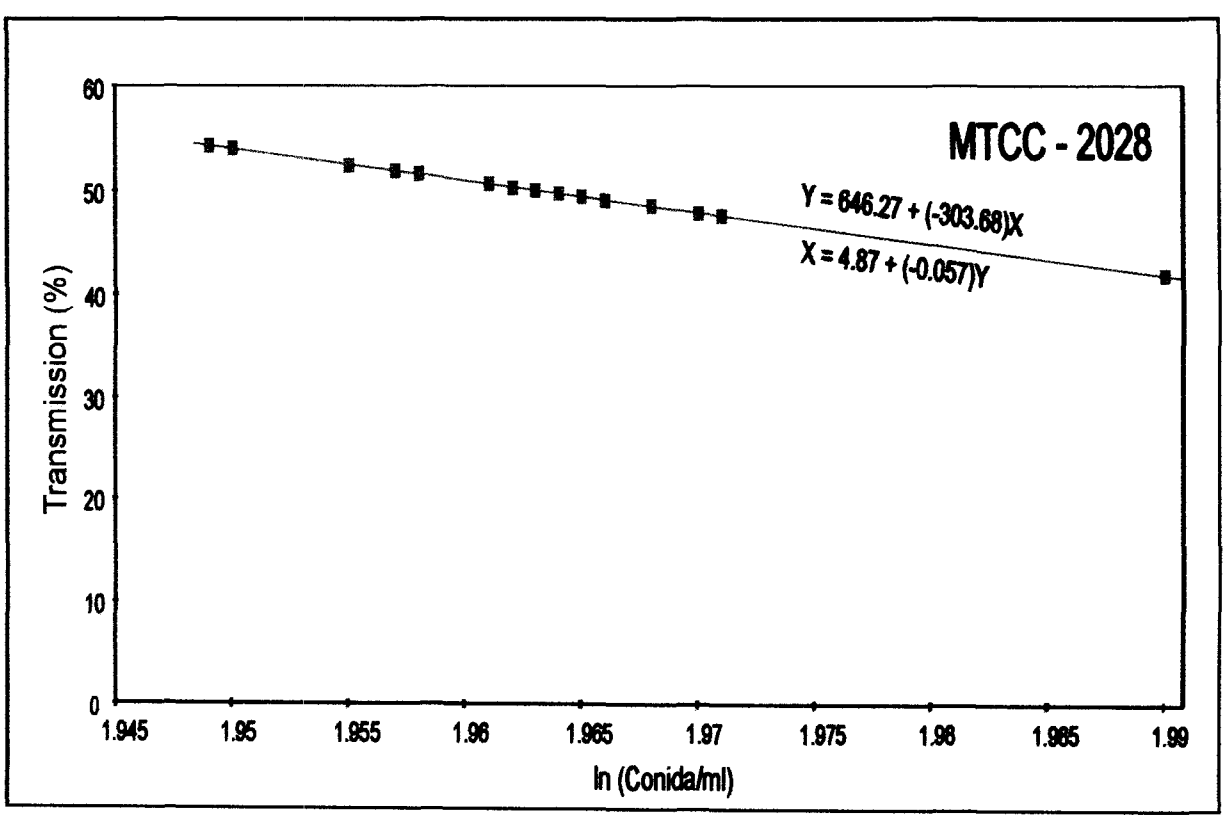
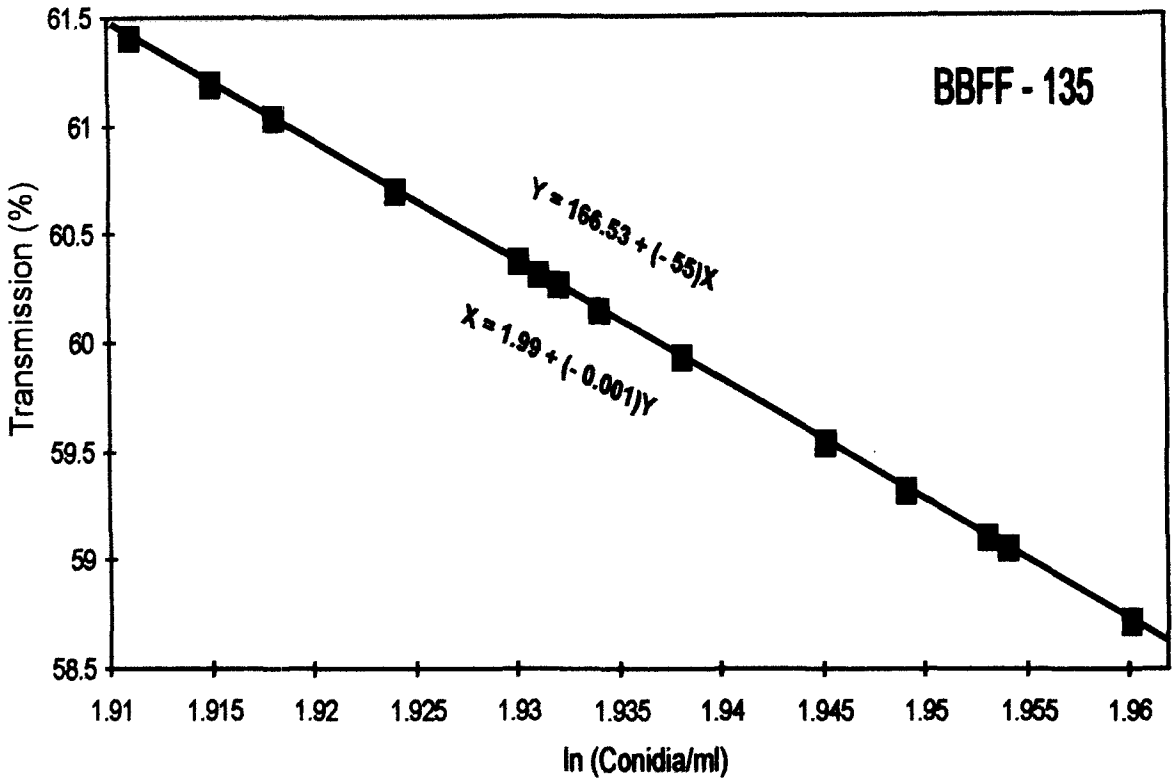


Figure 5. Regression line for *Beauveria bassiana* isolate for the relationship between transmission (%, Y) and numbers of conidia in suspension (X) (ln-value, based on 15 selected data points at different periods of 0-6, 0-12 and 6-12 months nearest to 50% transmission).



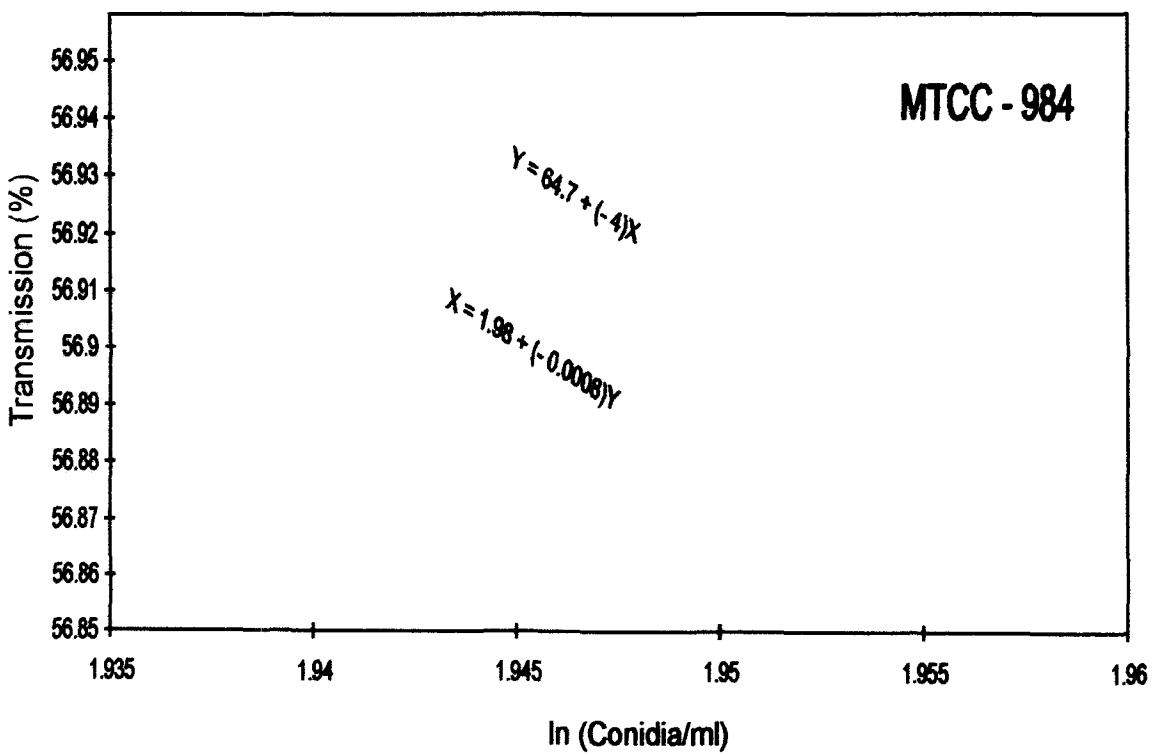


Figure 6. Regression line for *Beauveria bassiana* isolate for the relationship between transmission (%, Y) and numbers of conidia in suspension (X) (ln-value, based on 15 selected data points at different periods of 0-6, 0-12 and 6-12 months nearest to 50% transmission).

4.4 Determination of lethal concentration and lethal time of *B. bassiana* and *M.anisopliae.*

In order to determine the lethal concentration and lethal dose of entomopathogenic fungi contact/oral toxicity tests were performed using three various bioassays (Plate - 5). The bioassays were conducted using (a) individual termites, (b) group of termites without soil system and (c) group of termites in soil application. In case of individual termite bioassay, workers of *O.obesus* exposed to different fungal isolates such as 2028, 1216, 135, 984, 892 and 140 at different conncentrations ranging from 10^4 - 10^7 conidia ml^{-1} revealed that the mortality rate ranged from 62 - 97 %, 60 -96 %, 40 - 71 %, 43 - 84 %, 49 - 91 %, and 32 - 66 % respectively (Table - 7). While considering the effect of the isolates at different concentrations, the mortality rate ranged from 66 - 97 %, 55 - 92 %, 37 - 84 %, 32 - 62 % and 13 % at the concentrations of 10^7, 10^6, 10^5 and 10^4 conidia ml^{-1} and untreated control respectively. The critical difference (C.D.) values for different isolates and different concentrations at 5.0 % level were determined and presented in Table -7. Mortality rates of *B. bassiana* isolates 2028, 1216, 135, 984 were found to be 58-97%, 52-93%, 37-67%, 40-77% respectively and *M. anisopliae* isolates 892 and 140 were 46-85% and 22-62% respectively. These were significant at 5% level. The entomopathogenicity of different chosen fungal isolates in terms of fatality differed from 62 - 97 %, 48 - 92 %, 32 - 77 %, 22 - 58 % and 13 - 17 % at 10^7, 10^6, 10^5 and 10^4 conidia ml^{-1} concentrations and untreated control respectively, (Table - 10).

At different conidial concentrations of 10^7, 10^6, 10^5 and 10^4 conidia ml^{-1} the level of mortality obtained for the six fungal isolates ranged from 57.22 - 94.44 %, 32.77 - 83.88 %, 23.33 - 70.55 % and 18.33 - 55.55 % respectively whereas 11.65 - 16.55 % mortality was observed in untreated control. 55.55 - 94.44 %, 48.88 - 90.55 %, 26.66 - 61.11, 25.55 - 70 %, 40.55 - 75 %, and 18.33 - 57.22 % death rate of worker termites was registered at various chosen fungal isolates of *B.bassiana* and *M.anisopliae.* The critical difference values of 0.180, 0.166 and 0.406 was obtained for different strains, concentrations and both (Table - 13).

Probit analysis of dosage mortality as well as time mortality were worked out. Results have been presented in tables 8 and 9 for bioassays conducted to indivisual termites (Bioassay-I), tables 11 and 12 for group of termites (Bioassay- II) and tables 14 and 15 for groups of termites in soil application (Bioassay- III) Tables 8, 11 and 14 show the dosage - mortality responses of *O.obesus* to different fungal isolates in different bioassays (I, II & III).

In bioassay - I, the observed and expected responses were derived from chi square tests which ranged from 0.212 - 1.160. The lethal concentration to kill 50 % individuals ranged from 1.334×10^4 - 7.079×10^4 conidia / ml for *B.bassiana* and *M. anisopliae* isolates. The fiducial limits (95 %) for lower and higher limit ranged from 1.253×10^3 - 1.416×10^5 and 7.638×10^5 - 4.529×10^7 (Table - 8). In bioassay - II & III the chi square values were found to be between 0.106 - 2.451 conidia / ml (Table - 11) and 0.1185 - 3.0966 conidia / ml (Table - 14) respectively. The LC_{50} values for the strains of *B.bassiana* in bioassay II and III are between 1.175×10^6 to 2.818×10^4 and 2.630×10^7 to 5.012×10^4 conidia / ml whereas for *M. anisopliae* 1.862×10^5 to 1.318×10^7 and between 3.981×10^7 to 1.995×10^4 conidia / ml (Table 11 and 14). Probit analysis of time - mortality response of *O.obesus* to *B.bassiana* and *M.anisopliae* has been summarised in tables 9,12 and 15. Suitable results of observed and expected responses were obtained in all the three bioassays (I, II, and III) to different fungal isolates based on chi - square test . Results of three bioassays showed that the time required to kill 50 % of the worker termites (LT_{50}) ranged from 50.12 - 149.60h for *B.bassiana* and 72.44 - 158.50h for *M.anisopliae* (Table - 9,12 and 15).

Table 7: Mortality rate of termite workers of Odontotermes obesus exposed to different conidial concentrations of *B.bassiana* and *M. anisopliae.* Bioassay - I

Conidial Concentration (conidia ml^{-1})	Mortality (%)					
	Strains					
	B. bassiana				M. anisopliae	
	Bb - 2028	Bb - 1216	Bb - 135	Bb - 984	Ma - 892	Ma - 140
1×10^7	97.00 (4.46)	96.00 (4.44)	71.00 (3.84)	84.00 (4.16)	91.00 (4.32)	66.00 (3.70)
1×10^6	92.00 (4.35)	88.00 (4.25)	58.00 (3.48)	71.00 (3.83)	82.00 (4.11)	55.00 (3.39)
1×10^5	84.00 (4.16)	77.00 (3.99)	45.00 (3.08)	57.00 (3.45)	68.00 (3.75)	37.00 (2.81)
1×10^4	62.00 (3.58)	60.00 (3.53)	40.00 (2.92)	43.00 (3.01)	49.00 (3.20)	32.00 (2.62)
0 Control	13.00 (1.75)	13.00 (1.75)	13.00 (1.75)	13.00 (1.75)	13.00 (1.75)	13.00 (1.75)

	SEm+	C.D. (0.05)
Strain	0.0354	0.0830
Concentration	0.0323	0.0758
Strain x Concentration	0.0792	0.1856

Mean of 5 observations ; Bb - *Beauveria bassiana;* Ma - *Metarhizium anisopliae*

Figures represent percentage mean mortality after 7 days exposure.

Figures in parentheses are square root transformed values $X = \sqrt{X+0.05}$ of mean mortality.

Table 8: Probit analysis of dosage - mortality response of *Odontotermes obesus* to different strains of *B. bassiana* and *M. anisopliae*. Bioassay - I

Fungal isolate/ strain	Chi^2	Regression equation $Y=\bar{y}+b(x-\bar{x})$	LC_{50} conidia ml^{-1}	Fiducial limits (95%)
B. bassiana				
2028	0.296	Y=5.79+0.5568 (x-5.56)	1.334×10^4	1.253×10^3 - 1.416×10^5
1216	0.761	Y=5.66+0.4720 (x-5.66)	2.042×10^4	1.770×10^3 - 2.350×10^5
984	1.160	Y=5.20+0.4160 (x-5.97)	2.818×10^4	4.966×10^4 - 1.596×10^6
135	0.212	Y=4.91+0.3240 (x-5.98)	2.239×10^4	2.780×10^5 - 1.803×10^7
M. anisopliae				
892	0.900	Y=5.43+0.4820 (x-5.83)	7.079×10^4	1.057×10^4 - 4.732×10^7
140	1.006	Y=4.74+0.3770 (x-6.10)	5.888×10^4	7.638×10^5 - 4.529×10^7

Data taken after 7 days incubation at 27 - 30°C

Table 9: Probit analysis of Time - mortality response of *Odontotermes obesus* to different strains of *B.bassiana* and *M.anisopliae*. Bioassay - I

Fungal isolate/ strain	Chi^2	Regression equation $Y=\bar{y}+b(x-\bar{x})$	LT_{50} hrs (10^7 conidia ml^{-1})	Fiducial limits (95%)
B. bassiana				
2028	2.460	Y=5.61+3.44 (x-1.92)	56.23	41.69 - 75.68
1216	0.880	Y=5.51+3.59 (x-1.96)	63.10	48.42 - 82.04
984	2.270	Y=5.13+3.11 (x-2.01)	89.13	70.63 - 112.20
135	0.299	Y=4.84+2.70 (x-2.04)	114.80	89.13 - 147.60
M. anisopliae				
892	0.480	Y=5.35+3.43 (x-1.98)	72.44	56.62 - 92.47
140	0.136	Y=4.74+2.53 (x-2.05)	144.50	105.90 - 196.80

Data taken after 7 days incubation at 27 - 30°C

Table 10: Mortality rate of termite workers of *O. obesus* exposed to different conidial concentrations of *B.bassiana* (strains- 135, 984, 2028 and 1216) and *M. anisopliae* (Strains- 140 and 892). Bioassay - II

Conidial Concentration (conidia ml^{-1})	Mortality (%)					
	Strains					
	B. bassiana				*M. anisopliae*	
	Bb - 2028	Bb - 1216	Bb - 135	Bb - 984	Ma - 892	Ma - 140
1×10^7	97 (4.46)	93 (4.37)	67 (3.72)	77 (3.98)	85 (4.18)	62 (3.59)
1×10^6	92 (4.34)	81 (4.08)	54 (3.36)	64 (3.64)	75 (3.93)	48 (3.17)
1×10^5	77 (3.98)	70 (3.80)	44 (3.04)	51 (3.26)	59 (3.50)	32 (2.62)
1×10^4	58 (3.47)	52 (3.29)	37 (2.81)	40 (2.91)	46 (3.11)	22 (2.20)
0 Control	17 (1.95)	17 (1.95)	13 (1.75)	16 (1.91)	16 (1.91)	13 (1.75)

	SEm+	C.D. (0.05)
Strain	0.032	0.075
Concentration	0.030	0.070
Strain x Concentration	0.073	0.171

Mean of 5 observations ; Bb - *Beauveria bassiana;* Ma - *Metarhizium anisopliae*

Figures represent percentage mean mortality after 7 days exposure.

Figures in parentheses are square root transformed values $X = \sqrt{X+0.05}$ of mean mortality.

Table 11: Probit analysis of dosage - mortality response of *O.* obesus to different strains of *B.bassiana* and *M.anisopliae*. Bioassay - II

Fungal isolate/ strain	Chi^2	Regression equation $Y=\bar{y}+b(x-\bar{x})$	LC conidia ml^{-1}	Fiducial limits (95%)
B. bassiana				
2028	2.4510	Y=5.62+0.5269 (x-5.53)	2.818×10^4	3.631×10^3 - 2.138×10^5
1216	0.9000	Y=5.43+0.4686 (x-5.81)	6.310×10^4	5.675×10^3 - 6.918×10^5
984	0.4091	Y=5.00+0.3806 (x-6.05)	1.175×10^6	2.089×10^5 - 6.457×10^6
135	0.1060	Y=4.83+0.3077 (x-6.01)	2.951×10^6	2.979×10^5 - 2.917×10^7
M. anisopliae				
892	0.1140	Y=5.23+0.4211 (x-5.95)	1.862×10^5	2.944×10^4 - 1.175×10^6
140	0.6910	Y=4.60+0.4725 (x-6.29)	1.318×10^7	8.128×10^5 - 2.089×10^8

Data taken after 7 days incubation at 27 - 30°C

Table 12: Probit analysis of Time-mortality response of O. obesus to different strains of *B.bassiana* and *M.anisopliae*. Bioassay - II.

Fungal isolate/ strain	Chi^2	Regression equation $Y=\bar{y}+b(x-\bar{x})$	LT_{50} hrs (10^7 conidia ml^{-1})	Fiducial limits (95%)
B. bassiana				
2028	3.2500	Y=3.780+3.340 (x-1.91)	50.12	35.56 - 70.47
1216	2.4000	Y=5.590+3.100 (x-1.96)	60.26	44.16 - 82.04
984	3.4700	Y=5.024+2.870 (x-2.03)	104.70	81.28 - 131.80
135	0.3150	Y=4.760+3.045 (x-2.08)	141.30	109.60 - 177.80
M. anisopliae				
892	0.2271	Y=5.250+2.520 (x-2.00)	75.86	55.46 - 103.50
140	0.0210	Y=6.366+3.120 (x-2.08)	158.50	117.50 - 208.90

Data taken after 7 days incubation at 27 - 30°C

Table 13: Mortality rate of termite workers of *O.obesus* exposed to different conidial concentrations of *B. bassiana* (strains-135, 984, 2028 and 1216) and *M. anisopliae* (Strains-140 and 892).Bioassay - III.

Conidial Concentration (conidia ml^{-1})	Mortality (%)					
	Strains					
	B. bassiana				*M. anisopliae*	
	Bb - 2028	Bb - 1216	Bb - 135	Bb - 984	Ma - 892	Ma - 140
1×10^7	94.44 (4.40)	90.55 (4.31)	61.11 (3.19)	70.00 (3.75)	75.00 (3.93)	57.22 (3.45)
1×10^6	83.88 (4.15)	78.33 (4.01)	43.88 (3.03)	52.22 (3.30)	60.00 (3.53)	32.77 (2.65)
1×10^5	70.55 (3.82)	63.88 (3.64)	33.33 (2.66)	40.55 (2.93)	50.00 (3.23)	23.33 (2.27)
1×10^4	55.55 (3.40)	48.88 (3.20)	26.66 (2.41)	25.55 (2.36)	40.55 (2.57)	18.33 (2.03)
0 Control	15.55 (1.87)	16.65 (1.94)	12.80 (1.73)	11.65 (1.66)	15.55 (1.88)	11.65 (1.67)

	SEm+	C.D. (0.05)
Strain	0.077	0.180
Concentration	0.071	0.166
Strain x Concentration	0.1741	0.4065

Mean of 9 observations ; Bb - *Beauveria bassiana;* Ma - *Metarhizium anisopliae*

Figures represent percentage mean mortality after 7 days exposure.

Figures in parentheses are square root transformed values $X = \sqrt{X+0.05}$ of mean mortality.

Table 14: Probit analysis of dosage - mortality response of *O. obesus* to different strains of *B.bassiana* and *M. anisopliae*.Bioassay- III.

Fungal isolate/ strain	Chi^2	Regression equation $Y=\bar{y}+b(x-\bar{x})$	LC_{50} conidia ml^{-1}	Fiducial limits (95%)
B. bassiana				
2028	3.0966	Y=5.52+0.5010 (x-5.87)	5.012×10^4	1.133×10^4 - 2.216×10^5
1216	1.2750	Y=5.23+0.4440 (x-5.62)	8.913×10^4	2.973×10^4 - 3.286×10^5
984	0.2757	Y=9.77+0.4680 (x-6.21)	5.248×10^6	1.690×10^6 - 1.626×10^7
135	2.8080	Y=4.58+0.3842 (x-6.23)	2.630×10^7	4.169×10^6 - 1.622×10^4
M. anisopliae				
892	5.9600	Y=5.00+0.3650 (x-6.15)	1.995×10^4	5.495×10^5 - 7.097×10^6
140	0.1185	Y=4.40+0.5580 (x-6.41)	3.981×10^7	8.570×10^6 - 1.845×10^8

Data taken after 7 days incubation at 27 - 30°C

Table 15: Probit analysis of Time - mortality response of *O. obesus* to different strains of *B.bassiana* and *M.anisopliae*.Bioassay-II.

Fungal isolate/ strain	Chi^2	Regression equation $Y=\bar{y}+b(x-\bar{x})$	LT_{50} hrs. (10^7 conidia ml^{-1})	Fiducial limits (95%)
B. bassiana				
2028	0.817	Y=5.57+3.53 (x-1.87)	53.09	39.17 - 71.78
1216	1.400	Y=5.39+3.90 (x-1.98)	72.78	56.49 - 93.54
984	3.160	Y=4.86+2.96 (x-2.05)	122.20	96.16 - 154.90
135	1.610	Y=4.68+2.47 (x-2.05)	149.60	104.50 - 208.90
M. anisopliae				
892	2.520	Y=4.95+2.68 (x-2.03)	112.20	87.30 - 143.90
140	0.814	Y=4.62+2.25 (x-2.05)	158.50	104.50 - 234.40

Data taken after 7 days incubation at 27 - 30°C

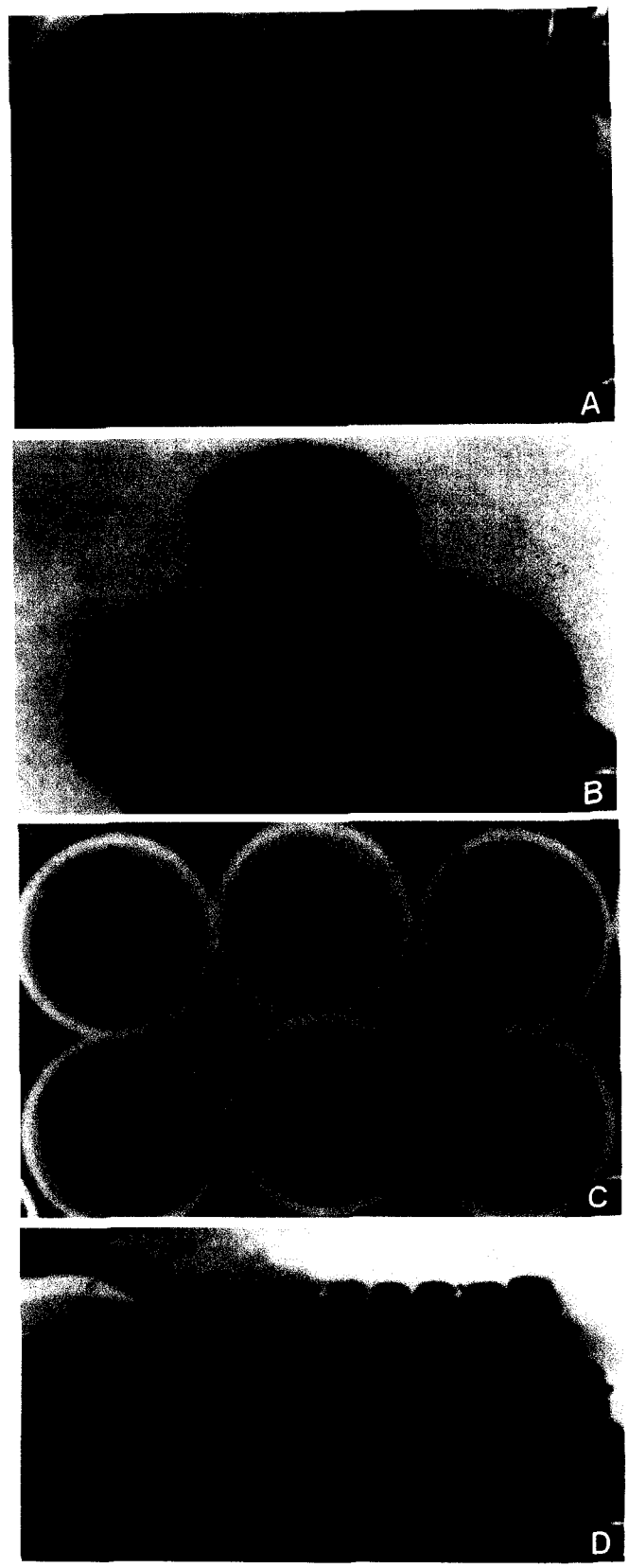


Plate 5 (A-D) : Termites collected from field (A) and acclimatized on a special set up (B).Experimental set up for pathogenicity tests of *M.anisopliae* and *B.bassiana* against *O.obesus* (C&D).

4.5 Pathogenicity of entomopathogenic fungi toward *Odontotermes obesus*

The cumulative percent mortality and LT_{50} and LT_{90} among workers of *O.obesus* at different days since treatment when exposed to different conidial concentrations of *B.bassiana* isolates 135 and 984 are given in tables 16 & 17. The isolate 135 required 5, 4, 4, 3 and 5 days exposure period to kill 50 % of the termite population by 1 x 10^6 conidial /ml conce ntration of conidia taking 10, 14, 20, 28, and 34 days old culture respectively.

Similarly, isolate 984 required 5, 4.5, 4, 3 and 5 days exposure period to kill 50 % of the termite population by 1 x 10^6 conidia / ml concentration of conidia taking 10, 14, 20, 28 and 34 days old cultures respectively. Results from the experiments showed that 90 % mortality was achived during 6 to 7 days exposure in isolate 135 and isolate 984 using 28 and 20 days old cultures. (Table 16 & 17).

The untreated controls had 19 - 21% mortality 7 days after treatment (Tables 16 & 17). The percentage mortality of *O.obesus* increased with age of the culture then decreased in treatments with the 34 - day - old culture. 98 - 100 % mortality was noticed with 28 days old culture at 10^6 conidia /ml concentration. Higher conidial concentrations were more lethal, and the mortality of all the treatments differed significantly (p = 0.05) from untreated controls. Conidial concentrations of 1 x 10^6 / ml, of 20 or 28 days old cultures, produced over 90 % worker termite mortality. Lethal time for 90 % and 50 % mortality decreased with increasing age of the cultures and conidial concentration up to 28 days.

Table 18 summarises the two-way analysis of variance for conidial concentration and different ages of the culture of *B.bassiana* isolate 984. The two-way ANOVA of the data revealed significant differences (P=0.05) between different ages of the culture, between conidial concentrations and their interations.

Table 19 summarises the results of a two-way analysis of variance for martality of *O.obesus* workeers treated with different concentrations of various ages of culture of *B.bassiana*. The mortality of *O.obesus* varied significantly (P=0.05) in different ages of the culture. conidial concentrations and their interactions.

Table 16: Cumulative percent mortality and LT_{50} and LT_{90} among termite workers of *O.obesus* at different days since treatment when exposed to different conidial concentrations of different aged culture of *B.bassiana* (135)#

Age of the culture (days)	Conidial concentration (condium/ml)	Percent Mortality - Day since treatment							Lethal time (in days)	
		1	2	3	4	5	6	7	LT_{50}	LT_{90}
	10^6	03 ± 0.6b	12 ± 0.4b	17 ± 0.8b	32 ± 0.1c	51 ± 0.0d	60 ± 0.3d	76 ± 0.5d	5	++
10	10^5	02 ± 0.2b	08 ± 0.0c	13 ± 0.8c	27 ± 0.6b	39 ± 0.6c	44 ± 0.5c	53 ± 2.9c	7	++
	10^4	02 ± 0.5b	10 ± 2.1b	18 ± 0.0b	28 ± 2.0b	33 ± 0.2b	41 ± 0.8b	44 ± 0.4b	+	++
	Control	0 a	0 a	03 ± 0.7a	07 ± 0.3a	10 ± 0.2a	13 ± 1.2a	21 ± 1.2a	+	++
	10^6	07 ± 0.9c	14 ± 1.9c	28 ± 0.5c	52 ± 0.0c	69 ± 0.5c	78 ± 0.2c	83 ± 0.0d	4	++
	10^5	03 ± 0.8b	11 ± 0.8b	22 ± 0.6b	33 ± 0.8b	39 ± 0.0b	52 ± 0.3b	59 ± 2.0c	6	++
14	10^4	03 ± 0.8b	10 ± 1.5b	20 ± 0.0b	27 ± 1.8b	38 ± 0.0b	47 ± 0.6b	52 ± 0.8b	7	++
	Control	0 a	0 a	03 ± 0.5a	10 ± 0.7a	13 ± 0.6a	17 ± 0.9a	19 ± 1.3a	+	++
	10^6	11 ± 0.5b	25 ± 0.3d	34 ± 2.2d	56 ± 1.8d	72 ± 0.9d	80 ± 0.0d	95 ± 0.4d	4	7
	10^5	08 ± 0.0b	28 ± 2.8c	30 ± 0.9c	42 ± 0.5c	54 ± 2.0c	64 ± 0.3c	69 ± 0.3c	5	++
20	10^4	07 ± 0.0b	23 ± 2.5b	28 ± 1.5b	33 ± 2.9b	42 ± 0.8b	50 ± 2.8b	55 ± 0.4b	6	++
	Control	0 a	03 ± 1.9a	03 ± 1.9a	07 ± 0.8a	10 ± 1.9a	17 ± 1.0a	19 ± 1.8a	+	++
	10^6	13 ± 2.8c	35 ± 0.8d	50 ± 1.0d	70 ± 1.9d	88 ± 2.0d	98 ± 2.0c	100 ± 0.8d	3	6
	10^5	08 ± 0.3b	22 ± 0.0c	36 ± 0.4c	52 ± 1.0c	58 ± 2.0c	72 ± 3.0b	83 ± 0.0c	4	++
28	10^4	08 ± 0.0b	19 ± 1.9b	28 ± 1.8b	38 ± 1.9b	50 ± 2.0b	67 ± 2.2b	69 ± 0.3b	5	++
	Control	0 a	0 a	03 ± 0.6a	07 ± 2.0a	13 ± 2.0a	17 ± 1.5a	21 ± 1.8a	+	++
	10^6	09 ± 2.0c	12 ± 1.8b	22 ± 2.8b	39 ± 0.9c	50 ± 2.8d	72 ± 0.6d	78 ± 2.8d	5	++
	10^5	03 ± 1.2b	10 ± 2.9b	20 ± 2.9b	28 ± 2.0b	39 ± 2.8c	50 ± 0.3c	58 ± 2.2c	6	++
34	10^4	03 ± 0.8b	10 ± 1.0b	20 ± 1.2b	25 ± 0.5b	36 ± 1.0b	45 ± 2.0b	50 ± 1.5b	7	++
	Control	0 a	03 ± 0.6a	07 ± 1.9a	10 ± 2.0a	10 ± 2.5a	17 ± 1.0a	19 ± 1.5a	+	++

Each figure is a mean of 5 observations.

Treatments were replicated five times with 30 termite workers per treatment. Means followed by same letter of a given column were not significantly different by Tukey's multiple range test (P=0.05)

\+ LT_{50} not achieved within time since treatment; ++ LT_{90} not achieved within time since treatement.

Table 17: Cumulative percent mortality and LT_{50} and LT_{90} among termite workers of *O.obesus* at different days since treatment when exposed to different conidial concentrations of different aged culture of *B.bassiana* (984)#

Age of the culture (days)	Conidial concentration (condium/ml)	Present Mortality - Day since treatment							Lethal time (in days)	
		1	2	3	4	5	6	7	LT_{50}	LT_{90}
10	10^6	03 ± 0.3b	10 ± 0.1b	17 ± 0.4b	30 ± 1.1c	50 ± 0.6d	60 ± 0.3d	73 ± 0.5d	5	++
	10^5	03 ± 0.4b	07 ± 0.4c	13 ± 0.4c	27 ± 0.4b	37 ± 0.5c	43 ± 0.5c	53 ± 2.1c	7	++
	10^4	03 ± 0.5b	10 ± 1.1b	17 ± 0.5b	27 ± 1.0b	33 ± 3.2b	40 ± 3.0b	41 ± 3.4b	+	++
	Control	0 a	0 a	03 ± 0.7a	07 ± 0.3a	10 ± 0.2a	13 ± 1.2a	21 ± 1.2a	+	++
14	10^6	07 ± 0.7c	13 ± 1.4c	27 ± 1.5c	47 ± 1.0c	67 ± 0.6c	77 ± 0.7c	82 ± 0.9d	4 5	++
	10^5	03 ± 0.5b	10 ± 0.6b	20 ± 0.8b	30 ± 0.8b	37 ± 0.9b	50 ± 2.3b	58 ± 2.6c	6	++
	10^4	03 ± 0.5b	10 ± 0.5b	20 ± 0.9b	27 ± 0.6b	37 ± 0.4b	47 ± 0.8b	51 ± 0.9b	7	++
	Control	0 a	0 a	03 ± 0.5a	10 ± 0.7a	13 ± 0.6a	17 ± 0.9a	19 ± 1.3a	+	++
20	10^6	10 ± 2.5b	20 ± 2.3d	33 ± 2.7d	50 ± 1.4d	70 ± 1.9d	80 ± 2.5d	94 ± 3.4d	4	7
	10^5	07 ± 0.9b	27 ± 1.9c	27 ± 1.9c	40 ± 1.5c	53 ± 2.8c	63 ± 3.3c	69 ± 1.3c	5	++
	10^4	07 ± 0.8b	23 ± 1.5b	23 ± 1.5b	33 ± 1.7b	40 ± 1.8b	50 ± 1.3b	54 ± 1.4b	6	++
	Control	0 a	03 ± 1.9a	03 ± 1.9a	07 ± 0.8a	10 ± 1.9a	17 ± 1.0a	19 ± 1.8a	+	++
28	10^6	13 ± 2.0c	33 ± 2.8d	50 ± 1.4d	70 ± 1.7d	80 ± 4.0d	93 ± 2.8C	100 ± 0.0d	3	6
	10^5	07 ± 2.3b	20 ± 3.0c	30 ± 2.4c	47 ± 1.6c	57 ± 2.6c	70 ± 3.6b	79 ± 3.6c	4 5	++
	10^4	07 ± 1.4b	17 ± 1.7b	23 ± 1.6b	37 ± 1.9b	50 ± 1.7b	67 ± 1.9b	67 ± 2.3b	5	++
	Control	0 a	0 a	03 ± 0.6a	07 ± 2.0a	13 ± 2.0a	17 ± 1.5a	21 ± 1.8a	+	++
34	10^6	07 ± 2.5c	10 ± 1.4b	20 ± 1.9b	37 ± 1.9c	50 ± 2.3d	70 ± 3.6d	78 ± 2.5d	5	++
	10^5	03 ± 1.0b	10 ± 1.7b	20 ± 0.9b	27 ± 2.0b	37 ± 2.9c	50 ± 2.3c	56 ± 2.7c	6	++
	10^4	03 ± 0.6b	10 ± 2.0b	20 ± 1.3b	27 ± 1.5b	33 ± 1.8b	43 ± 2.0b	49 ± 1.8b	7	++
	Control	0 a	03 ± 0.6a	07 ± 1.9a	10 ± 2.0a	10 ± 2.5a	17 ± 1.0a	19 ± 1.5a	-	++

Each figure is a mean of 5 observations.

Treatments were replicated five times with 30 termite workers per treatment. Means followed by same letter of a given column were not significantly different by Tukey's multiple range test (P=0.05)

\+ LT_{50} not achieved within time since treatment; ++ LT_{90} not achieved within time since treatement.

Table 18: Two way analysis of variance for conidial concentration and different ages of the culture of *B. bassiana* (984)

Source	SS	DF	MS	SEM±	CD(0.05)	CV%
Total	2454.0	99	24.8	-	-	-
Between ages of the culture (Factor A)	48.7	4	12.2	0.06	0.15	1.35
Between conidial concentrations (Factor B)	2339.9	3	780.0	0.05	0.13	0.05
Interaction (A x B)	59.0	12	4.9	0.13	0.29	5.41

Table 19: Two way analysis of variance for mortality of *O. obesus* exposed to different conidial concentration of different aged culture of *B. bassiana* (984)

Source	SS	DF	MS	SEM±	CD(0.05)	CV%
Total	327.1	99	3.3	-	-	-
Between ages of the culture (Factor A)	17.9	4	4.5	0.04	0.09	0.56
Between conidial concentrations (Factor B)	300.8	3	100.3	0.03	0.08	0.44
Interaction (A x B)	6.4	12	0.5	0.07	0.17	2.22

4.6 Mass production and formulation

4.6.1 Quantification of condial production of B.bassiana in different combinations of water and vegetable oil .

B.bassiana isolate 2028 grown in different substrates with different moisture and oil combinations and their respective conidial production are summarised in Table 20 and Figures - 7. The conidial concentration of the chosen isolate was found to vary from 10^7 - 10^9 in bajra. 10^7 - 10^9 in maize, 10^6 - 10^8 in wheat and wheat bran. Conidial concentration varied from 10^5 - 10^7 in ground nut and barley, 10^5 - 10^7 in rice bran at different percentage of moisture and oil. However, the isolate showed maximum sporulation at around 80% water and 4 - 8% oil addition. The highest yields varied between 6.55 x 10^8 and 1.15 x 10^9 conidia / ml of harvest in bajra substrate on addition of 70 - 80% of moisture and 8% of oil (Table 20).

4.6.2 Mass production

Solid / grain medium :

Entomopathogenic fungi were mass multiplied on grain media. *B.bassiana* isolates cultured for 30 days on 400g. crushed grains of Bajra (Pennisetum typhoides) in tubs yielded a grain spore dust of 4.43 x 10^7, 4.60 x 10^7, 4.97 x 10^7, and 4.33 x 10^8 conidia / g dry weight by isolates 984, 2028, 135, and 1216 respectively. The same isolates yielded 1.48 x 10^8, 1.82 x 10^8, 1.31 x 10^8 and 1.82 x 10^8 conidia / g dry weight when multiplied on 200g. crushed Bajra grains and cultured for 20 days, (Table - 21 and Plate - 6). Where as spore production in *M.anisopliae* isolates 892 and 140, mass multiplied on 400g. crushed Bajra and cultured for 30 days, were 4.54 x 10^7 and 1.94 x 10^8 conidia / g dry weight. The same isolates when grown on 200g. crushed Bajra and cultured for 20 days yielded 1.77 x 10^8 and 1.83 x 10^8 conidia / g dry weight of grain spore dust (Table 21). The spore concentration of different isolates were tested for its bioefficacy against termite workers and the results of the mortality of the termite workers are given on table 22. Highest mortality (88.8%) was achieved with *B.bassiana* 2028.

Table 20: Conidial production of *B. bassiana* (2028) in different combination of sunflower oil and water

Water (%)	Sunflower Oil (%)	* Conidial Production (conidia/gm)						
		Substrate						
		Bajra	Maize	Wheat	Wheatbran	Groundnut	Barley	Ricebran
50	0	4.8×10^7	1.2×10^7	1.1×10^6	2.6×10^6	1.0×10^6	2.3×10^6	3.5×10^5
	2	7.1×10^7	1.8×10^7	1.3×10^6	1.1×10^6	1.2×10^6	5.0×10^6	2.8×10^5
	4	1.5×10^8	7.9×10^7	4.0×10^6	2.2×10^6	4.9×10^6	4.3×10^6	4.3×10^5
	8	3.1×10^8	3.8×10^7	6.5×10^6	4.2×10^6	5.3×10^6	4.0×10^6	6.0×10^5
	16	8.7×10^7	2.2×10^7	4.3×10^6	3.4×10^6	3.3×10^6	1.3×10^6	1.5×10^5
60	0	2.6×10^8	5.8×10^7	1.6×10^6	1.4×10^6	1.0×10^6	2.3×10^6	3.5×10^5
	2	8.3×10^7	2.4×10^7	2.5×10^6	2.9×10^6	1.2×10^6	5.0×10^6	2.8×10^5
	4	1.2×10^8	7.8×10^8	1.1×10^7	3.4×10^6	4.9×10^6	4.3×10^6	4.2×10^5
	8	1.6×10^8	3.8×10^8	4.6×10^7	5.3×10^7	5.3×10^6	4.0×10^6	6.0×10^5
	16	3.8×10^8	5.4×10^8	2.8×10^7	1.6×10^7	3.3×10^6	1.2×10^6	1.5×10^5
70	0	2.9×10^8	2.8×10^7	1.1×10^7	3.4×10^6	2.3×10^6	3.0×10^6	2.5×10^6
	2	1.5×10^8	3.5×10^7	6.1×10^7	2.5×10^6	3.5×10^6	1.5×10^6	2.0×10^6
	4	5.5×10^7	1.3×10^9	2.8×10^8	1.9×10^8	2.2×10^7	4.7×10^6	5.8×10^6
	8	6.5×10^8	7.8×10^8	3.7×10^8	2.6×10^8	8.0×10^7	7.8×10^6	2.5×10^6
	16	2.9×10^8	4.0×10^8	7.2×10^7	3.3×10^7	5.6×10^7	5.8×10^6	2.0×10^6
80	0	1.5×10^8	4.3×10^7	2.3×10^7	2.5×10^7	2.5×10^6	8.8×10^6	4.2×10^6
	2	6.8×10^7	2.0×10^8	5.4×10^7	4.1×10^7	4.1×10^6	7.0×10^6	3.9×10^6
	4	1.8×10^8	3.7×10^8	2.1×10^8	2.5×10^8	1.2×10^7	5.0×10^6	1.4×10^7
	8	1.2×10^9	6.4×10^8	5.4×10^8	3.2×10^8	4.1×10^7	1.1×10^7	3.4×10^7
	16	6.3×10^8	4.4×10^8	6.6×10^7	6.8×10^7	3.3×10^7	4.0×10^6	6.9×10^6
90	0	5.3×10^7	1.5×10^7	2.4×10^7	2.6×10^7	1.6×10^6	3.3×10^6	4.2×10^6
	2	2.8×10^7	7.5×10^7	2.2×10^7	1.6×10^7	3.2×10^5	4.5×10^6	3.0×10^6
	4	9.2×10^7	2.5×10^8	1.4×10^8	5.8×10^7	1.0×10^7	3.3×10^6	6.8×10^6
	8	1.7×10^8	1.0×10^8	5.5×10^7	1.1×10^8	3.6×10^7	6.5×10^6	2.8×10^7
	16	7.5×10^7	3.5×10^7	4.4×10^7	4.8×10^7	1.2×10^7	2.0×10^6	8.0×10^6

* Mean of three observations

Table 21: Mass culture of different isolates of *B. bassiana* and *M. anisopliae* in solid substrate (Bajra)

Fungus	Strain	*Count(conidia /gm)	
		Age of culture	
		20 days[a]	30 days[b]
Beauveria bassiana	984	1.48×10^8	4.43×10^7
	2028	1.82×10^8	4.60×10^7
	135	1.31×10^8	4.97×10^7
	1216	1.82×10^8	2.33×10^8
Metarhizium anisopliae	892	1.77×10^8	4.54×10^7
	140	1.83×10^8	1.94×10^8

* Haemocytometer measurements

a 200 g substrate

b 400 g substrate

Table 22: Mortality of termite workers of *O.obesus* treated with different isolates of *B.bassiana* and *M.anisopliae* mass produced in solid substrate (Bajra)

Fungus	Strain	Connection (conidia/gm)	Mortality(%)*
Beauveria bassiana	984	1×10^7	72.6c
	2028	1×10^7	88.8e
	135	1×10^7	64.4b
	1216	1×10^7	80.2d
Metarhizium anisopliae	892	1×10^7	72.6c
	140	1×10^7	61.6b
Control		Untreated	16.0a

* Mortality after 7 days exposure; Figures represent the mean of 5 replicates. Means followed by same letter are not significantly different by Duncan's multiple range test (P=0.05)

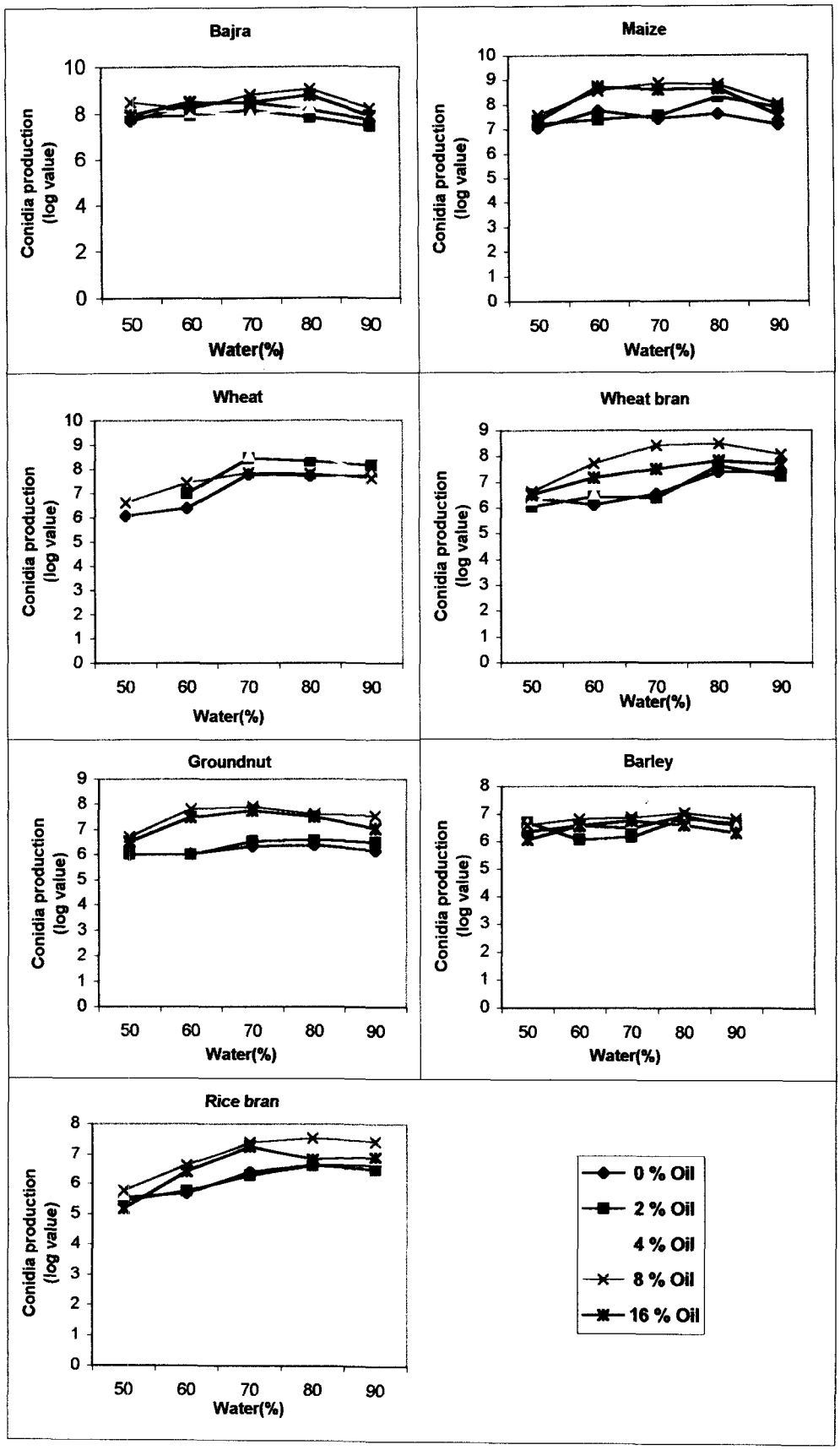


Figure 7: Conidia production of *B.bassiana* (2028) in various substrates in different combinations of sunflower oil and water

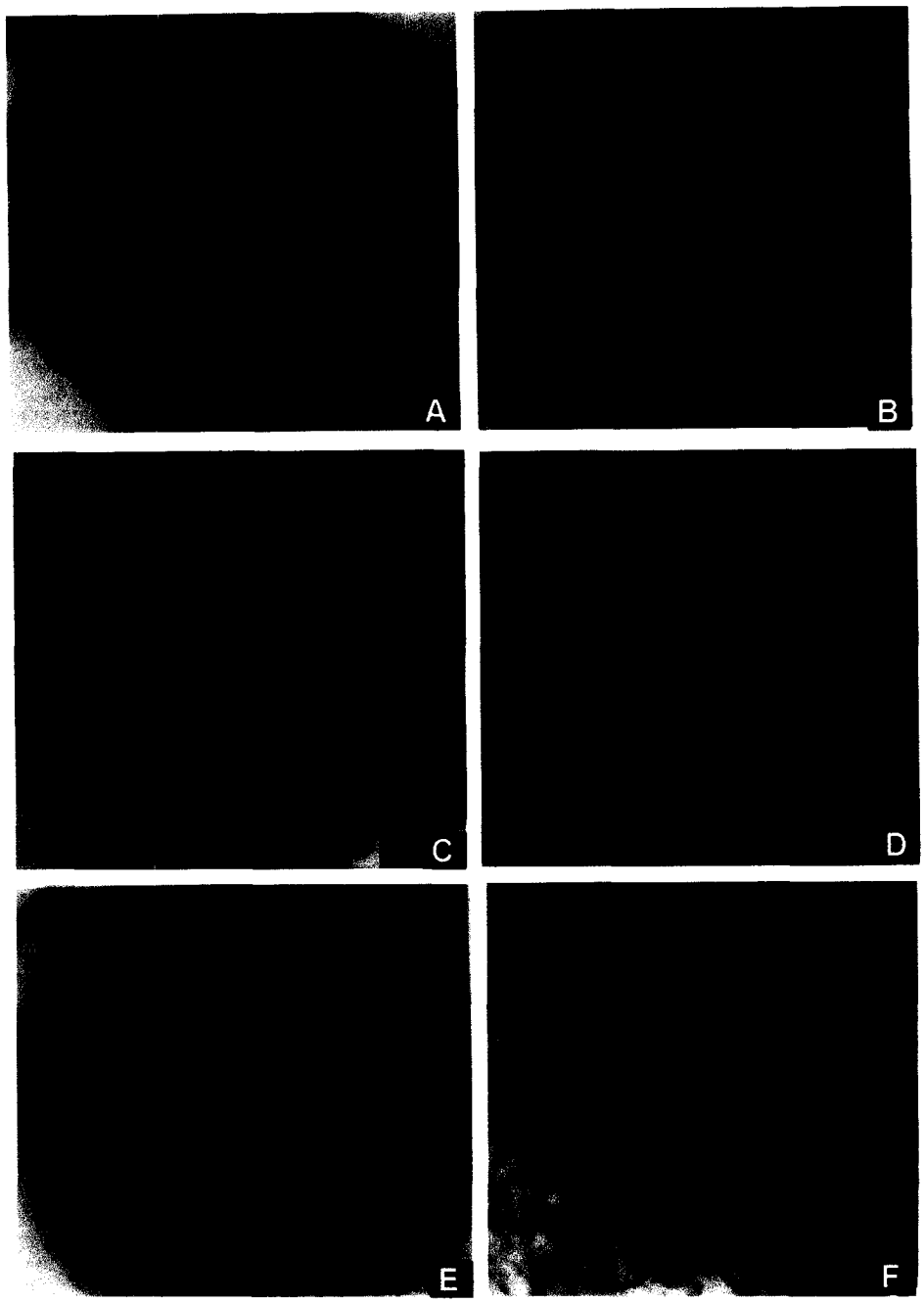


Plate 6 (A-F) : Development of fungal mass culture in solid medium. *B.bassiana* isolates - 2028 (A), 984 (B), 1216 (D) & 135 (F) and *M.anisopliae* isolates - 892 (C) & 140 (E).

Liquid / broth medium

Culturing of entomopathogenic fungus on two types of broth medium (i.e. molasses yeast and yeast glucose) contained in 5 litre capacity tubs (Plate - 7) revealed that *B. bassiana* isolate 2028 produced more number of conidia followed by isolates 1216,135 and 984 in molasses yeast (MY) media, while *M.anisopliae* isolate 892 produced more conidia than isolate 140. In yeast glucose (YG) media, *B.bassiana* isolate 2028 produced more number of conidia followed by isolates 135, 984 and 1216. *M.anisopliae* isolate 140 produced more number of conidia than isolate 892. The conidial counts in the slurry of MY media of isolates 2028, 1216, 135, 984, 892 and 140 were noted to be 1.85×10^7, 1.67×10^7, 1.50×10^7, 1.07×10^7, $1.80 \times 10^7$and 1.62×10^7 conidia / ml, respectively (Table 23)

Pilot scale mass production

Cutterin of entomopathogenic fungus on 200 litre capacity synthetic tanks on large scale revealed that *B.bassiana* isolate 2028 produced more number of spores followed by 1216, 135 and 892 which were recorded as 2.42×10^7, 2.26×10^7, 2.15×10^7 and 2.00×10^7 conidia/ ml respectively (Table24). Whereas *M. anisopliae* isolate 892 produced 2.39×10^7 conidia / ml and isolate 140 produced 2.22×10^7 conidia / ml respectively (Table 24). The conidial suspensions of different isolates were used to confirm the efficacy against worker termites and the results are given on (Table 25). The mortality of termite workers were highest (89.6%) with *B. bassiana* 2028. Mortality percent ranged from 62 to 89.6 while 20% mortality was observed for the untreated control .

Dust and Liquid formulations

The dust and liquid formulations which were used in field experiments against the termiles had spore strength about 1×10^7 gm / ml. The efficacy of dust and liquid formulations of *B.bassiana* and *M.anisopliae* (applied together) against termite workers is given on Table 26. The mortality rate of termites achieved was 87.2% with dust formulated product compared to 89.2% unformulated solid grain culture product. With liquid formulated product (Metabass), 91.4% mortality was recorded compared to 87.8% mortality with unformulated liquid culture product.

Table 23: Mass culture of different isolates of *B. bassiana* and *M.anisopliae* in different liquid media

Fungus	Strain	Media	
		MY	YG
		* Count (conidia/ml)	
Beauveria bassiana	984	1.07×10^7	2.59×10^7
	2028	1.85×10^7	3.34×10^7
	135	1.50×10^7	2.76×10^7
	1216	1.67×10^7	2.40×10^7
Metarhizium anisopliae	892	1.80×10^7	2.53×10^7
	140	1.62×10^7	2.68×10^7

MY = Molasses and Yeast ; YG = Yeast and Glucose

* Haemocytometer measurements

Age of cultures (used as inoculum) : **20 days**

Incubation period of mass cultures : **15 days**

Table 24: Pilot scale mass production of spores of the entomopathogens cultured on molasses - yeast extract media

Entomopathogen	Isolate	* Spore strength in the product (conidia/ml)
Beauveria bassiana	984	2.00×10^7
	2028	2.42×10^7
	135	2.15×10^7
	1216	2.26×10^7
Metarhizium anisopliae	892	2.39×10^7
	140	2.22×10^7

* Haemocytometer measurements

Table 25: Mortality of termite workers of *O.obesus* treated with different isolates of *B.bassiana* and *M.anisopliae* mass produced in liquid medium (MY)

Fungus	Strain	Concentration (Conidia/ml)	Mortality(%)
Beauveria bassiana	984	1×10^7	73.8c
	2028	1×10^7	89.6e
	135	1×10^7	64.8b
	1216	1×10^7	81.6d
Metarhizium anisopliae	892	1×10^7	73.4c
	140	1×10^7	62.0b
Control		Untreated	20.0a

* Mortality after 7 days exposure. Figures represent the mean of 5 replicates.
Means followed by same letter are not significantly different by Duncan's multiple range test (P=0.05)

Table 26: Efficacy of dust and liquid formulations of *B.bassiana* and *M.anisopliae* against termite workers of *O.obesus*.

Treatment	Conidia (gm/ml)	Mortality (%)*
Muscardine fungal dust (dust formulation)	1×10^7	87.2b
Solid culture(unformulated)	1×10^7	89.2b
Control	Untreated	19.0a
Metabass(liquid formulation)	1×10^7	91.4b
Liquid culture(unformulated)	1×10^7	87.8b
Control	Untreated	20.0a

* Mortality after 7 days exposure ; Figures represent the mean of 5 replicates.
Means followed by same letter with in a column are not significantly different by Duncan's multiple range test (P=0.05)

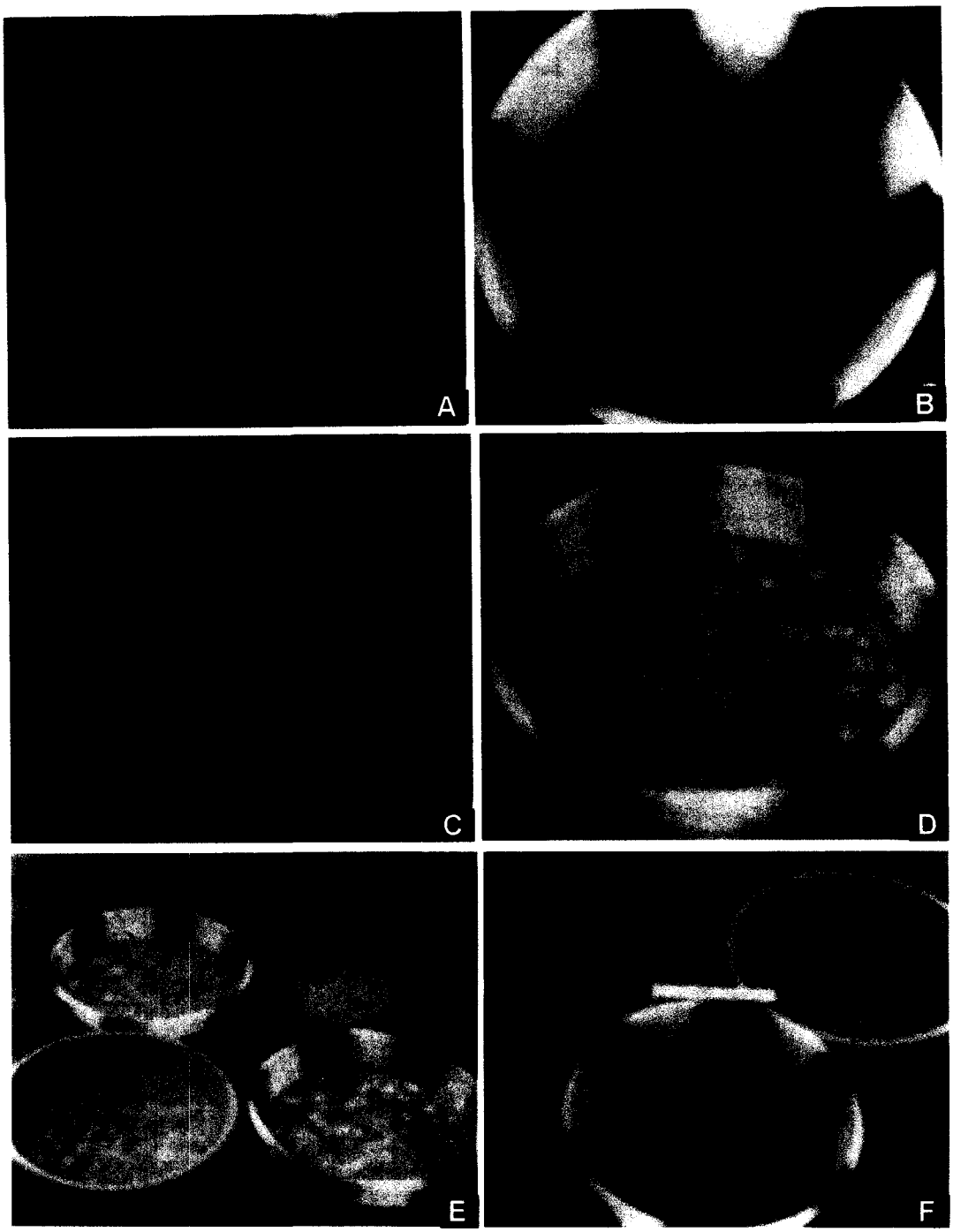


Plate 7 (A-F) : Development of fungal mass culture in liquid medium. *M.anisopliae* isolates - 892 (A) & 140 (B) and *B.bassiana* isolates - 2028 (C), 1216 (D), 135 (E) & 984 (F).

4.6.3 Test on substrate variation and carbon source on conidia production

The growth of the fungus depended mainly on the types of substrates and carbon source (dextrose). Different substrates namely bajra, maize, wheat bran, rice bran, groundnut, and wheat differed in the rate of spore production. However in some cases addition of 2% dextrose to each substrates slightly increased the amount of spore production (Table 27 - 32). Bajra substrate gave highest yield of spore production compaired to other substrates. *B.bassiana* isolate 1216 showed the highest yield 4.87 x 10^8 conidia / ml of harvest in bajra (Table 27). Lowest yield of conidia production was observed in rice bran substrate (Table 30).

Pathogenicity

Potentiality of the isolates were evaluated on the basis of their pathogenic effect on the termite population. Higher conidial concentration of the isolates caused higher percentage of mortality of workers termites (Tables 27-32). The pathogenic effect of the isolates grown in rice bran substrate retarded due to yield of lower conidial concentration (Table 30). Here percentage of mortality of termites varied from 30-45%. The pathogenicity of the isolates grown in bajra substrate caused the highest percentage mortality of termites, 60-95% (Table 27). Isolates grown in maize substrate also caused high percentage of mortality, 55-80%(Table 28). There was no sighificant difference in percentage mortality of termites between substrates having dextrose or without dextrose, where isolates were grown (Table 27-32).

4.7 Food preference and deterence test for termites

4.7.1 Food preference test :

Food preference test of *O.Obesus* was found following two methods such as glass trough test and four arm glass chamber test (Plate - 8). The chosen food substances for the above tests are filter paper,rice bran,wheat bran,*Glyricidia* powder,maize,and bajra. The mean numbers of workers of *O.obesus* attracted towards different food substances in feeding tests are shown in Table -33.The test results revealed that the maximum number of termites found to be attracted towards bajra, (260.8) and *Glyricidia* powder (256.9) followed by wheat bran(167.5),rice bran(126.8), maize (69.8),and finally filter paper(35.6)in glass through test. In glass chamber test, the maximum number of termites attracted toward *Glyricidia* powder (231.8) followled by bajra (205.6),wheat bran(112.8),maize(98.4),rice bran(86.5) and filter paper (58.2), (table 33).

Table 27: Inoculum density and potentiality of different isolates of *B.bassiana* and *M.anisopliae* in Bajra substrate against termite workers of *O.obesus*.

Fungus	Strain	With Dextrose		Without Dextrose	
		Conidial population (conidia/ml) *	Potentiality **	Conidial population (conidia/ml) *	Potentiality *
Beauveria bassiana	2028	2.07×10^8	90	2.77×10^8	90
		2.05×10^8	90	2.39×10^8	85
		2.98×10^8	95	2.88×10^8	90
	1216	4.87×10^8	90	3.24×10^8	90
		4.21×10^8	85	2.43×10^8	80
		3.46×10^8	80	1.96×10^8	80
	984	1.82×10^8	70	1.74×10^8	80
		1.79×10^8	80	1.98×10^8	75
		2.16×10^8	80	2.19×10^8	70
	135	1.85×10^8	80	1.27×10^8	70
		1.27×10^8	70	1.04×10^8	65
		1.09×10^8	70	7.97×10^7	65
Metarhizium	892	8.25×10^7	80	1.29×10^8	75
anisopliae		9.00×10^7	80	7.45×10^7	85
		1.28×10^8	90	7.97×10^7	70
	140	1.29×10^8	75	1.35×10^8	75
		8.02×10^7	60	1.16×10^8	75
		9.80×10^7	75	9.60×10^7	65

* Mean count of twenty samples; ** Percentage Mortality after 7 days exposure.

Table 28: Inoculum density and potentiality of different isolates of *B.bassiana* and *M.anisopliae* in Maize substrate against termite workers of *O.obesus*.

Fungus	Strain	With Dextrose		Without Dextrose	
		Conidial population (conidia/ml) *	Potentiality **	Conidial population (conidia/ml) *	Potentiality *
Beauveria bassiana	2028	1.80×10^7	70	8.27×10^7	75
		1.74×10^8	80	3.00×10^8	80
		4.85×10^7	70	3.35×10^7	75
	1216	3.55×10^7	65	3.46×10^8	80
		3.49×10^8	80	3.30×10^8	75
		9.50×10^7	70	3.72×10^7	60
	984	6.15×10^7	60	7.17×10^7	60
		1.49×10^8	65	5.67×10^7	60
		2.97×10^7	60	1.92×10^7	55
	135	8.82×10^7	65	1.34×10^8	60
		6.35×10^7	55	5.25×10^7	60
		8.90×10^7	60	4.55×10^7	55
Metarhizium	892	1.33×10^8	80	1.12×10^8	65
anisopliae		4.92×10^7	60	2.37×10^8	75
		1.14×10^8	65	9.45×10^7	60
	140	2.88×10^8	60	3.58×10^8	70
		3.11×10^8	75	1.08×10^8	55
		9.17×10^7	55	4.52×10^7	55

* Mean count of twenty samples; ** Percentage Mortality after 7 days exposure.

Table 29: Inoculum density and potentiality of different isolates of *B.bassiana* and *M.anisopliae* in Wheat Bran substrate against termite workers of *O.obesus*.

Fungus	Strain	With Dextrose		Without Dextrose	
		Conidial population (conidia/ml) *	Potentiality **	Conidial population (conidia/ml) *	Potentiality *
Beauveria bassiana	2028	8.75×10^7	65	1.10×10^8	60
		8.25×10^7	60	1.17×10^8	70
		3.50×10^7	55	5.50×10^7	60
	1216	11.00×10^7	70	7.50×10^7	65
		8.75×10^7	60	5.00×10^7	60
		6.00×10^7	55	6.50×10^7	55
	984	9.50×10^7	60	6.50×10^7	50
		5.00×10^7	55	6.25×10^7	50
		4.00×10^7	40	3.25×10^7	45
	135	1.75×10^8	45	3.17×10^8	55
		2.02×10^8	55	1.90×10^8	50
		1.65×10^7	45	1.90×10^8	50
Metarhizium	892	6.50×10^7	50	1.75×10^7	40
anisopliae		6.75×10^7	50	2.50×10^7	60
		5.50×10^7	45	2.25×10^7	40
	140	6.00×10^7	50	2.50×10^7	40
		5.50×10^7	45	2.75×10^7	40
		4.75×10^7	40	3.25×10^7	45

* Mean count of twenty samples; ** Percentage Mortality after 7 days exposure.

Table 30: Inoculum density and potentiality of different isolates of *B.bassiana* and *M.anisopliae* in Rice Bran substrate against termite workers of *O.obesus*.

Fungus	Strain	With Dextrose		Without Dextrose	
		Conidial population (conidia/ml) *	Potentiality **	Conidial population (conidia/ml) *	Potentiality *
Beauveria bassiana	2028	5.25×10^6	40	5.00×10^6	40
		6.75×10^6	40	4.75×10^6	40
		8.25×10^6	45	4.75×10^6	35
	1216	7.75×10^6	35	4.50×10^6	35
		8.00×10^6	45	5.75×10^6	40
		7.75×10^6	45	5.50×10^6	35
	984	5.25×10^6	30	3.50×10^6	30
		5.75×10^6	30	3.25×10^6	30
		6.00×10^6	40	4.00×10^6	40
	135	4.75×10^6	40	3.25×10^6	45
		3.50×10^6	35	3.00×10^6	30
		4.50×10^6	40	2.50×10^6	30
Metarhizium	892	4.50×10^6	30	4.50×10^6	45
anisopliae		5.50×10^6	50	3.50×10^6	35
		3.50×10^6	35	3.00×10^6	35
	140	4.75×10^6	35	3.50×10^6	35
		3.50×10^6	30	5.00×10^6	35
		5.25×10^6	30	3.25×10^6	25

* Mean count of twenty samples; ** Percentage Mortality after 7 days exposure.

Table 31: Inoculum density and potentiality of different isolates of *B.bassiana* and *M.anisopliae* in Groundnut substrate against termite workers of *O.obesus.*

Fungus	Strain	With Dextrose		Without Dextrose	
		Conidial population (conidia/ml) *	Potentiality **	Conidial population (conidia/ml) *	Potentiality *
Beauveria bassiana	2028	7.20×10^7	55	4.18×10^7	55
		7.42×10^7	60	6.05×10^7	60
		1.15×10^8	65	3.03×10^7	55
	1216	6.28×10^7	60	4.06×10^7	35
		6.73×10^7	50	3.96×10^7	45
		6.09×10^7	50	4.91×10^7	55
	984	2.41×10^7	45	2.60×10^7	55
		3.30×10^7	50	8.75×10^6	40
		4.12×10^7	60	1.78×10^7	45
	135	9.20×10^6	45	3.77×10^6	45
		6.12×10^6	45	3.60×10^6	45
		3.92×10^6	40	2.40×10^6	35
Metarhizium	892	3.40×10^7	60	1.01×10^7	50
anisopliae		3.24×10^7	50	2.24×10^7	55
		2.35×10^7	45	9.52×10^6	40
	140	9.20×10^6	45	5.57×10^6	50
		6.67×10^6	40	5.75×10^6	45
		1.07×10^7	55	4.45×10^6	35

* Mean count of twenty samples; ** Percentage Mortality after 7 days exposure.

Table 32: Inoculum density and potentiality of different isolates of *B.bassiana* and *M.anisopliae* in Wheat substrate against termite workers of *O.obesus*.

Fungus	Strain	With Dextrose		Without Dextrose	
		Conidial population (conidia/ml) *	Potentiality **	Conidial population (conidia/ml) *	Potentiality *
Beauveria bassiana	2028	1.43×10^8	75	1.17×10^8	70
		1.07×10^8	65	5.87×10^7	60
		6.97×10^7	60	2.32×10^7	60
	1216	7.62×10^7	55	3.80×10^7	60
		1.87×10^8	65	1.46×10^8	65
		9.07×10^7	55	3.02×10^7	55
	984	7.06×10^7	60	6.30×10^7	55
		5.12×10^7	55	3.77×10^7	50
		4.92×10^7	55	2.02×10^7	60
	135	2.32×10^7	45	2.44×10^7	45
		3.45×10^7	55	2.36×10^7	55
		2.87×10^7	50	1.35×10^7	45
Metarhizium	892	5.50×10^7	60	2.86×10^7	60
anisopliae		6.01×10^7	60	4.65×10^7	65
		5.37×10^7	50	3.64×10^7	55
	140	4.90×10^7	45	2.65×10^7	45
		5.77×10^7	55	3.55×10^7	55
		6.27×10^7	60	2.39×10^7	50

* Mean count of twenty samples; ** Percentage Mortality after 7 days exposure.

4.7.2 Food deterence test:

The chosen isolates of *B.bassiana* and *M.anisopliae* produce certain metabolites which act as deterrents towards workers of *O.obesus*.The tests were conducted in two ways one in glass trough and another in four arm glass chamber and there results are furnished in table 34. In both the tests the lesser number of termites (38.64-212.8) are oriented towards substrate treated with isolates of *B.bassiana* and *M.anisopliae* than untreated control (325.4-423.7). The deterrency of the isolates are in the following descending order: BB-984 (38.64), BB-1216 (74.67), Ma-140 (82.6), Ma-892 (105.4), Bb--2028 (126.3) and Bb-135 (198.67) in glass trough test. The number of termites in contact with treated substances in four arm glass chamber test are in the ascending order of Bb-984 (45.7), Ma-140 (76.3), Bb-1216 (83.5), Bb-2028 (108.4), Bb-892 (125.8) and Bb-135 (212.8).Highest and lowest deterrency are found in the *B.bassiana* isolates of 984 and 135 in glass trough test and in four arm glass chamber test (table-34).

4.8 Effect of soil factors on the pathogenicity of the fungi

A study on the effect of soil factors on *B.bassiana* and *M.anisopliae* showed that soil pH and soil sterility did not affect the pathogenicity of the different isolates of the fungi to the *O.obesus*,unlike soil moisture (Table-35). There was no significant difference in the mortality of workers of *0.obesus* on acid and alkaline soil treated with the isolates of muscardine fungi. 57.67-86.33 percent of mortality was observed under higher moisture content(60 and 90%)whereas 10.00-29.67% mortality was noticed under relatively lower moisture content (15 & 30%),(Table-35). High humidity favoured spore germination and rapid development of mycosis. The fact that *B.Bassiana* and *M.anisopliae* isolates applied to the soil with very low moisture content can still cause infection to termites is an encouraging finding .The results of this experiment show no interaction among the effect of soil ptl,moisture content and soil sterility on the pathogenecity of *M.anisopliae* and *B.bassiana* (Table - 35).

Table 33: Mean numbers of *O.obesus* workers in contact with different substrate in feeding tests.

Substrate	Mean No. of termites in contact with substrate	
	Glass through test *	Four arm glass chamber test **
Filter Paper	35.6 ± 2.8 a	58.2 ± 12.6a
Rice bran	126.8 ± 10.1b	86.5 ± 12.2b
Glyricidia Powder	256.9 ± 12.5c	231.8 ± 6.9c
Maize	69.8 ± 17.3d	98.4 ± 9.5b
Wheat bran	167.5 ± 12.5c	112.8 ± 11.5b
Bajra	260.8 ± 16.9c	205.6 ± 16.3c

* Mean number of termites in contact with substrate summed for all replicates at each 10 minutes for one hour and forty minutes (n=10 counts)

** Mean number of termites in contact with substrate summed for all replicates at each 10 minutes for 1 hr (n= 6 counts)

Mean followed by same letter within a column are not significantly different by DMRT (P=0.05)

Table 34:Mean numbers of *O.obesus* workers in contact with the substrate treated with different isolates of *B.bassiana* and *M.anisopliae* in feeding tests.

Fungus	Strain	Glass through test[a]		Four arm glass chamber test[b]	
		No. of termites in contact with Substrate		No. of termites in contact with Substrate	
		Treatment	Control	Treatment	Control
Beauveria	2028	126.30 ± 20.5 *	423.70 ± 6.2	108.40 ± 23.4 *	325.40 ± 16.5
bassiana	1216	74.67 ± 12.3 *	416.80 ± 15.8	83.50 ± 14.2 *	380.10 ± 9.9
	984	38.64 ± 16.4 *	392.60 ± 12.8	45.70 ± 6.9 *	413.30 ± 6.9
	135	198.67 ± 19.1 *	411.90 ± 12.6	212.80 ± 10.8 *	418.60 ± 12.7
Metarhizium	892	105.40 ± 10.1 *	350.60 ± 15.2	125.80 ± 8.2 *	398.60 ± 15.6
anisopliae	140	82.60 ± 9.8 *	409.50 ± 6.9	76.30 ± 9.6 *	384.20 ± 9.4

a Mean number of termites in contact with substrate summed for all replicates at each 15 minutes for 1 hr (n=4 counts)

b Mean number of termites in contact with substrate summed for all replicates at each 10 minutes for 1 hr (n= 6 counts)

* $P<0.01$ (t-test)

Table 35:Percentage mortality of workers of *O.obesus exposed to 1 x 10^7 conidia/ml of different isolates of B.bassiana* and *M.anisopliae* as affected by different soil factors under laboratory conditions.

Soil factor	Mortality (%)*					
	Beauveria bassiana			*Metarhizium anisopliae*		
	MTCC-2028	NCIM - 1216	MTCC-984	BBFF-135	MTCC-892	BBFF-140
Soil pH						
Acid (4.80)	89.00a	87.67a	79.00a	70.33a	82.33a	64.33a
Alkaline (7.30)	87.67a	85.00a	79.67a	68.00a	81.67a	63.67a
Moisture content						
15%	24.00a	18.67a	13.67a	11.33a	17.00a	10.00a
30%	29.67a	29.67b	29.33b	24.00b	30.33b	18.33a
60%	67.67b	68.33c	64.33c	68.33c	63.33c	57.67b
90%	86.33c	83.33d	71.33c	70.00c	75.00d	60.67b
Soil sterility						
Sterilized	89.00a	87.67a	72.33a	64.33a	75.33a	62.67a
Unsterilized	88.33a	86.00a	70.00a	65.67a	74.67a	63.33a

* Mortality after 7 days exposure; Mean followed by same letter within a column are not significantly different by DMRT (P=0.05)

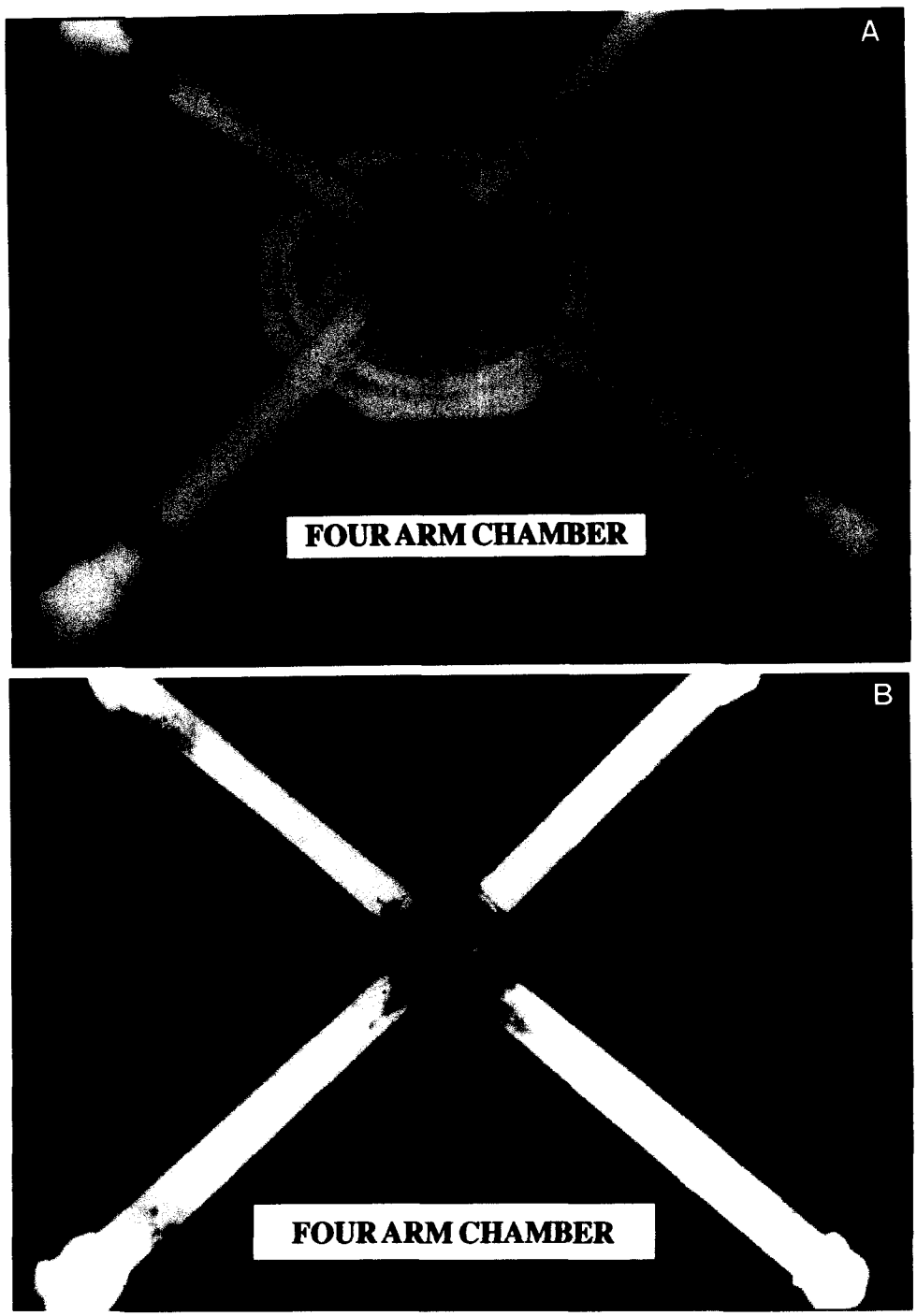


Plate 8(A& B): Four arm chambers for food preference and deterrence tests of termites.

4.9 Formulation and its efficacy

4.9.1 Evaluation of formulations and pathogenicity of the entomopathogenic fungi.

Evaluation of different formulations of muscardine fungi as dust, wettable powder and liquid solutions in comparison with unformulated muscardine fungi (*M. anisopliae* and *B. bassiana*) revealed that the dust formulations exhibited lesser mortality of the termites than the unformulated muscardine fungi while liquid formulations exhibited higher mortality than the unformulated fungi , (Table - 36 and figure - 8). Among the dust formulations, clay formulation was found to give a mortality of 75.3% followed by tale (74.7%) and China clay (68.0%), Table - 37 and Figure - 8

In case of wettable powder formulation, Dedenol formulation recorded highest mortality (80%) followed by Fuller's earth (78.7%) and Arrowroot powder (63.3%), Table - 37, Figure - 8

In liquid formulations, Tween 80 formulation showed higher mortality (90.7%) than lavolin (87.3%) and detergent cake (Hogla) (72.7%), Table - 37, Figure - 8.

The results obtained from the addition of certain adjuvants and UV protectants to the muscardine fungi highlighted a higher mortality rate in the case of sunflower oil (92.7%), Molasses (92%), Coconut oil (91.3%) and Crude Sugar and Robin blue (90%) than unformulated muscardine fungi whereas lower mortality was registered in palm oil (79.3%), Mustard oil (72.7%), Soybean oil (81.3%) and Ranipal (85.3%) (Table - 38, Figure - 8)

4.9.2 Field evaluation of liquid formulation

A field trial was conducted to evaluate the efficacy of the liquid formulated mycopesticide, as alone and in different combinations with chlorpyriphos against live wood termite, *Microcerotermes* sp. on infested tea bushes. The infestation ratio and population size of *Microcerotermes* sp. in different treatments and untreated control before and after spraying of the biocide as well as biocide plus chemical are summarised in tables 39 and 40.

The experimental result shows that the percentage infestation ratio of the termites ranged from 71-98% before spraying of biocide and biocide plus chlorpyriphos. The percentage

Table 36: Efficacy of dust and liquid formulations of *B.bassiana* and *M.anisopliae* against termite workers of *O.obesus*.

Treatment	Conidia/gm or ml	Mortality (%)*
Muscardine fungal dust (dust formulation)	1×10^7	87.2b
Solid culture (unformulated)	1×10^7	89.2b
Control	Untreated	19.0a
Metabass (liquid formulation)	1×10^7	91.4b
Liquid culture (unformulated)	1×10^7	87.8b
Control	Untreated	20.0a

* Mortality after 7 days exposure; Figures represent the mean of 5 replicates

Means followed by same letter within a column are not significantly different by Duncan's multiple range test (P=0.05)

Table 37: Laboratory evaluation of different formulations of entomopathogenic fungi *B. bassiana* and *M. anisopliae* against workers of *Odontotermes obesus* **

Treatment	Mortality (%)*
Muscardine fungi (Unformulated)	89.3
Muscardine Fungal Dust	
Talc	74.7
Clay	75.3
China clay	68.0
Muscardine Fungi Wettable Powder	
Fuller's earth	78.7
Arrowroot powder	63.3
Dedenol	80.0
Liquid Muscardine Fungal Solution	
Tween 80 (0.5%)	90.7
Lavolin (0.01%)	87.3
Hogla (0.2%)	72.7

Muscardine fungi- a mixture of four isolates of *Beauveria bassiana* and *two isolates of* Metarhizium anisopliae (1 x 10^7 conidia/gm or ml).

* Mean of 5 observations

** 30 termites taken for each replicates.

Table 38:Effect of certain Adjuvants and UV protectants on efficacy of entomopathogenic fungi(liquid formulated product) against workers of *O.obesus***.

Treatment (formulation +adjuvants)	Adjuvant/ UV protectant	Mortality** (%)
Muscardine fungi + Sunflower oil	0.2%	92.7
Muscardine fungi + Coconut oil	0.2%	91.3
Muscardine fungi + Palm oil	0.2%	79.3
Muscardine fungi + Mustard oil	0.2%	72.7
Muscardine fungi + Soyabean oil	0.2%	81.3
Muscardine fungi + Crude Sugar	20.0%	90.0
Muscardine fungi + Molasses	20.0%	92.0
Muscardine fungi + Robin blue	1.0%	90.0
Muscardine fungi + Ranipal	1.0%	85.3
Muscardine fungi alone (control)	-	88.7

Muscardine fungi - a mixture of four isolates of *Beauveria bassiana* and two isolates of *Metarhizium anisopliae* (1 x 10^7 conidia/ml)

* Mean of 5 observations

** 30 termites taken for each replicates.

Table 39: Field evaluation of a fungal formulation (Metabass) in relation to percentage infestation ratio of *O. obesus* workers on tea bushes at weekly inspections.

Treatment	Block & Plot	Percentage of infestation ratio/week								
		*Pretreatment	# Post treatment							
			I	II	III	IV	V	VI	VII	VIII
Chlorpyriphos	IA	71	42	29	5	-	-	-	-	-
	IB	77	46	33	7	3	-	-	-	-
	IC	84	50	40	18	5	14	-	-	-
	ID	80	44	39	9	3	-	-	-	-
	IE	82	56	21	14	-	-	-	-	-
Chlorpyriphos	IIA	74	54	36	28	14	9	3	-	-
(Half dose)	IIB	81	60	44	23	11	3	1	-	-
+	IIC	70	49	30	12	4	-	-	-	-
Metabass	IID	72	50	33	14	5	1	-	-	-
	IIE	89	62	41	22	13	5	-	-	-
Chlorpyriphos	IIIA	79	68	50	32	19	6	-	-	-
(Quarter dose)	IIIB	83	72	61	47	21	11	5	-	-
+	IIIC	81	75	63	56	35	22	13	7	-
Metabass	IIID	75	67	54	39	27	13	2	-	-
	IIIE	80	63	49	30	11	9	-	-	-
Metabass	IVA	72	69	60	51	35	12	6	2	-
	IVB	86	80	72	65	27	19	4	-	-
	IVC	79	75	68	42	22	15	-	-	-
	IVD	82	76	70	61	43	28	14	5	-
	IVE	75	68	52	43	31	26	18	3	-
Control	VA	90	85	81	90	89	95	71	72	61
	VB	95	86	80	86	80	88	79	82	62
	VC	98	81	92	92	91	86	72	64	70
	VD	86	92	90	89	90	95	75	73	68
	VE	81	83	96	92	86	90	70	77	83

* Datas recorded before spray of chlorpyriphos (Chemical) or Metabass (formulated mycopesticide) on tea bushes, + Untreated plot

Table 40: Field evaluation of a fungal formulation (Metabass) in relation to population size of workers of O. obesus workers on tea bushes at weekly inspections.

Treatment	Block & Plot	Population of *O. obesus* /week on tea bushes							
		*Pretreatment	# Post treatment						
			I	II	III	IV	V	VI	VII	VIII
Chlorpyriphos	IA	2111	1065	758	62	-	-	-	-	-
	IB	1978	802	473	90	31	-	-	-	-
	IC	2128	716	527	202	86	11	-	-	-
	ID	2056	735	518	109	23	-	-	-	-
	IE	1827	917	256	86	-	-	-	-	-
Chlorpyriphos	IIA	2056	985	659	481	187	116	36	-	-
(Half dose)	IIB	1879	912	716	379	154	31	9	-	-
+	IIC	1965	886	591	168	42	-	-	-	-
Metabass	IID	2132	955	584	216	71	12	-	-	-
	IIE	1908	863	590	300	144	34	-	-	-
Chlorpyriphos	IIIA	1885	1187	811	498	301	67	-	-	-
(Quarter dose)	IIIB	1962	1009	944	681	293	109	42	-	-
+	IIIC	1705	1121	1008	933	515	284	111	37	-
Metabass	IIID	2164	1200	997	668	474	159	11	-	-
	IIIE	2009	1058	765	498	166	89	-	-	-
Metabass	IVA	2169	1269	1011	983	667	204	97	21	-
	IVB	2028	1300	1165	1040	381	296	33	-	-
	IVC	1965	1299	1008	668	361	202	-	-	-
	IVD	1856	1315	1115	995	568	357	109	61	-
	IVE	1790	1200	918	652	412	320	212	32	-
Control	VA	1899	1933	1921	1830	1918	1844	1433	1024	1059
	VB	1946	2200	1875	1774	2138	1931	1421	1057	958
	VC	2141	1986	1838	1963	2043	2163	1684	1174	1625
	VD	2012	1843	1348	1752	1829	1970	1269	1040	1118
	VE	1867	1903	1884	2044	1946	1900	1280	1974	1021

* Datas on *O.obesus* population were recorded from the tea bushes before spray of chlorpyriphes (chemical) or Metabass (formulated mycopesticide). # Datas for I to VIII weeks on *O.obesus* population were recorded after

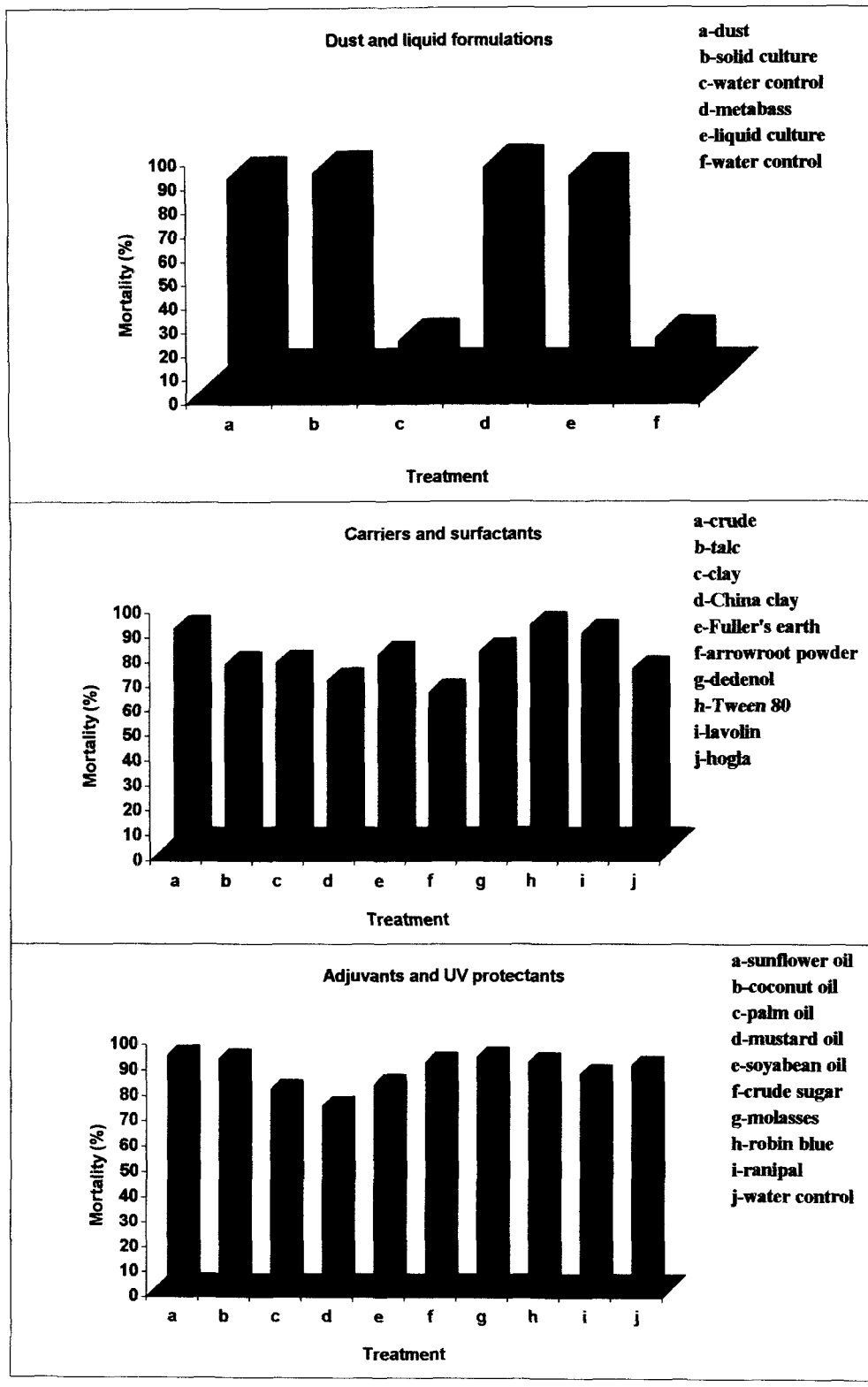


Figure 8: Efficacy of different formulations of *B.bassiana* and *M.anisopliae* against *O.obesus*

infestation ratio was found throughout the study period of two months in untreated control plots. The infestation ratio reduced within 3 weeks in chlorpyriphos alone sprayed plots followed by half dose of chlorpyriphos and Metabass (formulated mycopesticide) (4 weeks) and quarter dose of chlorpyriphos and Metabass (5 weeks) whereas in liquid formulation (Metabass) treated plots the infestation ratio was brought under control within 6-7 weeks time (Table - 39). The population size of *Microcerotermes* sp. was 1705 - 2169 per plot before treating the tea plants with biocide and biocide plus chlorpyriphos at different combinations.

The population size decreased gradually in the same manner as described in the findings of infestation ratio (Table - 40). Observations on the infestation ratio and population size showed that the fungal formulation, Metabass could effectively control termite population alone as well as in combination with chlorpyriphos.

4.10 Field evaluation of bait for population suppression of subterranean Termites

The field colonies of *O.obesus* and *O. distans* were identified, w ooden stake survey and monitoring station application methods are being followed to estimate the foraging populations and the activity of *O.Obesus* and *O. distans.*

By following the wooden stake survey and triple mark-recapture method, the foraging territory and the population were estimated before the introduction of baits which is furnished in Table - 41 for colonies of *O.obesus* and *O. distans* (Plate - 9, 10 and 11).

The estimated foraging populations of *O.obesus* (Colony I & II) and *O. distans* (Colony III) in shade trees plantation area (colony I, 10058.5 m^2 area with 130 survey stakes at a distance of 10 m with 8 monitoring stations; Colony - II, 8562.6 m^2 area with 104 survey stakes at a distance of 10m with 12 monitoring stations) and teak plantation area (2418.15 m^2 area with 88 survey stakes at 10m distance with 14 monitoring stations) was determined (Mean + SEM) to the tune of 742495.79 + 42099.5, 126689.44 + 66131.8 and 674025.54 + 51849.83 respectively for colony I,II and III (Table - 41). The mean worker weight, maximum distance travelled during foraging, their foraging territory and biomass of *O.obesus* and *O. distans* ranged from 4.12 - 6.93mg, 61-112m, 654-1900m^2 and 4.34 - 5.22 Kg respectively (Table - 41).

Of the 88-130 survey stakes placed in the ground, typically only some were infested by termites

Table 41: Foraging populations of *O. obesus* and *O.distans* colonies before the introduction of fungal baits

Species	Colony	Worker wt (mg) Mean + SEM	No. of active monitoring stations	Estimated foraging population Mean + SEM	Max distance[a] m	Foraging territory m^2	Foraging biomass[b] Kg
Odontotermes obesus	I	5.85 ± 1.5	8	742495.79 ± 42099.5	112	1900	4 34
	II	4.12 ± 2.6	12	1266894 ± 66131.8	61	1282	5 22
Odontotermes distans	III	6.93 ± 4.8	14	674025.54 ± 51849.83	85	654	4.67

[a] Maximum linear distance between two monitoring stations containg marked termites

[b] Foraging biomass = estimated number of foraging termites X mean worker weight

Table 42: Number of marked termites released (*ri*), number of termites captured (*ni*), and number of marked termites among those captured (*mi*) during a triple mark-recaptured programme before and after introduction of muscardine fungal baits.

Species	Colony	*ith* Mark recapture	*ri* beforer bait	*ri* after bait	*ni* before bait	*ni* after bait	*mi* before bait	*mi* after bait
Odontotermes obesus	I	1	2678 (2)	456 (1,4,6)	4205 (1,2,3,5)	892 (1,2,4,6)	82	11
		2	3011 (1,2,3,5)	605 (1,2,4,6)	6816 (1,2,3,4,5,8)	415 (1,2,4,6)	154	5
		3	6207 (1,2,3,4,5,8)	307 (1,2,4,6)	8203 (2,4,5,6,7,8)	117 (1,2,4,6)	71	2
	II	1	3051 (1)	774 (1,5)	8122 (1,3,4,5,7,8,10,11,12)	928 (1,5,8,10)	129	22
		2	7808 (1,3,4,5,7,8,10,11,12)	511 (1,5,8,10)	10056 (1,2,3,4,7,8,11,12)	637 (1,2,5,8,10)	211	14
		3	9872 (1,2,3,4,7,8,11,12)	512 (1,2,5,8,10)	4372 (2,4,5,6,7,9,11,12)	115 (1,2,5,8,10)	28	5
Odontotermes distans	III	1	2061 (8)	583 (1,3,5,6,7,8,14)	6069 (1,4,9,12,13,14)	1052 (1,4,5,10,14)	54	21
		2	5802 (1,4,9,12,13,14)	785 (1,4,5,10,14)	1062 (1,2,3,5,6,9,10)	607 (1,4,5,6,10,12)	21	10
		3	965 (1,2,3,5,6,9,10)	452 (1,4,5,6,10,12)	5925 (2,3,4,8,9,10,12,14)	368 (1,4,5,6,10,12)	95	18

Identification number of monitoring stations from which marked termites were captured and released are listed in parenthesis.

Table 43: Foraging activities as expressed by the total number of *O. obesus* collected from colony-I and total wood consumed (grams per colony) before and after introduction of the muscardine fungal baits.

	Month	Total termites catch / colony	Total wood cosumed g / colony
1st. year			
Before	July	2247	126
introduction of	Aug	4363	312
muscardine	Sep	6879	456
fungal bait	Oct	10012	848
	Nov	25612	1216
	Dec	37565	1578
2nd. year			
After	Jan	42654	1602
introduction	Feb	39862	1056
of muscardine	Mar	21162	859
fungal bait	Apr	10620	657
	May	12547	608
	June	5622	295
	July	1604	98
	Aug	260	20
	Sep	28	2

Table 44: Foraging activities as expressed by the total number of *O. obesus* collected from colony II and total wood consumed (grams per colony) before and after introduction of the muscardine fungal baits.

	Month	Total termites catch / colony	Total wood cosumed g / colony
1st. year			
Before	Sep	5289	359
introduction of	Oct	9257	601
muscardine	Nov	39566	985
fungal bait	Dec	46025	1305
	Jan	45163	1268
	Feb	30162	1005
2nd. year			
After	Mar	15167	892
introduction	Apr	17743	568
of muscardine	May	10062	344
fungal bait	June	563	110
	July	612	86
	Aug	118	15
	Sep	96	8

Table 45: Foraging activities as expressed by the total number of *O. obesus* collected from colony III and total wood consumed (grams per colony) before and after introduction of the muscardine fungal baits.

	Month	Total termites catch / colony	Total wood cosumed g / colony
1st. year			
Before	July	3271	282
introduction of	Aug	6550	388
muscardine	Sep	9577	735
fungal bait	Oct	19262	1012
	Nov	26615	1305
	Dec	42216	1709
2nd. year			
After	Jan	40745	1502
introduction	Feb	29865	1208
of muscardine	Mar	19864	953
fungal bait	Apr	9654	552
	May	1563	127
	June	356	76
	July	120	12

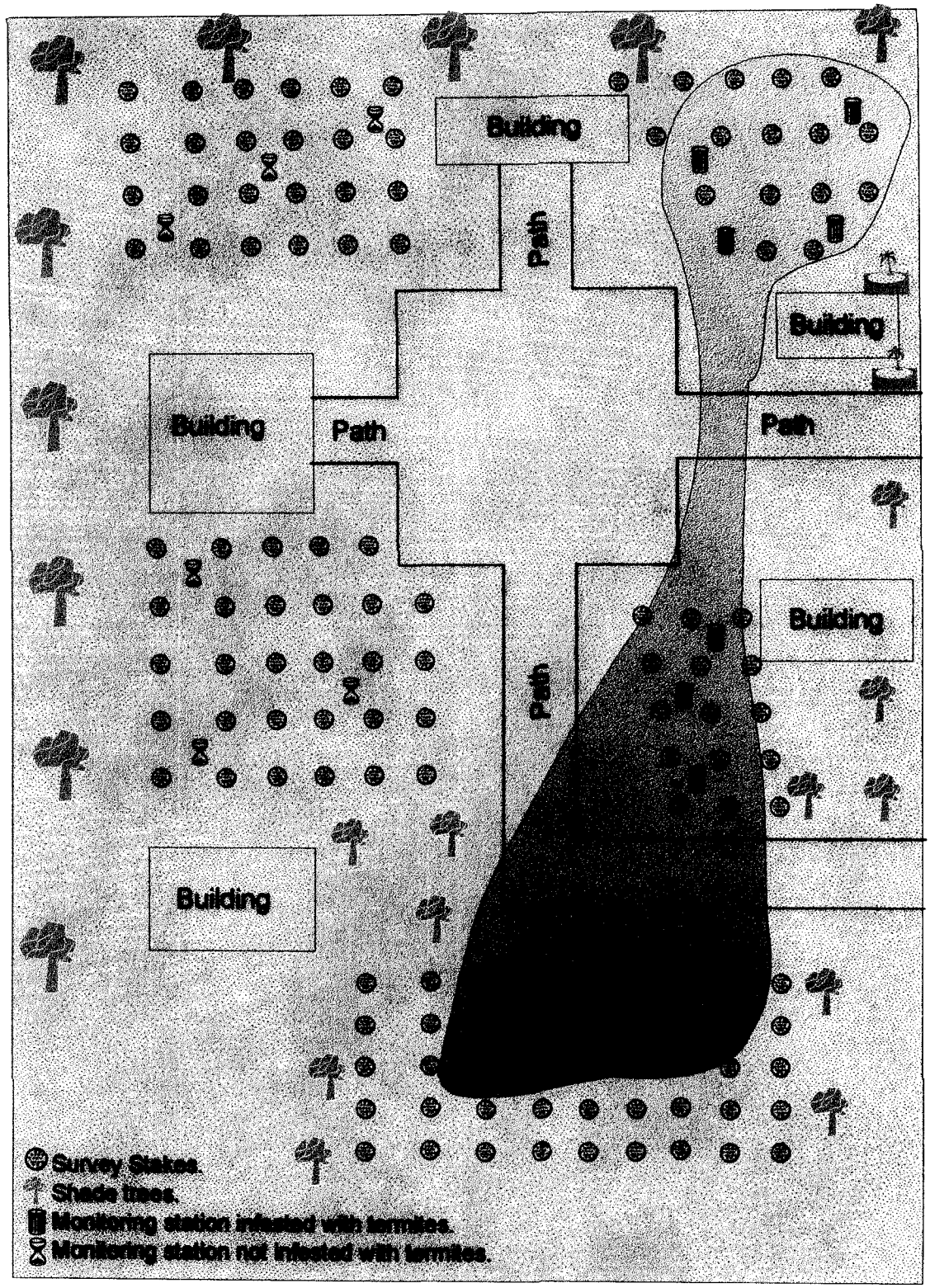


Figure 9 : Foraging territories (Shaded areas) of field colony - I of *Odontotermes obesus* before the introduction of fungal bait matrix.

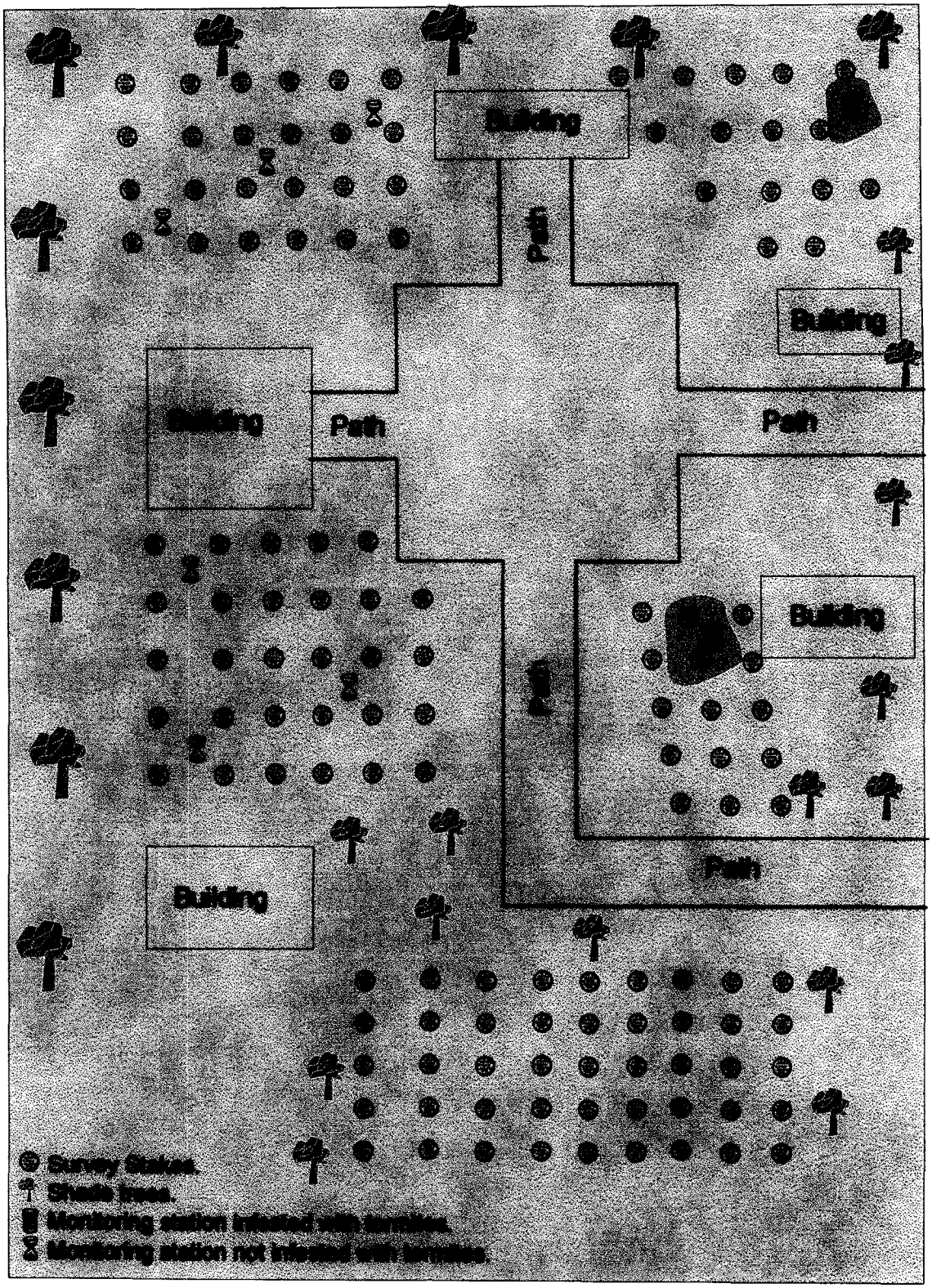


Figure 10 : Foraging territories (Shaded areas) of field colony - I of *Odontotermes obesus* after the introduction of fungal bait matrix.

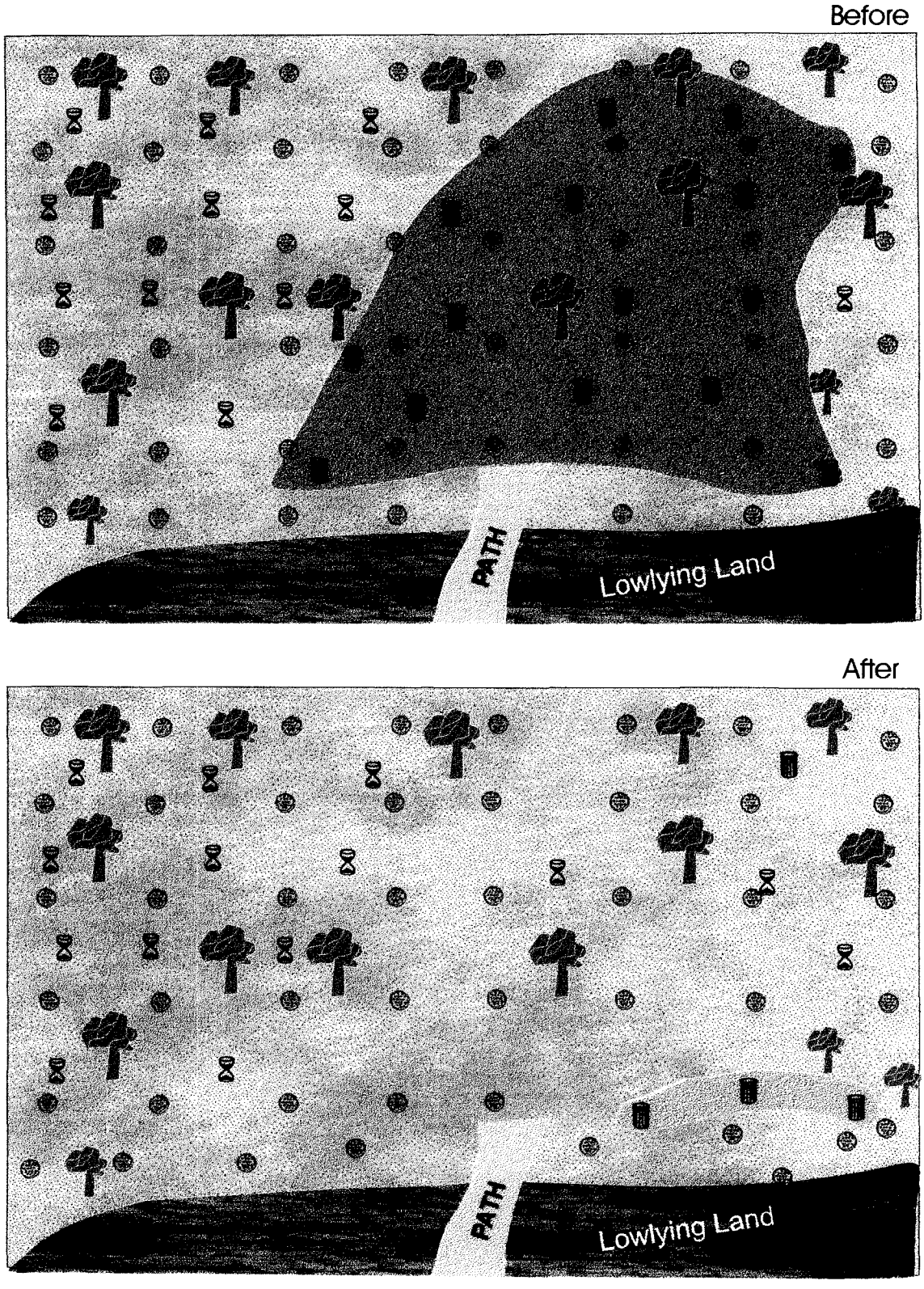
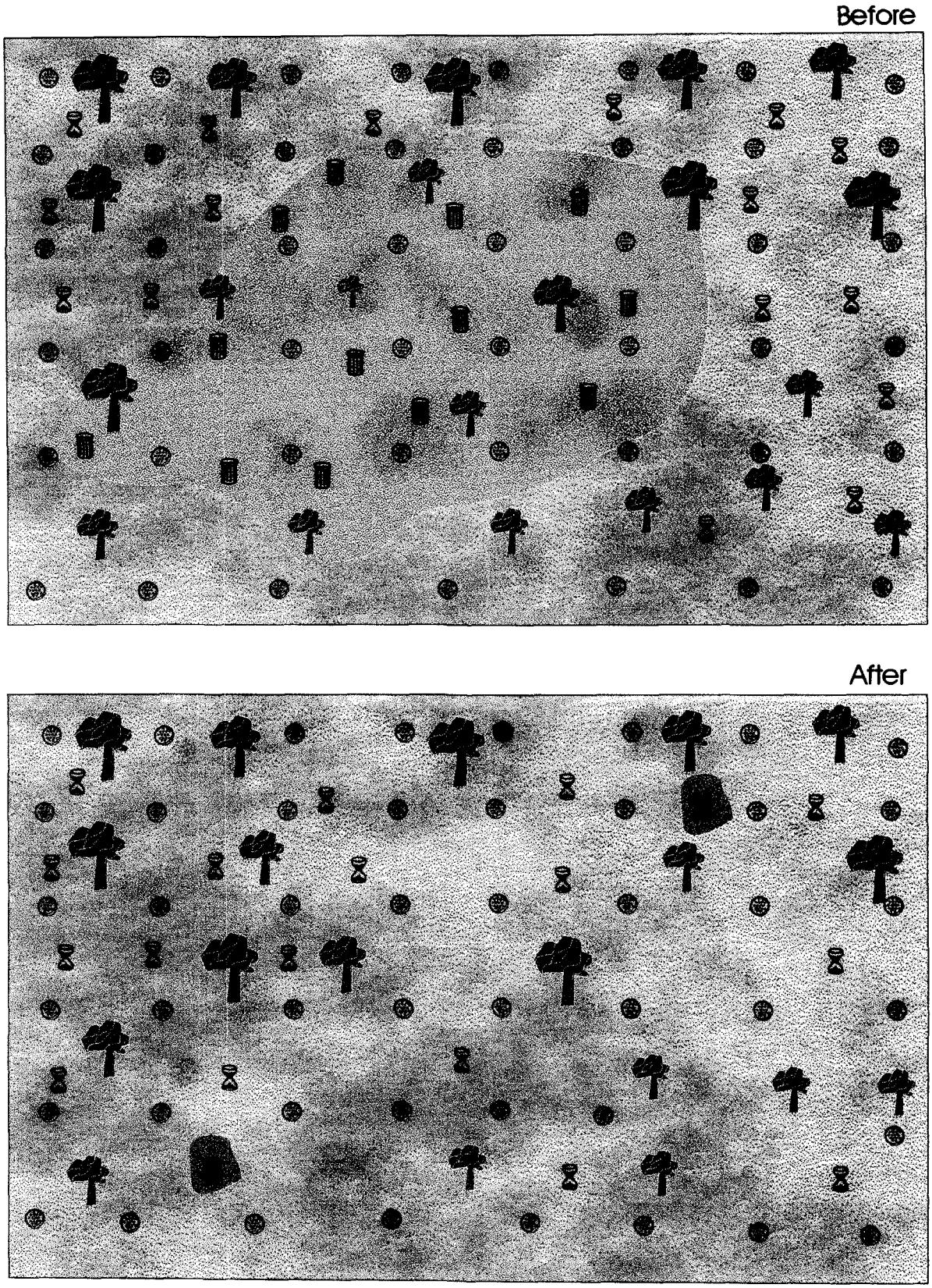


Figure 11 : Foraging territories (Shaded areas) of field colony - II of *Odontotermes obesus* before and after the introduction of fungal bait matrix.



Survey Stakes.
Shade trees.
Monitoring station infested with termites.
Monitoring station not infested with termites.

Figure 12 : Foraging territories (Shaded areas) of field colony - III of *Odontotermes distans* before and after the introduction of fungal bait matrix.

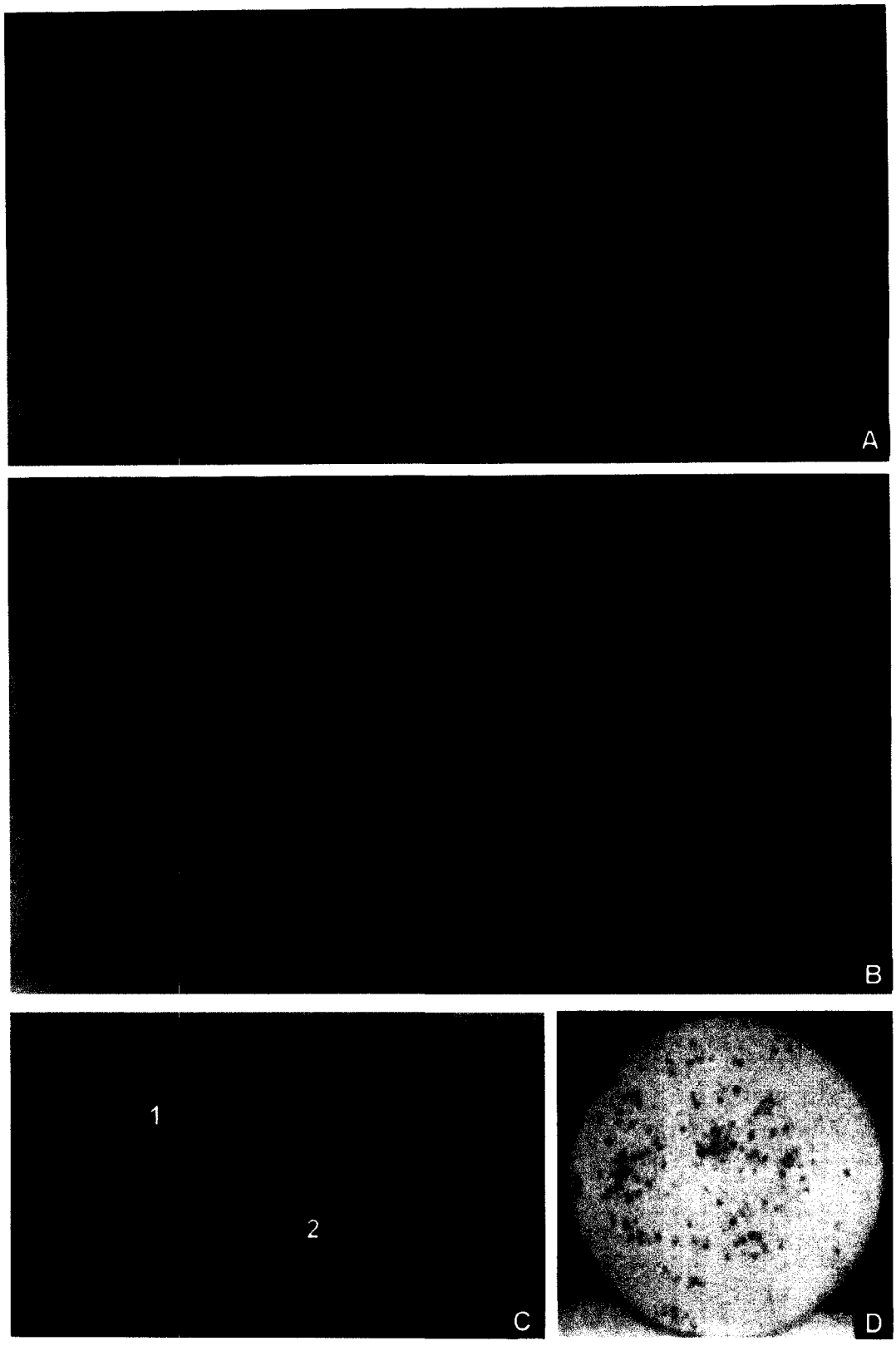


Plate 9 (A-D) : Wooden-stake survey in termite prone areas (A); Introduction of bait tubes in the soil (B);Uninfested (C-1) and infested (C-2) wooden survey stakes; Termites treated with Nile blue (D).

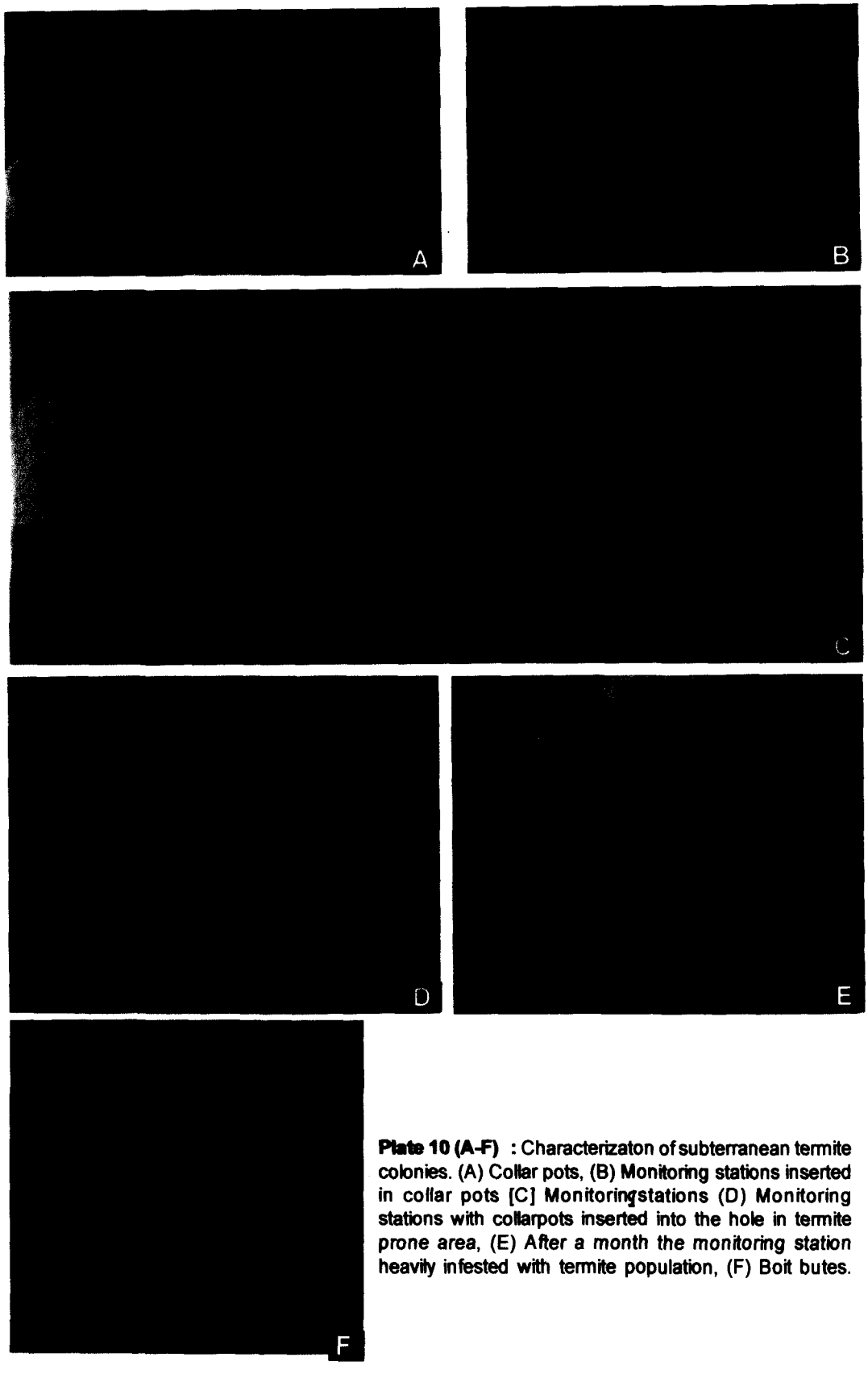


Plate 10 (A-F) : Characterizaton of subterranean termite colonies. (A) Collar pots, (B) Monitoring stations inserted in collar pots [C] Monitoringstations (D) Monitoring stations with collarpots inserted into the hole in termite prone area, (E) After a month the monitoring station heavily infested with termite population, (F) Boit butes.

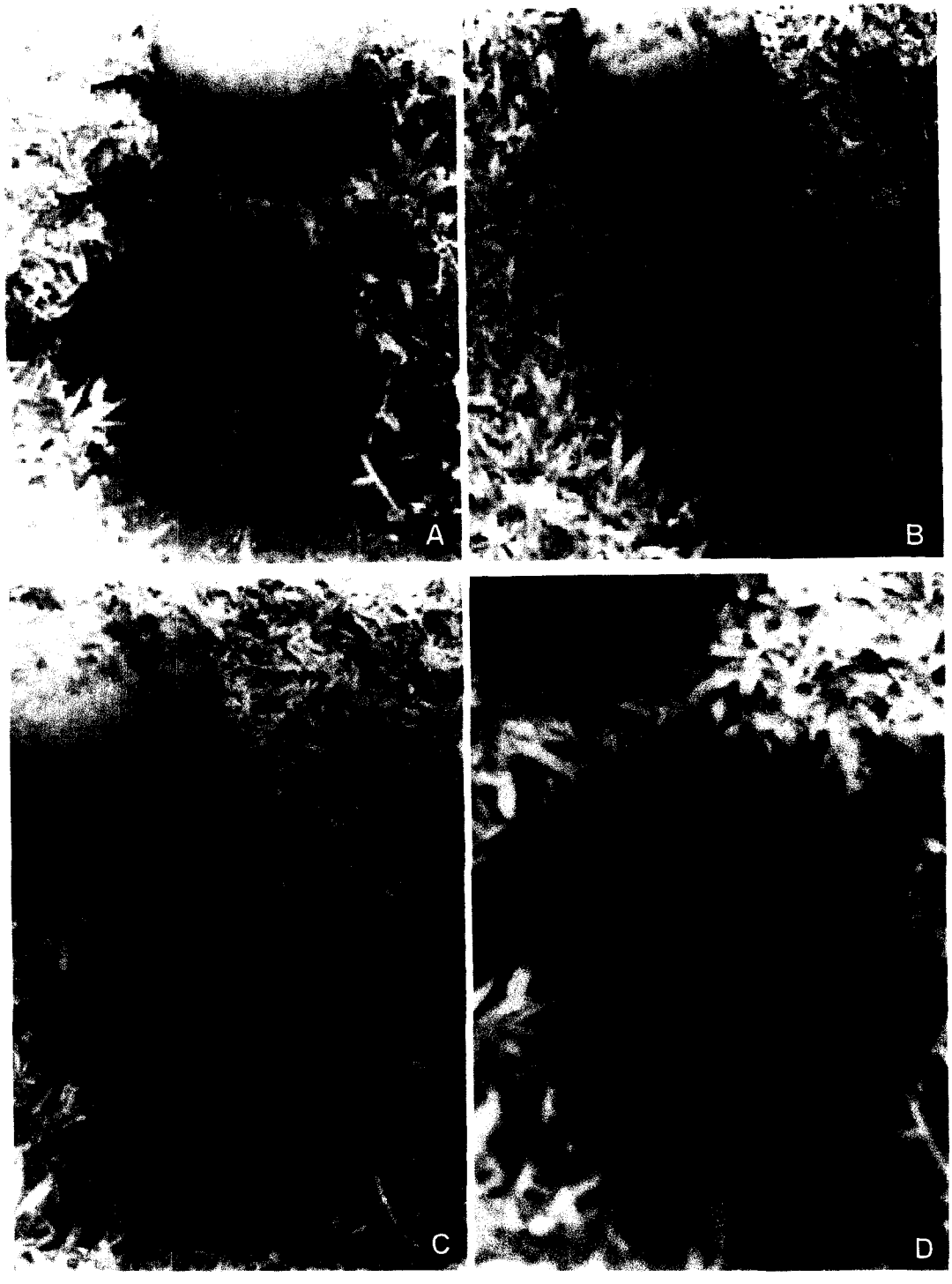


Plate 11 (A-D): Termite infestations and nests visible after removing underground monitoring stations.

and were replaced with the underground monitoring stations(Figs-9,11 & 12). The number of marked termites released, termites recaptured, marked termites and marked termites among recaptured during the triple mark-recapture procedure before and after introduction of mascardine fungal bait are listed in Table - 42.

Tables 43-45 exhibit the total wood consumption and total number of termites collected per colony before and after introduction of muscardine fungal bait between first and second year, from colonies I,II and III of *O. obesus* and *O. distans*. The wood consumption and the total number of termites per colony before the introduction of fungal bait ranged from 126-1578 g/ colony and 2247 - 37565 termites/colony, in colony - I; 359 - 1305 g/colony and 5289-46025 termites/colony in colony-II; and 282-1709 g/colony and 3271 - 42216 termites/colony in colony - III (Tables - 43-45)

After the introduction of fungal bait (Plate - 9) into the colonies the wood consumption and the total number of termites collected per colony declined drastically to the tune of 1602-295 g/colony and 42654-5622 termites/colony in colony - I; 892-110 g/colony and 17543-563 termites/colony in colony - II; 1502 - 76 g/colony and 40745-356 termites/colony in colony - III (Tables 43-45).

The summary of the baiting programmes using bait matrix containing muscardine fungi and their effects on the foraging populations of *O.obesus* and *O. distans* is given in Table-46

The population of *O.obesus* in colony - I reduced from 742495 to 102528 after 9 months baiting period. Colony - II of *O.obesus* showed a reduction in population size from 1266894 to 71880 after 7 months of baiting period while the population of *O.distans* in colony - III reduced from 674025 to 73782 after 7 months baiting period (Table 46 and Figures 10,11,12).

4.11 Determination of the efficacy of formulated mycoinsecticide through different application procedures

The formulated mycoinsecticide was tested for its efficacy against the termites in the plantation areas of *Dalbergia sissoo, Albizia lebbeck, Michelia champaca* and *Lagerstroemia speciosa* . The mortality of the saplings of the mentioned plant species due to termite infestation and their growth under different application procedures is given on Table 47 and Plate - 12. Mortality of the saplings due to termite infestation was not observed for all application procedures. Only 32-38% mortality was observed for untreated control. The mean increase of plant height in 12 months was comparatively greater in case of *L. Speciosa* , where mean increase was about 109.2cm in container application procedure. Mean increase of 102.5cm

Table 46: Summary of baiting programmes using bait matrix containing muscardine fungi and their effects on the foraging populations of *O. obesus* and *O. distans*

Species	Colony	Initial population Mean ± SEM	No. bait tubes used	No. baiting required	Bait matrix consumed g	AI consumed mg	Final population	Bait period (months)
Odontotermes obesus	I	742495.79 ± 42099.5	65	9	957	97.5	102528 ± 24913.77	9
	II	1266894.44 ± 66131.8	83	7	1494	149.4	71880 ± 11370.75	7
Odontotermes distans	III	674025.54 ± 51849.83	71	7	1207	120.7	73782.8 ± 10653.37	7

Table 47: The mortality of the saplings of different plant species due to termite infestation and their growth under different application procedures.

*Application Procedures	No of Saplings	Mortality Percent (%)	Mean increase of Plant height in 12 months (CM)			
			Michelia Champaca	***Dalbergia Sissoo***	***Lagerstraemia Speciosa***	***Albizia lebbeck***
Planting Pit Application	100	0	96.5	65.7	102.5	64.8
Container Application	100	0	92.2	67.8	109.2	65.5
Post Planting Application	100	0	88.1	59.2	94.6	57.7
Root Dip Treatment	100	0	86.6	54.7	96.1	55.0
Untreated+	100	32-38	95.4	58.5	90.5	58.2

* Application of formulated mycoinsecticide by different procdures to four plant species in the plantation area.

+ Saplings untreated with the mycoinsecticide.

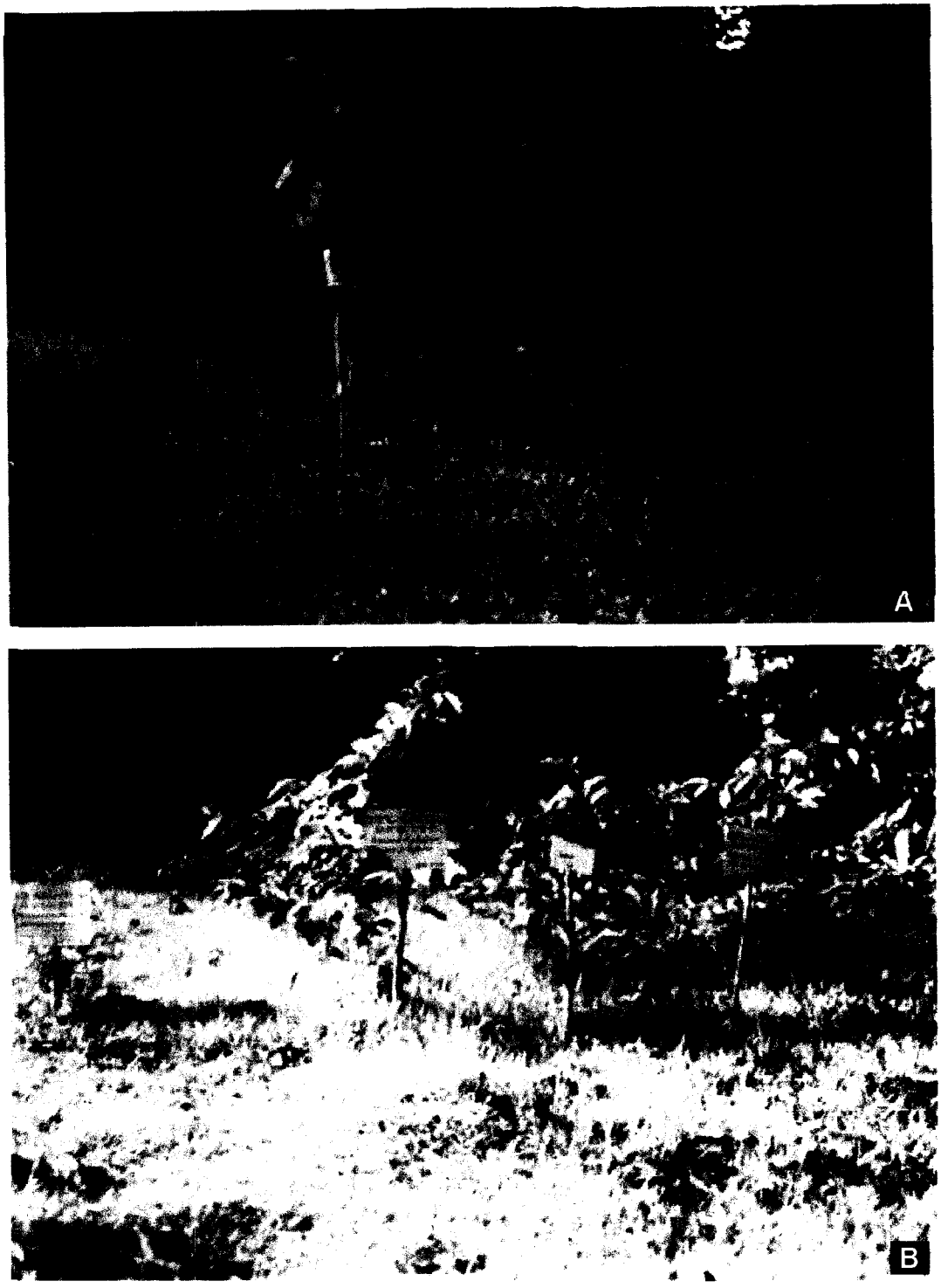


Plate 12 (A&B) : Mycoinsecticide formulations and their application on growth of *Michelia champaca* (A) and *Lagerstoemia speciosa* (B). Post planting application (1st row), root dip treatment (2nd row), planting pit application (3rd row) and container application (4th row).

was observed under planting pit application for *L.speciosa*. Relatively low increase of 55 cm was observed for *A.lebbeck* in root dip treatment.

4.12 Evaluation of Storage life and Pathogenicity of the fungi

Storage life

The storage life for the formulated strains of *B.bassiana* and *M.anisopliae* was measured by compairing the number of conidia sporulated after storage at temperature $4^{o}C$ for three intervals 6,12 and 18 months. The number of conidia sporulated from the formulation of two entomopathogenic fungi at storage intervals and storage temperature are given in Figure 13. The result shows that the conidial concentration is highest for the fresh formulated strains, i.e. 'O'month interval. As storage period increased, the conidial concentration decreased gradually conidial concentration was found to reduced from 1.8×10^{8} to 1.4×10^{5} conidia / ml within 18 months interval for the isolate 2028. Isolate 140 showed highest reduction of conidial concentration within 18 months interval which is about 1.5×10^{8} to 1.1×10^{4} conidia / ml.

Pathogenicity

Pathogenicity of fresh and stored formulated isolates of *B.bassiana* and *M.anisopliae* against *Odontotermes obesus* workers is shown on table 48 and Figure 14 . The result shows that the highest percentage of mortality was achieved by applying the isolate 2028 i.e. 88.3% (fresh culture) and 61.7% (6 months stored culture). When the four isolates of *B.bassiana* were mixed the mortality percentage was more i.e. about 66.7% (6 months stored culture). Mixture of all the six isolates showed the highest mortality percentage.

4.13 SDS-PAGE analysis

SDS-PAGE was performed for the detailed analysis of the protein profile of different isolates of *Metarhizium anisopliae* and *Beauveria bassiana*. On the gets, different coomassie blue stained bands were observed for the mycelial proteins of the isolates. In *M.anisopliae* isolate 892 the number of bands observed was seven and their molecular weight ranged from 72 6-22.4KDa (Table-49, plate -13). In *M.anisopliae* isolate 140, tem bands were recorded and the molecular weight ranged from 83.6-18.4. In *B.bassiana* isolates 2028, 984 and 135 the number of protein bands recorded were 14,11.12 and 11respectively. Their molecular weights were dipicted on table - 49.

Table 48: Pathogenicity of fresh and stored formulated isolates of *B. bassiana* and *M.anisopliae* against *O. obesus* workers*.

Fungus	Isolates	Period of storage in (months)	Mortality of the workers	
			Mycosis (%)	Efficacy (%)
Beauveria bassiana	2028	0	88.3	82.48
		6	61.7	55.88
		12	46.7	40.88
		18	30.0	24.18
	1216	0	83.3	77.48
		6	58.3	52.48
		12	40.0	34.18
		18	31.7	25.88
	984	0	75.0	69.18
		6	50.0	44.18
		12	31.7	25.88
		18	26.7	20.88
	135	0	66.7	60.88
		6	46.7	40.88
		12	28.3	22.48
		18	23.3	17.48
	Mixture of the above four isolates	0	91.7	85.88
		6	66.7	60.88
		12	51.7	45.88
		18	35.0	29.18
Metarhizium anisopliae	892	0	81.7	75.88
		6	60.0	54.18
		12	48.3	42.48
		18	31.7	25.88
	140	0	73.3	67.48
		6	51.7	45.88
		12	35.0	29.18
		18	20.0	14.18
	Mixture of the above two isolates	0	86.7	80.88
		6	61.7	55.88
		12	53.3	47.48
		18	40.0	34.18
Beauveria bassiana and *Metarhizium anisopliae*	Mixture of all the six isolates	0	93.3	87.48
		6	70.0	64.18
		12	56.7	50.88
		18	41.7	35.88

*Mean of three observations

Table 49: Molecular weights of protein-bands in S.D.S - PAGE analysis of mycelial proteins of *M.anisopliae* and *B. bassiana* isolates.

Isolates	* Number of observed bands	Molecular weight (KDa)
M892	7	72.6, 59, 50,37.4, 29.4,28.2 and 22.4
M140	10	83.6, 79.4, 68,59,54,40.8,29.4,26.6, 19.6, 18.4
B 2028	14	54.5, 50, 34.5, 29.8, 29.4, 26.1,25.4, 24.5, 23.2, 22.4, 21.6, 19.6, 17.7 and 15.8
B1216	11	54.2, 50, 47.5, 43, 28.2, 26.6, 26, 23.2, 22.4, 19.6 and 17.6
B984	12	59, 50, 43, 37.4, 29.8, 29.4, 29, 28.2, 26, 24.2, 20.8 and 17.7
B135	11	68, 59, 50, 47.5, 38.6, 34.5, 24.5, 21.6, 18.4, 16.5,14.8

M - *Metarhizium anisopliae* , B- *Beauveria bassiana*

* Coomassie blue stained bands.

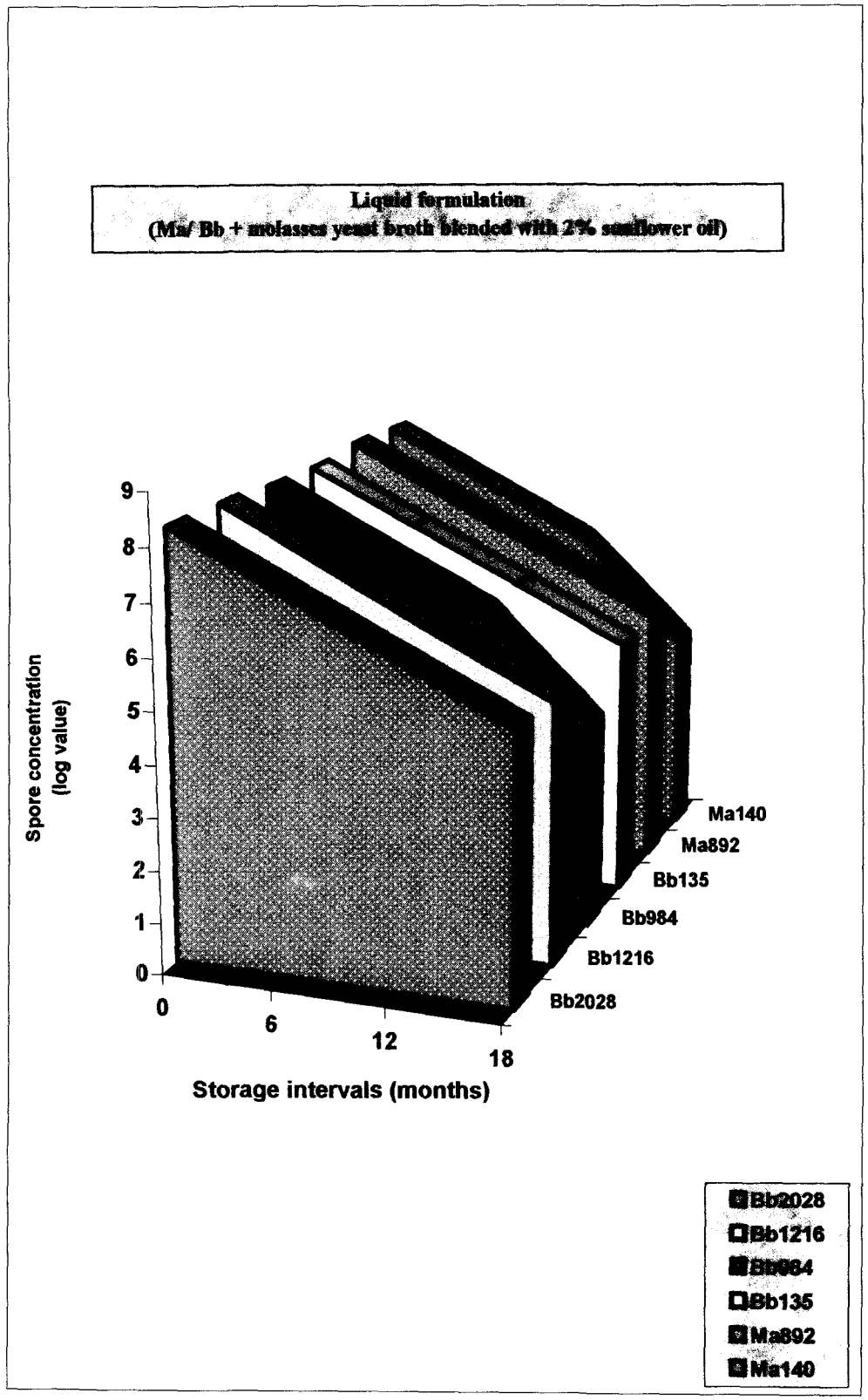


Figure 13: Effect of storage intervals on conidiation of M.anisopliae and B.bassiana isolates

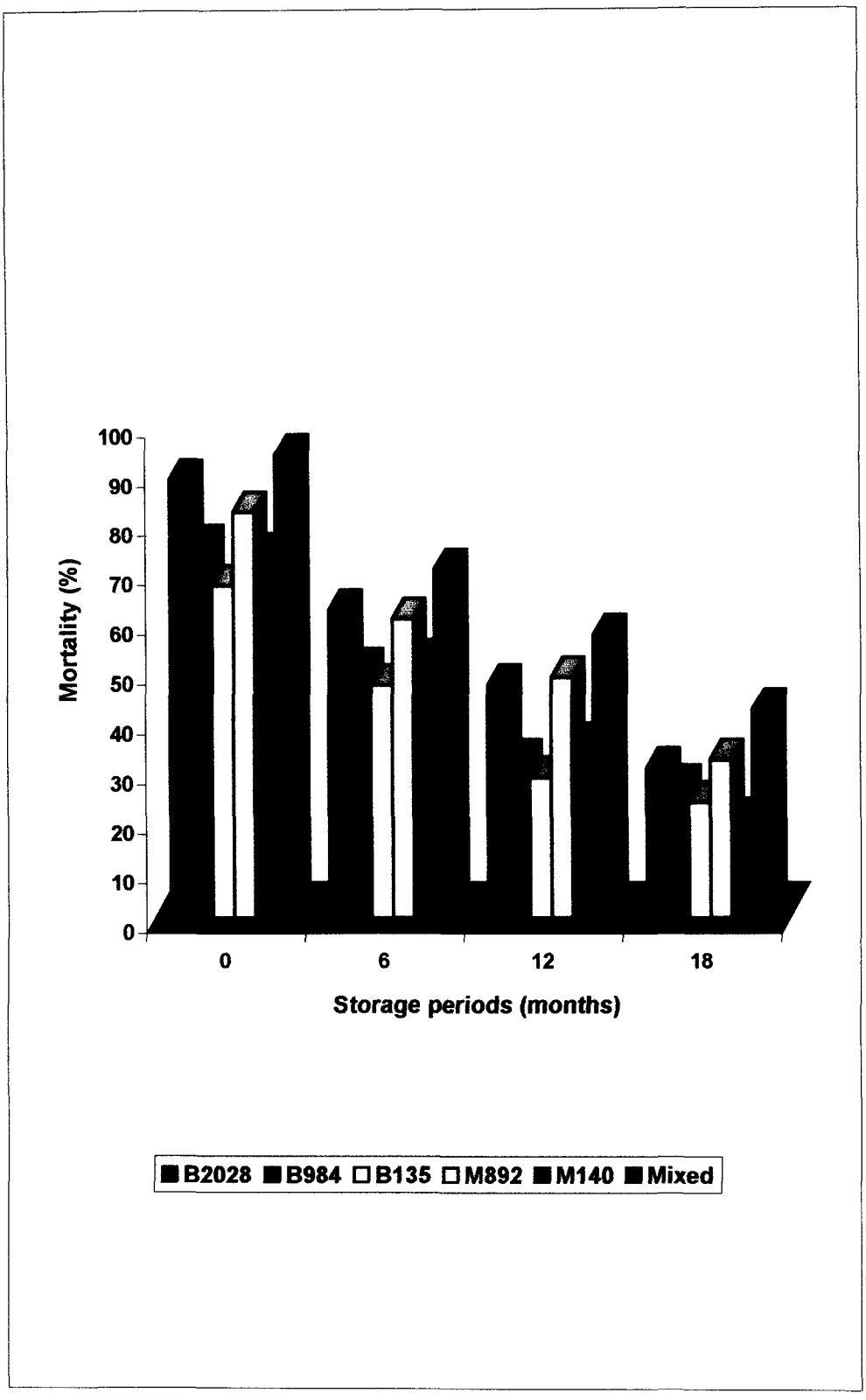


Figure 14: Effect of storage periods on the efficacy of formulations (Ma / Bb in MYB blended with 2% sunflower oil) against mortality of *O.obesus*

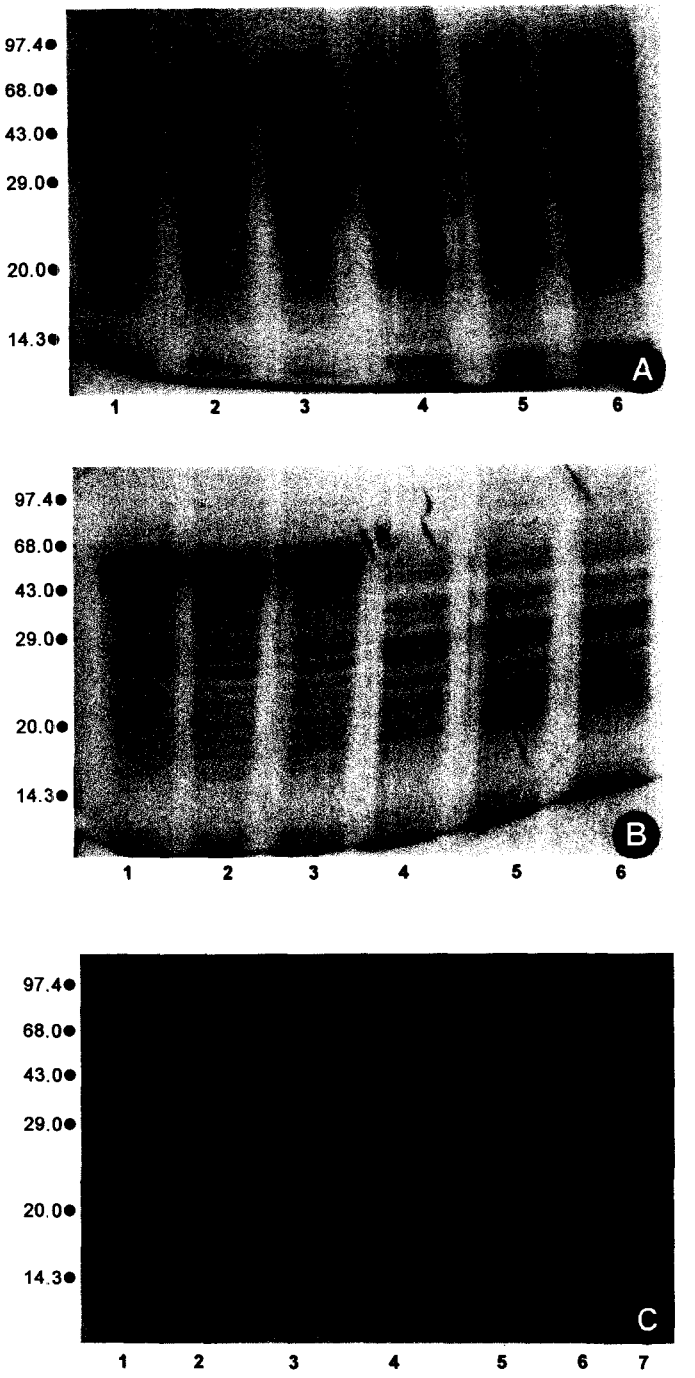


Plate 13 (A - C): SDS-PAGE analysis of mycelial protein (A) *M.anisopliae* isolate - 892 (Lanes: 1-3) and *B. bassiana* isolate-2028 (Lanes: 4-6). (B) *B. bassiana* isolates - 1216 (Lanes: 1-3), 984 (Lanes: 4-6). (C) *B.bassiana* isolate 135 (Lanes: 1, 3, 5 & 7) and *M. anisopliae* isolate -140 (Lanes: 2, 4 & 6).

4.14 Immunodiffusion test

The effectiveness of antigen preparation from the mycelia of *M. anisopliae* (isolate 892) and *B. bassiana* (isolate 2028) in raising antibodies was checked by homologue cross reaction following agar gel diffusion technique.

Initially from the crude mycelial protein preparation of *M. anisopliae* (isolate 892) and *B. bassiana* (isolate 2028) ammonium sulphate purification were carried out as mentioned earlier. The ammonium sulphate saturated (80-100%) fraction were tested for cross reactivity by agar get double diffusion tests. Tests involving antisera of either *M. anisopliae* (isolate 892) or *B. bassiana*(isolate 2028) showed precipitin bands with purified mycelial antigen of different isolates (plate - 14)

The results showed strong precipitin reactions when antiserum raised against mycelia of *M. anisopliae* (isolate 892) and ***B. bassiana*** (isolate 2028) was reacted against its own antigen and weak or no precipitin reaction were observed with antigen of other isolate (Plate - 14 . Table-50)

4.15 Optimization of ELISA

The optimum conditions for ELISA reaction were determined initially as a number of experiments in this investigation have been carried out using ELISA. Optimization of ELISA was done using antisera raised against antigen preparations from mycelia of *M. anisopliae*(isolate - 892) and *B. bassiana* (isolate - 2028).

Antigen dilution

To determine the effect of antigen dilution a series of dilution of *M. anisopliae*(isolate 892) mycelial antigen from 10,000 ng to 625 ng was made and tested against two antiserum dilution (1:125 and 1:250). Results (Table - 51 & 52 and figure 15) revealed that ELISA values decreased with increasing dilution of antigen. The absorbance values of 1:125 antiserum dilution was relatively higher than 1:250 antiserum dilution.

Similarly, to determine the effect of antigen dilution a series of dilution of *B. bassiana* (isolate-2028) mycelial antogen from 10,000 ng to 625 ng was made and tested against two antiserum dilution (1:125 and 1:250). Results (Table - 53 & 54 and Figure - 15) revealed that ELISA values decreased with increasing dilution of antigen. However, concentration lower than 625 ng can also be detected by ELISA. Absorbance values at 1:125 antiserum dilution was relatively higher than 1:250 antiserum dilution.

Table 50: Detection of cross reactive antigens among different isolates of *B. bassiana* and *M. anisopliae* in agar gel double diffusion test using anti *M.anisopliae* (isolate 892) and *B. bassiana* (isolate 2028) antisera

Antigens	Antiserum of M. anisopliae (isolate 892) Precipitin band	Antigens	Antiserum of *B. bassiana* (isolate 2028) Precipitin band
M 892	+	M 892	-
M 140	±	M 140	-
B 135	-	B 135	±
B 1028	-	B 2028	+

(+) Common Precipitin band present;

(±) Week precipitin band absent.

(-) Common precipitin band absent.

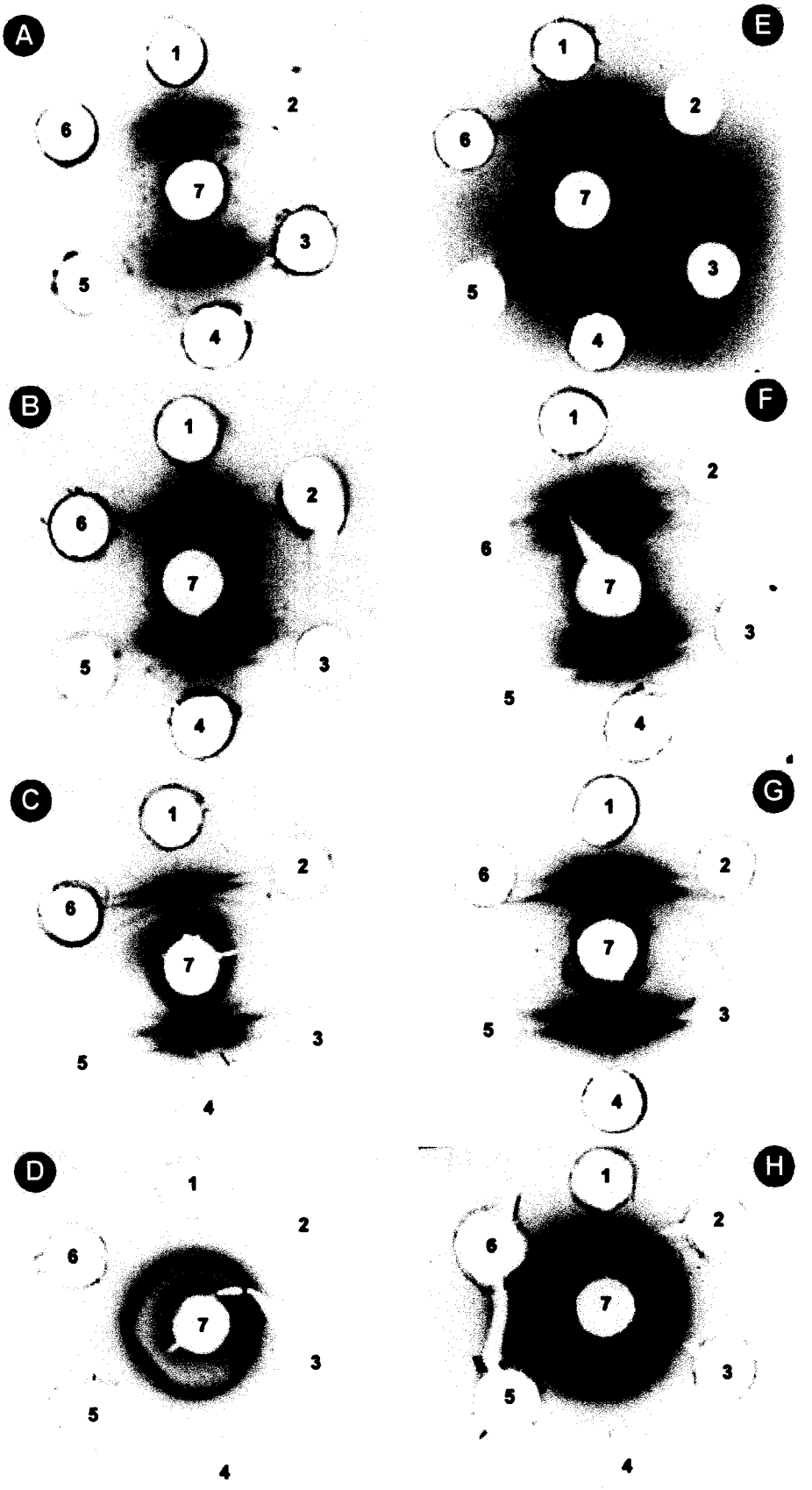


Plate 14 (A-H): Agar gel double diffussion tests with PAb of *M.anisopliae* isolate 892 (A-D) & *B.bassiana* isolate 2028 (E-H). Peripheral wells were loaded with mycelial antigens of *M. anisopliae* isolates 892 (wells 1&4) & 140 (wells 2&5) and *B. bassiana* isolates 135 (well 3) & 2028 (well 6). Central wells were loaded with PAb [A&E - 1st bleeding, F- 2nd bleeding, B&G - 3rd bleeding and C, D & H - 4th bleeding]

Table 51: Optimization of Polyclonal antibody raised against mycelial antigen of *M.anisopliae* (892) in respect of different antigen concentration.

Antigens Conc. (ng/ml)	Antiserum dilution 1:125 Absorbance at 405 nm			
	Expt 1	Expt 2	Expt 3	Mean
10,000	1.350	1.186	1.345	1.293
5,000	1.347	1.205	1.310	1.287
2,500	1.278	1.160	1.250	1.229
1,250	1.128	0.956	1.003	1.029
625	1.103	1.066	1.116	1.095

Table 52: Optimization of Polyclonal antibody raised against mycelial antigen of *M. anisopliae* (892) in respect of different antigen concentration.

Antigens Conc. (ng/ml)	Antiserum dilution 1:250 Absorbance at 405 nm			
	Expt 1	Expt 2	Expt 3	Mean
10,000	1.249	1.200	1.248	1.232
5,000	1.054	1.134	1.238	1.142
2,500	0.794	0.946	0.801	0.847
1,250	0.845	0.675	0.683	0.734
625	0.511	0.678	0.828	0.672

Table 53: Optimization of Polyclonal antibody raised against mycelial antigen of *B.bassiana*(2028) in respect of different antigen concentration.

Antigens Conc. (ng/ml)	Antiserum dilution 1:125 Absorbance at 405 nm			
	Expt 1	Expt 2	Expt 3	Mean
10,000	1.382	1.215	1.355	1.317
5,000	1.232	1.172	1.314	1.239
2,500	1.222	1.151	1.265	1.212
1,250	1.111	1.108	1.191	1.136
625	0.816	1.011	1.046	0.957

Table 54: Optimization of Polyclonal antibody raised against mycelial antigen of *B.bassiana*(2028) in respect of different antigen concentration.

Antigens Conc. (ng/ml)	Antiserum dilution 1:250 Absorbance at 405 nm			
	Expt 1	Expt 2	Expt 3	Mean
10,000	1.263	1.232	1.263	1.252
5,000	1.234	1.217	1.234	1.228
2,500	1.210	1.217	1.229	1.218
1,250	0.914	1.128	1.165	1.069
625	1.057	1.011	0.967	1.011

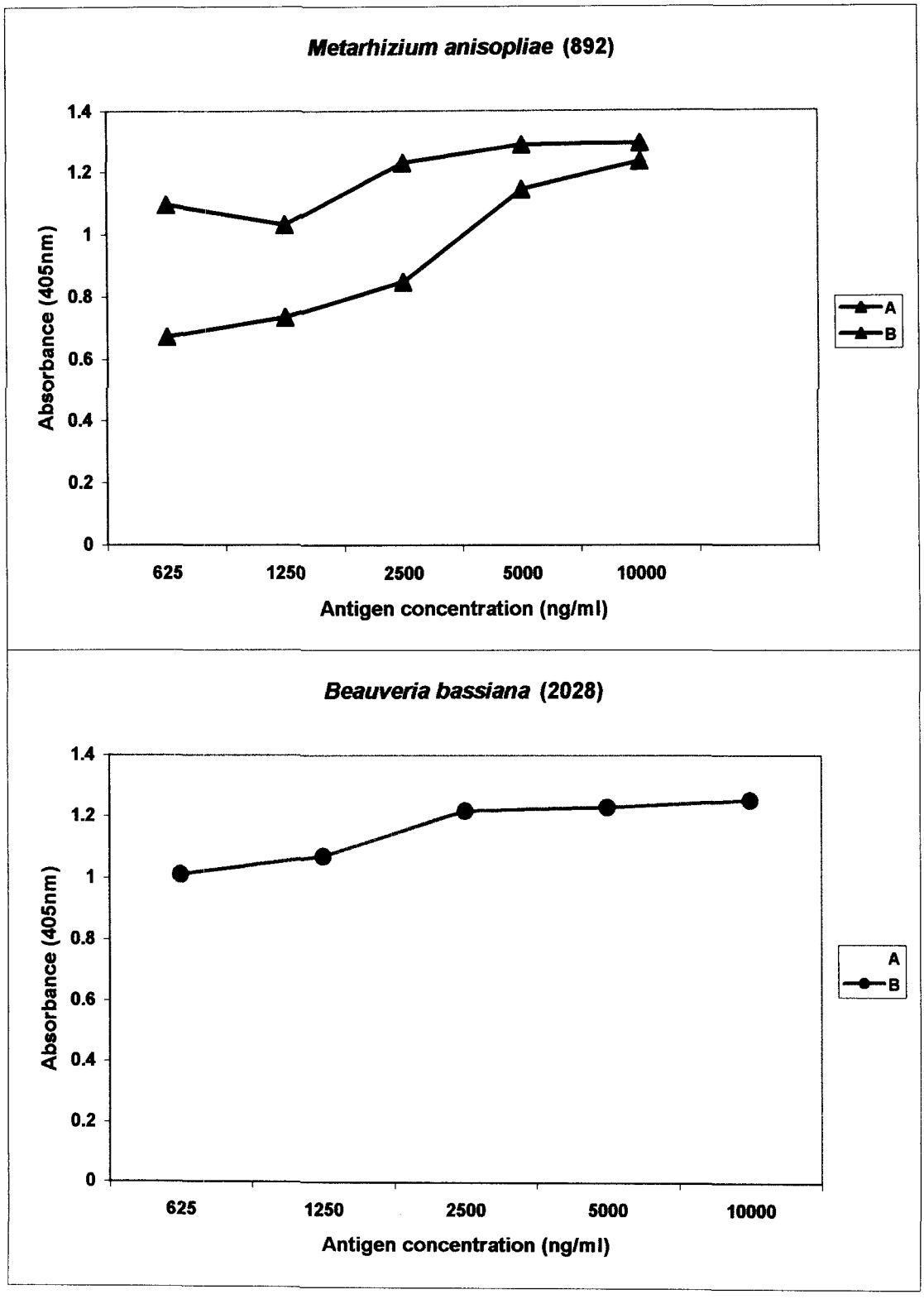


Figure 15: Optimization of mycelial antigen concentrations of *M.anisopliae* and *B.bassiana* by DAC-ELISA [A-1:125; B-1:250]

4.16 Determination of Serological cross reactivity of anti *M.anisopliae* and *B.bassiana* antisera

Cross reactivity of the antiserum raised against *M.anisopliae* (892) was tested against different fungi. For cross reactivety tests, antigens were prepared from the mycelia of all the mentioned fungal species and isolates were tested against anti *M.anisopliae* antiserum. Results (Figure 16) revealed that among the different fungi tested with antiserum raised against *M.anisopliae* (isolate 892), maximum homology was observed with *M.anisopliae* (892) antigen. Of all the fungi, minimum homology was exhibited by *Sclrotium* antigen. Similarly cross reactivity of the antiserum raised against *B.bassiana* (isolate 2028) was also tested against different fungi.

Among different fungi tested with antiserum raised against *B.bassiana* (2028), maximum homology was observed with *B.bassiana* (2028) antigen. Minimum homolog was exhibited by *Sclrotium* (Figure 16). There was a positive reactions in ELISA with higher absorbance obtained from the homologous fungal antigens.

4.17 Immuno Blotting

4.17.1 Dot - Blot

Dot immunobinding technique is a rapid and sensitive method for detection of pathogens. In the present study, dot blot was used to detect the antigen-antibody reactions in different combinations. Homologous reactions of mycelial antigens showed positive reaction i.e. deep pink coloured dots when fast red was used as substrate. When Nitro Blue Tetrazolium Chloride and Bromo-4-Chloro-3 Indolyl phospate Disodium was used as substrate, bright violet colour developed (Table-55, plate -16). PAbs used in the experiment were raised against mycelial antigens of *M.anisopliae* isolate 892 *B.bassiana* isolates 2028. When *M.anisopliae* 892 and *B.bassiana* 2028 amended soil antigens were probed with PAb of *M.anisopliae* 892 *B.bassiana* 2028 respectively, dots of high colour intensity were developed (Table - 55 , plate -15).

4.17.2 Western Blotting

Molecular probing of different antigens with PAb raised against mycelial antigen of *M.anisopliae* isolate 892 were performed through Western blotting technique. Homologous mycelial antigen of *M.anisopliae* isolate 892 exhibited six bands with molecular weights between 55-16 KDa (Table 56, plate - 15). Amended soil antigen of *M.anisopliae* isolate 892 was probed with mycelial PAb of *M.anisopliae* isolate 892 where four bands were revealed with molecular weights between 37.5-21 KDa (Table- 56 , Plate -15). To detect the cross reactivity of *M.anisopliae* 892 with other isolates, mycelial antigen of *M.anisopliae* 140 was probed with the PAb of *M.anisopliae* 892 where no clear bands were observed.

4.18 Immuno fluorescence

Fluorescent antibody labelling with fluoresin isothiocyanate (FITC) is known to be one of the powerful techniques to determine the cell or tissue location of major cross reactive antigen (CRA) shared by host and pathogen. In the present study, to determine the tissue and cellular location of CRA in mycelia and spores of *M.anisopliae* and *B.bassiana* isolates immunofluorescence was performed. Detailed methods of antibody staining of mycelia and spore have already been discussed under materials and methods. Mycelial preparations were photographed under UV-fluorescence (plate- 16). The mycelia of *M.anisopliae* (892) and *B.bassiana* (2025) fluoresce when treated with homologous antiserum followed by FITC(plate).

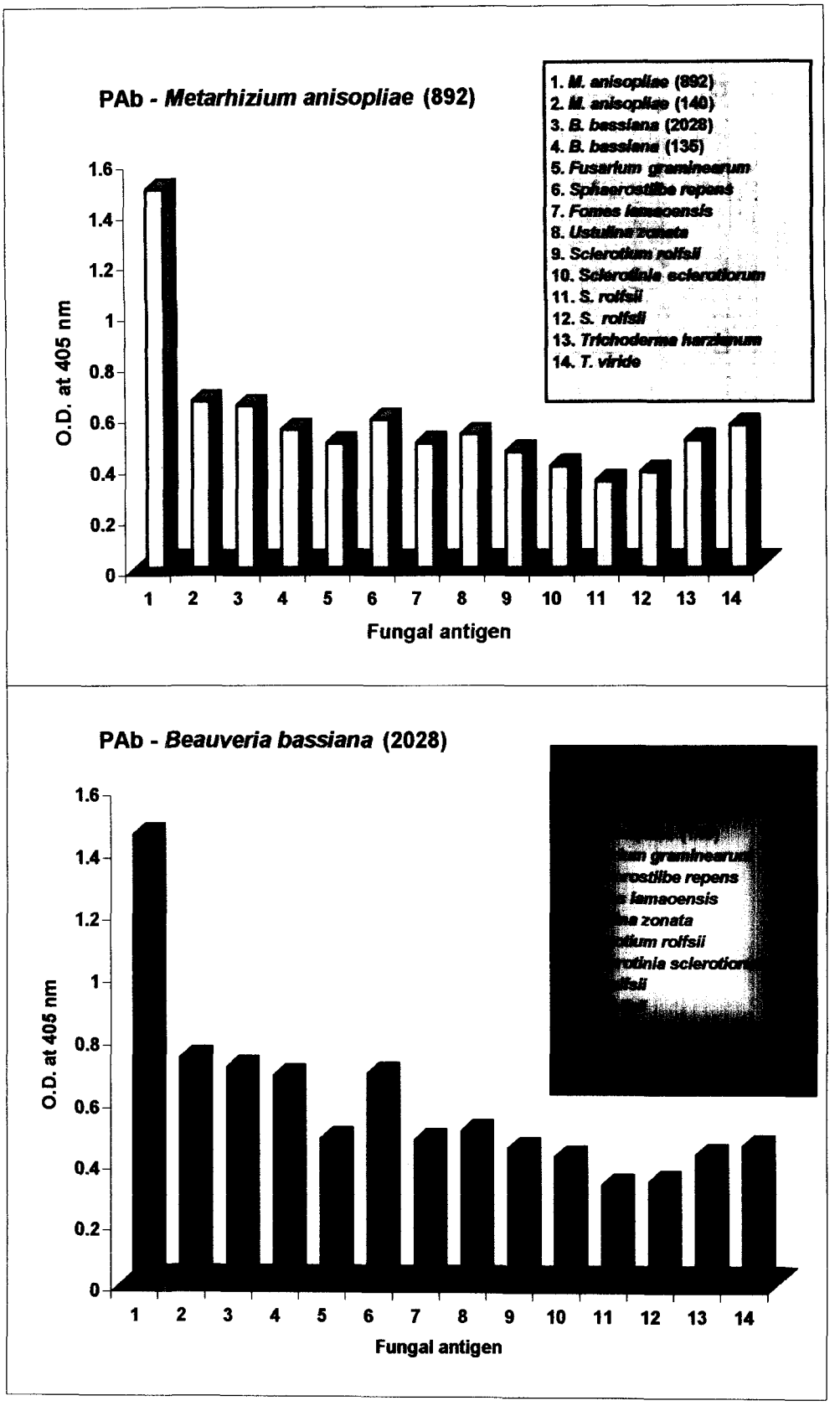


Figure 16: Cross reactivity of PAbs of *M.anisopliae* and *B.bassiana* with other fungi in DAC-ELISA

Table 55: Dot-blot reaction of PAbs of *M. anisopliae* isolate 892 and *B. bassiana* isolates 2028 with different mycelial antigens of *M.anisopliae* and *B.bassiana* isolates

	Colour intensity *	
Antigen	[a]PAb of *M. anisopliae* (892)	[a] PAb of *B.bassiana* (2028)
M892	++++	+++
M140	+	++
M892(soil amended)[b]	++++	++
B2028	+++	++++
B1216	++	++
B135	++	±
B984	-	+
B2028(soil amended)[b]	+++	++++

* Fast Red colour intensity: Bright (++++), High (+++), Low (++), Faint (+), not well detected (±), and no color (-)

[a] Mycelial PAb, M - *Metarhizium anisopliae* and B- *Beauveria bassiana*

[b] Antigen prepared from soil amended with spores and mycelia.

Table 56:Western blot analysis of fungal antigens of *M.anisopliae* isolate with polyclonal antibody (PAb) of *M. anisopliae* isolate 892

	Molecular weight (KDa) of observed bands	
	Antigen	
M892	* M892(ammended soil)	M140
54.5	37.4	-
43.0	21.6	-
37.4	26.5	-
26.6	21.6	-

Mycelial PAb of *M. anisopliae* used as probe

- Bands not observed, M=*Metarhizium anisopliae*

* Antigen prepared from soil ammended with spores and mycelial of *M. anisopliae*

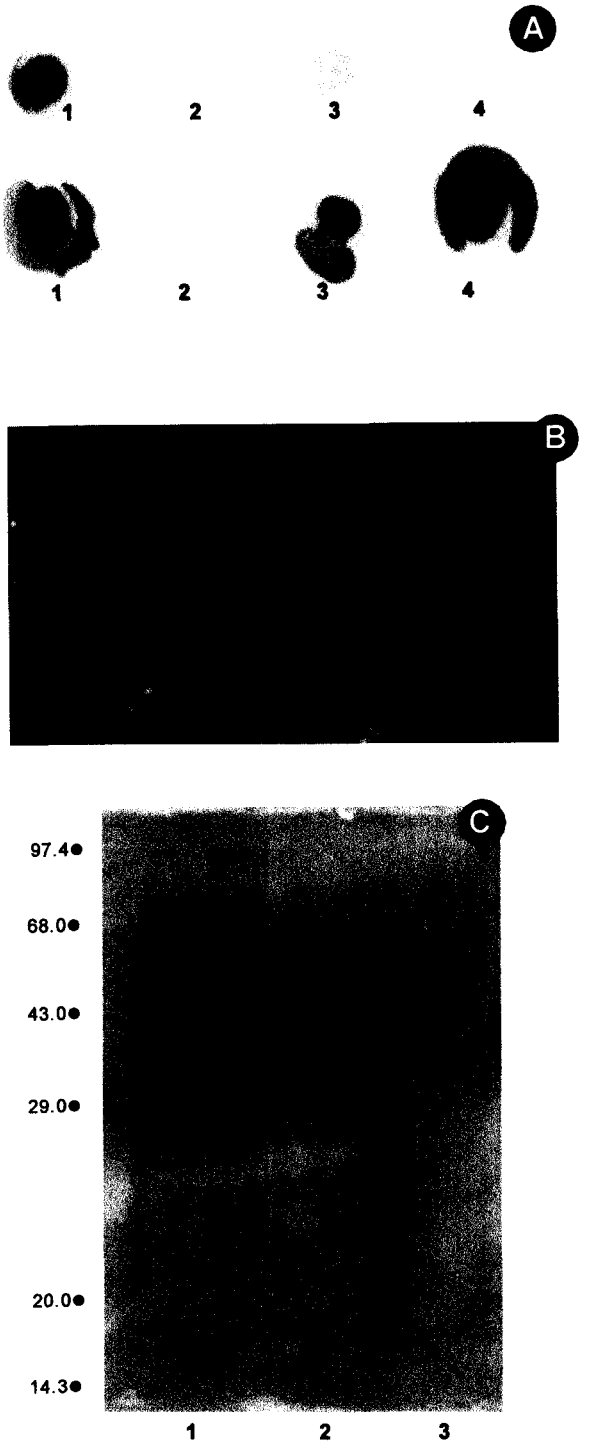


Plate 15 (A - B) : [A] Dot immunobindings of antigens and antibody on nitrocellulose paper. Antigens were mycelial proteins of *B. bassiana* isolate 2028 (1), isolate 1216 (2), isolate 135 (3), isolate 984 (4) and *M. anisopliae* isolate 892 (5), isolate 140 (6); Soil amended antigens of *M. anisopliae* isolate 892 (7) and *B. bassiana* isolate 2028 (8). Blots were probed with PAb prepared from mycelia of *M. anisopliae* isolate 892 (Fig A) and mycelia of *B. bassiana* isolate 2028 (Fig.B). (Fig. C) : Western blot analysis of mycelial antigens of *M. anisopliae* isolate 892 (lane1), soil amended antigens of *M. anisopliae* isolate 892 (lane2) and mycelial antigen of *M. anisopliae* isolate 140 (lane3). The blot was probed with PAb prepared from mycelial antigen of *M. anisopliae* isolate 892, on nitrocellulose paper.

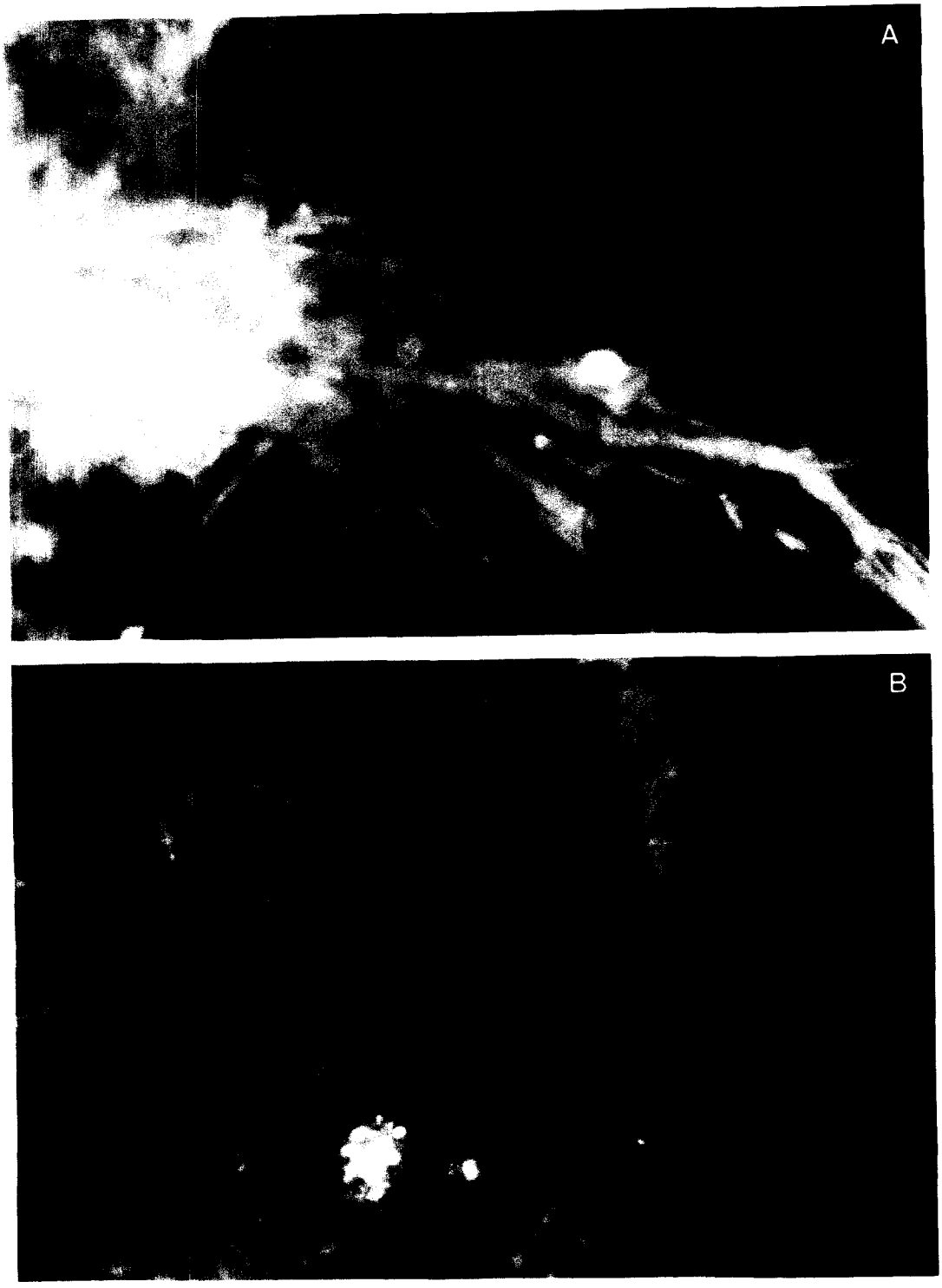


Plate 16 (A - B) : Indirect immunofluorescence of hyphae treated with homologous PAb and stained with FITC labelled antibodies of goat specific for rabbit globulin. (A) *M. anisopliae* isolate 892 (B) *B. basiana* isolate 2028.

DISCUSSION

Metarhizium anisopliae and *Beauveria bassiana* are wide spread entomopathogenic fungi on many insect species. Their high pathogenicity to termites was already recognized and their suitability in pest control was verified. However, all attempts to use the fungi as biological pest control agents failed for various reasons.

M.anisopliae and *B.bassiana* produce two types of spores, blastospores in the living host and conidia on the surface of the dead host. Blastospores are susceptible to environmental conditions and consequently less persistent than conidia. Artificial culturing of the fungi offer no particular difficulties; a utilizable source of carbon for germination, a nitrogen source for continuous hyphal growth, high humidity (80-100% R.H.), and temperatures between 20^0C and 28°C, with an optimum at 25°C - 28°C, are necessary. The most common species, *M. anisopliae* and *B. bassiana* are already being successfully reared in mass cultures and formulated. In the present study the mass production of the fungi, their formulation and their molecular characterization are described. The mass production was necessary for field assay.

Isolates of *B. bassiana* and *M. anisopliae* showed high virulence towards the termites (*O. obesus*) as evidenced from the present laboratory and field evaluations. From the bioassays with spore concentrations of the isolates it was possible to determine accurately the LC_{50} value for each isolate.

Previous studies have indicated that *B. bassiana* isolates induced>90% mortality by direct inoculation of *H. hampei* adult females at 1% spore concentration, whereas *M. anisopliae* isolates caused *H. hampei* mortality between 57% (Ma4) and 89% (MA3,Ma5) under similar conditions (Rosa *et al.* 2000). This difference may be related in

part to the origin of the various isolates; all *B. bassiana* materials were isolated from local populations of *H. hampei*, whereas the *M. anisopliae* materials originated from Lepidoptera and Homoptera from northern Mexico and the United States.

The LC_{50} values of Bb25 and Ma4 towards the coffee berry borer were 4.1×10^6 and 4.2×10^6 spores per ml , respectively (Rosa *et al.* 2000). These figures represent half the LC_{50} value of Bb25 to *P. nasuta* and a nearly identical value for the LC_{50} of Ma4 to *P. nasuta.* It is therefore evident that these isolates are highly virulent to both the coffee berry borer and the parasitoid *P. nasuta.* In another study with *C. stephanoderis* , LC_{50} values were found to be greater than that of *P. nasuta* which reflected that *C. stephanoderis* was substantially more resistant to fungal infection compared with *P. nasuta.* Niranjana (2002) also made an attempt to use *B.bassiana* in managing coffee berry borer.

Field evaluations on the impact of applications of bio-insecticides for the control of insects natural enemy populations are very limited in number. An evaluation of the impact of aerial applications of *Beauveria brongniartii* spores to forest habitat for control of *Melolontha melolontha* L. reported that only 1.1% of non target insects and spiders were infected by the pathogen of >10,000 individuals sampled.

In the present investigation it was clear that surface contamination had good effect on the incidence of fungal infection in the worker termite populations. Thus, it would be appropriate to apply the fungus in the field during the phase of termite infestation. With the field application of *B. bassiana* and *M. anisopliae* isolates the infestation ratio of the plants was significantly reduced.

In the present study, four isolates of *B.bassiana* and two isolates of *M.anisopliae* were tested against the worker termites (*O. obesus*) under laboratory condition. All the isolates proved to be pathogenic to the termites at different degrees. *B.bassiana* isolate 2028 gave the lowest lethal concentration (LC_{50}) of 1.334 x 10^4, 2.818 x 10^4 and 5.012 x 10^4 conidia / ml at bioassay - I, II and III respectively. The lethal time for 50% mortality (LT_{50}) was also recorded to be lowest (56.23 hrs, 50.12 hrs and 53.09 hrs respectively) when treated with isolate 2028 of *B.bassiana.*

In a bioassay test with the conidial suspension of five isolates of three entomopathogenic fungi for their infectivity to second instar larvae of *Spodoptera litura , B. bassiana* was found to be the most virulent (Prasad *et,al.*1989). The data on the dosage-mortality response of the larvae to the different fungal isolates following direct application of the conidial suspension indicated a good fit of the observed and expected responses based on a chi-square test. The regression coefficients in general were very low with all the fungal isolates. The comparison of LC_{50} however, revealed the differential susceptibility of the pest to the fungal isolates. There was a sharp increase in the values of LC_{50} in other isolates . When second, third and fourth instar larvae of the test insect were bioassayed for their susceptibility to *B.bassiana,* it was observed that the susceptibility to infection decreased with the age of the larvae.

In the present investigation, dosage-mortality responses of *O. obesus* to the isolates of *M.anisopliae* and *B.bassiana* in different bioassays (I, II, III) showed the observed and expected responses which were achieved from chi square tests. The values ranged from 0.212 - 1.160 in bioassay - I, while in bioassay - II and III the chi square values were found between 0.160 - 2.451 and 0.1185 - 3.0966 respectively.

The lethal concentrations, LC_{50} and LC_{90} of 12 fungal isolates were determined by Burdeos and Villacarlos (1989). All the isolates of *M.anisopliae* were highly pathogenic to adult sweet potato weevils, while those of *B.bassiana* were moderately pathogenic. They observed that the spore germination of *M.anisopliae was* 96% and *B.bassiana* was 95%.

Marked differences were observed in the virulence of the various isolates. The isolate that required the lowest concentration of spores to cause 50% mortality of the weevil population was considered the most virulent. Thus, the isolates arranged in decreasing order of virulence were Ma1 for *M.anisopliae* (8.42×10^5) and Bb1 for *B.bassiana* (1.54×10^7). Although Bb1 was relatively virulent, it was not comparable to any of the isolates of *M.anisopliae*. Unlike the latter, the two isolates of *B.bassiana* differed considerably in their pathogenicity to the sweet potato weevil. This is probably a common characteristic of the group as shown also by other workers. For instance, Fargues (1972) in testing seven strains of *B.bassiana* against the Colorado potato beetle, *Leptinotarsa decemlineata*, also found great variation in pathogenicity so that the most virulent strain caused 100% mortality in 5 days, while the least virulent caused only 10% mortality after 26 days. *B. bassiana* had been reported to infect adult sweet potato weevils but it appeared to have contributed very little to the mortality of the weevils in the field. Barson (1977) reported that varying conditions of temperatures and humidity determine the success or failure of a pathogenic fungus. In their study, the adult sweet potato weevils inoculated with *M.anisopliae* and *B. bassiana* were incubated at 27^0C-30^0C and 70-90% RH. The upper temperature ranges were within the optimum range of 27^0C-28^0C reported for *M.anisopliae* and 20^0C-30^0C for *B.bassiana*.

The experiment of Burdeos and Villacarlos (1989) indicated that virulence was a function of fungal species. However, spore concentration was also found to be an important factor in the expression of the virulence of the best isolates that were previously selected. For instance, at 1 x 10^5 spores / ml, mortality of the treated weevils was significantly greater than that of the uninoculated control in Ma1 and Bb1 treatments. However, a concentration of 1 x 10^8 spores / ml of all species significantly reduced weevil population under laboratory conditions.

The calculated LC_{50} values for the three entomopathogens were probably an overestimate because the assay did not consider the actual number of spores which came in contact with the weevil. In a related study using *B. bassiana*, Fargues (1972) estimated 5 x 10^4 to 1 x 10^5 spores / ml were required to cause 85 - 90% mortality among fourth - instar larvae of the Colorado potato beetle, *L.decemlineata*. Barson (1977) on the other hand, obtained 100% mortality of *Scolytus scolytus* larvae after 7 days exposure to more than 1 x 10^6 spores/ml of *B. bassiana*. The highly sclerotized cuticle in the adult weevils used in the present work probably formed a barrier against fungal infection by *B. bassiana*, so that higher concentration was required to cause infection compared with the above works.

At 1x10^8 spores/ml, the highest daily mortality of the insects exposed to Ma1 (41%) and Bb1 (47%) occurred on the third and fourth day after exposure, respectively. In all the treatments, percent daily mortality gradually declined until the seventh day when almost all the insects had died. In the present study, the *B. bassiana* isolate 135 required 5,4,4,3 and 5 days exposure to kill 50% of the termite (*O. obesus*) population while isolate 984 required 5,4.5,4,3 and 5 days exposure to kill 50% of the population of *O. obesus* and 1 x 10^6 conidia / ml concentration taking 10,14,20,28 and 34 day-old cultures.

In the present investigation, the varying effectiveness of the isolates of entomopathogens is reflected in the appearance of mycelial growth and the sporulation of the fungus on the body surface of the dead insect. Like many other entomopathogenic fungi, *M. anisopliae* and *B. bassiana* kill their host insect through the action of the hyphae which germinates from spores outside the body, penetrate the exocuticle, invade and ramify inside the body to subsequently destroy the internal tissues. Infected insects became restless, weakened and finally ceased feeding. Death due to mycosis occurred three days after inoculation. Dead termites became hard and their appendages turned brittle. About 24-48 hrs after death, whitish mycelia began to appear on the intersegmental regions and joints of the appendages of the dead insect. Sporulation on the body surface of the termite occurred on the fourth and fifth days due to infection by *M. anisopliae* and *B. bassiana*, respectively. By this time, the dead insects were almost covered with mycelial growth.

The present investigation proved that *B. bassiana* isolates 984, 2028, 1216 and 135 caused mycosis on *O. obesus* workers which was confirmed by direct microscopical examination and by culturing of the infected workers in water-agar plates. *M. anisopliae* isolates 892 and 140 also showed a similar response. The present observations agree with those of Burdeos and Villacarlos (1989)

The initial stages of insect infection by entomopathogenic fungi include the penetration of the host cuticle (Hajek and Leger, 1994). Cuticle solubilisation and subsequent hyphal penetration occured by the action of extracellular enzymes and acid metabolites (Bidochka and Khachatourians 1987 and 1990). Following cuticle penetration, the fungus proliferates within the body of its host.

Burdeos and Villacarlos (1989) working with *Scolytus scolytu* larvae and *Cylas formicarius* adult weevils found that *B. bassiana*

required a higher spore concentration and a shorter post-treatment period to kill 50 percent of the worker population. Workers of *O. obesus* infected with *B. bassiana* and incubated at 27-30°C and 70-90% RH; experienced fast growth of the white muscardine fungus, thus causing high mortalities in the termites. They reported that the entomopathogenic fungi normally require about 100% RH and 20-30°C temperature for germination, growth and sporulation.The present study confirms the pathogenicity of *B. bassiana* and *M. anisopliae* against *O. obesus* workers.

The susceptibility of *Megalurothrips sjostedti* to *M. anisopliae* were evaluated on cowpea varieties by Ekesi *et al.* (2000). The result of their study showed that the susceptibility of *M. sjostedti* to *M.* anisopliae were dependent on the cowpea varieties. Mortality of the legume flower thrips was higher on the moderately resistant variety at all concentrations of inoculum and at all temperatures compared to the tolerant and susceptible varieties. Lethal time and lethal concentration values on the moderately resistant variety were shorter and lower, respectively, compared to the susceptible and tolerant varieties. These results suggest a faster kill of thrips on the moderately resistant variety with a low concentration of inoculum compared to the tolerant and susceptible varieties. Thrips feeding on moderately resistant cowpea varieties have longer developmental periods, lower body weights and lower reproduction potential (Ekesi *et al.*, 1998). These effects could increase the susceptibility of thrips to the fungal pathogen due to the physiological and metabolic stress imposed on the insect by feeding upon a suboptimal host (Butt and Brownbridge, 1997).

Temperatures of 15°C and 20°C caused a significant decrease in development of fungal infections but did not significantly affect the percentage mortality caused by any concentration of the fungus on the

susceptible and moderately resistant varieties. But in the present investigation it was observed that *M. anisopliae* and *B. bassiana* were more virulent when kept at 28 + 2°C and incubated for 28 days. The mortality percent of termites was also increased. Most authors agree that incubation period of most fungal diseases in insects is temperature-dependent (Ferron, 1978; Carruthers and Soper, 1987).

For mass multiplication of *B. bassiana* and *M. anisopliae* isolates, in the present investigation, molasses yeast broth was selected. *B. bassiana* isolates 2028, 1216, 135 and 984 produced $2.42x10^7$, $2.26x10^7$, $2.15x10^7$ and $2x10^7$ conidia /ml respectively when cultured on 200 litre broth in large scale production tanks. *M. anisopliae* isolates 892 and 140 produced $2.39x\ 10^7$ and $2.22\ x\ 10^7$ conidia/ml respectively on similar capacity tanks. Sharma *et al.*, (1999) also mass multiplied *M. anisopliae* and *B. bassiana* in molasess yeast broth (200ml) in 1 litre capacity Erlenmeyer flasks. *M. anisopliae* produced $8x10^8$ conidia/ml while *B. bassiana* produced $1x10^9$ conidia/ml which are relatively higher than the present findings. The results of the present study are also in consonance with the findings of Rombach *et al.*, (1987) who investigated that a liquid medium containing sucrose and yeast extract produced maximum mycelial growth and many conidia of *B. bassiana.*

In the present study, entomopathogenic fungi were also mass multiplied on different grains. Crushed grains of Bajra (*Pennisetum typhoides*) yielded spore dust of $4.43x10^7$, $4.6x10^7$,$4.97x10^7$ and $4.33x10^8$ conidia/gm by *B. bassiana* isolates 984,2028, 135 and 1216 respectively. The spore production by *M. anisopliae* isolates 892 and 140 in crushed Bajra were $4.54x10^7$ and $1.94x10^8$ conidia/gm dry weight. The mass multiplication of entomopathogenic fungi by Sharma *et. al.* (1999) on various grains indicated that *M. anisopliae* produced maximum

yield of conidia (2 x 10^9 g^{-1}) on crushed maize grain medium. In their study, though pearl millet grain produced higher conidia but, due to its sticky nature, the harvesting of conidia from this medium became a little difficult. Therefore, the next best maize grain medium was selected for mass multiplication of *M. anisopliae*. Secondly, use of whole grain of maize in the grain media experiment may also be responsible for comparatively less conidial production. Therefore, use of crushed maize grain instead of whole grain in the mass multiplication might have accelerated the conidial production.

Considering the above results, in the present investigation, maize grains were also used for mass multiplication and spore production .Suitability of a particular grain for preparation of medium may vary with differential requirements of the strains or due to difference in production technology used. Nevertheless, crushed Bajra grains in the present study was comparable with that of other grains, yet Bajra grain was preferred because of its low cost, high yield and economic feasibility over other grains.

Grain media requirement varied among different species of fungal pathogens. For *Beauveria* spp, cowpea grain medium proved best for their conidial production (Sharma *et al.*,1999). *B. bassiana* and *B. brongniartii* mass cultured on cowpea grains filled in two kg capacity polypropylene bags yielded 1.5x10^9 and 1.8 x 10^9 conidia g^{-1} of dry grain weight, respectively. In their investigation, *B. brongniartii* was also cultured on cowpea whole grains, which produced 1.8 x 10^9 conidia g^{-1} of dry weight. Aregger (1992) reported high yield of 1x10^8 to 2x10^9 conidia g^{-1} on white grains of barley and documented that the yield of conidia depended mainly on the addition of water and the length of incubation. In the present study, bajra grains were preferred for mass multiplication of *B. bassiana* as the grains of barley in our experiment

provided very low conidial production, however, spore production varied among the isolates .

Esenther and Beal (1978) conducted a field trial in Southern Mississippi to determine if placing decayed wood bait blocks impregnated with merix could suppress termite (*Reticulitermest sp.*) populations when placed around the perimeter of an area. Bait blocks treated with 10mg merix / block were buried at 1.5 m spacing at the perimeter of six 7.5m-square plots, 3 of which also had treatments along the perimeter of an outer 30m square. Non-insecticidal bait blocks were used by them to monitor termite activity within the treatment lines. They observed that during a 3.5 yr period termite activity on the treated plots was suppressed.

Baits treated with diiodomethyl para-tolyl sulfone at 600ppm (wt/wt) were introduced into selected trap stations of 3 colonies of the Formosan subterranean termite, *Coptotermes formosanus* Shiraki in Hallandale, between November 1987 and November 1988 (Su and Scheffrahn ,1991). They observed that termites neither avoided foraging at sites containing treatment nor was feeding significantly reduced on the treated versus untreated baits. One year after bait application, foraging populations of 3 colonies that received A-9248 baits were reduced 65-98%. Their results demonstrated that a toxicant bait can be used to suppress foraging populations of subterranean termite colonies and hence reduced their damage potential. Su and Scheffrahn (1991) surveyed the foraging populations of colonies of the eastern subterranean termite, *Reticulitermes flavipes*, in residential and undeveloped environments of Southern Florida. A triple mark-recapture program using the dye marker Nile Blue A indicated foraging population of *R. flavipes* contain 0.2-5.0 million termites per colony, and the foraging territories encompass an area of up to 2,361 m^2 and a linear

foraging distance of 71m. Habitat type was not correlated with foraging population size.

Subterranean termites (Isoptera: Rhinotermitidae) were found to be ubiquitous in both wooded and desert regions of North America which were important contributors to nutrient cycling, energy flow, and ecosystem productivity (Grace ,1994). With the use of microbiological pesticides or microbial pest control agents (MPCAs) , termites might suffer unintended exposure to these pathogenic agents.

Cornelius and Grace(1994) performed bioassays to test the responses of the Formosan subterranean termite, *Coptotermes formosanus* Shiraki, to dichloromethane extracts of whole *Iridomyrmex glaber* (Mayr) workers. They observed that termite workers were strongly repelled by filter papers treated with *I.glaber* extracts. In a choice test, termite workers fed significantly less on filter papers treated with *I.glaber* extract compared with solvent treated controls within a 24h period. Bioassays also were performed by them to evaluate the effect of different concentrations of ant extracts on termite tunneling behavior. The rate at which termites penetrated the treated sand was concentration dependent. They also observed that in 10d, termites failed to penetrate any of the sand barriers treated with 500 ant equivalents per gram of sand. Their results suggested that ant extracts may also be considered as a potential source of natural products for termite control.

A baiting procedure that incorporated a matrix containing a chitin synthesis inhibitor, hexaflunmaron, was evaluated by Su (1994) against field colonies of the eastern subterranean termite, *Reticulitermes flavipes*, and Formosan subterranean termite, *Coptotermes formosanus* shiraki. Wooden stakes were first

driven into the soil to detect the presence of termites. Bait tubes were placed in the soil where termites were detected. A self-recruiting procedure, in which termites collected from wooden stakes were forced to tunnel through the matrix in bait tubes, significantly increased bait intake by termites.

In the present investigation the suppression of termite population in the field was based on different experimental procedures. Field colonies of *O.obesus* and *O.distans* were identified, wooden stake survey and monitoring station application methods were followed. This was done to estimate the foraging territory and the population of termites before the introduction of baits in field to suppress their population. In this investigation the fungal bait matrix used was muscardine fungi which was in contrast to bait matrix of acetone solution of hexaflumuron used by Su (1994). However, the bait application with muscardine fungi in the present study resulted in the reduction of termite population in the field which was in accordance with the findings of Su(1994) where hexaflumuron bait application in field suppressed the population of subterranean termites. The only difference was that the present study was based on biocontrol agents while investigation of Su (1994) was based on the application of chemical.

Kenne *et.al.*(2000) studied the hunting behavior of *Myrmicaria opaciventris* in order to evaluate if it can be used as biological control agent against the termites that damage sugarcane plantations. Hunting workers foraged in groups and recruited nest mates at short-range when they encountered large termite soldiers or groups of small termite workers. Differences in prey capture concerned may be considered as (1) means of detection; (2) termite body part seized; (3) percentages of prey abandoned and (4) use of venom. Large termites were stretched by several workers whose adherence to the substrate is facilitated by well-developed aralias and claws on the legs while others spread venom on the body and carved it up. An adaptation to termite capture was noted

with a distribution of tasks between the workers which subdued prey, and those which transported it. In the former case, the workers easily eliminated termite soldiers, successively attacked several termite workers and even captured new individuals while holding the first ones captured between their mandibles before retrieving them all at once. The remaining individuals were retrieved by the transporting workers. Given this particularly effective predatory strategy, they concluded that, under certain conditions, *M.opaciventris* can be used as a biological control agent against termites.

Verkerk *et.al* (1999) has described an outline of the most important novel techniques which would be feasibly developed for use in an agricultural context (e.g. chemical and microbial baiting, transmissible coatings). These methods have been developed primarily for control of 'lower' termites in buildings in industrialized countries. They are not directly transferable to agriculture in the tropics where 'higher' termites cause the vast majority of damage to crops and pest management decisions are made largely by resource-poor, small-holder farmers. The three most serious obstacles to the development of bio rational termite management in tropical agriculture probably relate to cost, availability of active ingredients or pathogens and the lack of research on station or trap design appropriate for 'pest' termite species. Based on their own experiences with tropical pest management and baiting technologies, they considered the characteristics required for the development of baiting systems, transmissible repellants for use in agriculture in developing countries. This includes the adaptation of these systems to allow the transference of biological control agents, such as entomopathogenic fungi and nematodes. They argued that it is important that such developments pose a negligible hazard to applicators or farmers, and cause no significant harm to non-target organisms, including beneficial fauna such as non-pest termites, earthworms and natural enemies important in the regulation of crop pests.

For the molecular characterization of the entomopathogenic fungi *M. anisopliae* and *B. bassiana* different experimental techniques have been adopted in the present investigation. Antigens were prepared from mycelia and conidia of all the isolates of *M.anisopliae* and *B.bassiana* as well as other soil fungi. Purified antigens prepared from the isolates of *M.anisopliae* and *B.bassiana* were resolved in SDS-PAGE and finally polyclonal antibodies (PAb) were generated against *M. anisopliae* (892) and *B. bassiana*(2028). These PAbs were tested against homologous antigens as well as antigens prepared from other isolates of *M.anisopliae*, *B.bassiana* and also other soil fungi using agar gel double diffusion tests. The cross-reactions were evident in several immunoenzymatic assays such as Direct antigen coated enzyme linked immunosorbent assay (DAC-ELISA), Dot immunobinding assay and Western blottings.

In the present investigation, cross reactivity of PAbs raised against *M. anisopliae* and *B. bassiana* were tested with other soil fungi, such as *Fusarium graminearum*, *Sphaerostilbe repens*, *Fomes lamaoensis*, *Sclerotium rolfsii*, *Sclerotiana sclerotiorum*, *Trichoderma harzianum* and *Trichoderma viride* . Results revealed that among all the above fungi tested, PAb of *M. anisopliae* reacted to some extent with isolates of *B.bassiana* and *S.repens* while PAb of *B. bassiana* reacted with isolates of *M.anisopliae* and *S.repens.* Positive reactions in ELISA with higher absorbance were always obtained from the homologous fungal antigens of *M. anisopliae* (892) and *B. bassiana* (2028). Absorbance values were lower for other fungi. Mohan (1988) successfully raised antiserum against pooled mycelial antigens of five *Phytophthora fragariae* races. In indirect ELISA, it detected homologous soluble mycelial antigens. PAb of *Phytophthora fragariae* reacted strongly with antigens from several *Phytophthora* species.The sensitivity of detection was high and concentrations as low as 2ng proteins / ml were detectable.

The present experiment showed that ELISA positive material can be detected at early infestation stages of the entomopathogens. Therefore, the ELISA test is sufficiently sensitive to find important applications in detecting *B. bassiana* and *M. anisopliae* on termites. It could overcome the difficulty observed in visually detecting slight infection of termites in their colony. The ELISA test might prove useful in monitoring field samples. The sensitivity achieved in the present study by no means reflects the limit of detection possible in ELISA. The ELISA test described should prove valuable in screening field collected samples of entomopathogens for identification and ensure their infection potential.

Molecular probing of amended soil antigen was also performed with PAb raised against mycelial antigen of *M. anisopliae*(892). Soil amended with spores and mycelia of *M. anisopliae* (892) was probed with PAb of *M. anisopliae*(892) where bands were revealed with same molecular weights and patterns as the bands present in homologous mycelial antigen of *M. anisopliae*(892). This kind of test is very helpful in detection and identification of the entomopathogen(s) from the soil. This test also helped us to investigate the soil persistence of the entomopathogen(s) generally after the field application of the formulated mycoinsecticide. This kind of assay may be used to fix up spray schedule of the mycoinsecticide and monitor their persistence.

The experiments presented by Pendland and Boucias (1990) showed that the polyclonal antibodies generated against *Spodoptera exigua* hemocytes and hemolymph and those produced against cell wall surfaces of an entomopathogenic fungus (*Nomurace rileyi*) not only reacted to their own antigens but also showed activity against heterologous antigens. The cross-reactions were evident in their assays using both fluorescent microscopy and Western blotting techniques.

In the present study, using indirect immunofluorescence technique , cellular location of cross reactive antigen in mycelia and spores of *M.anisopliae* and *B.bassiana* were determined. This is an excellent technique for detection and identification of the entomopathogens with their PAbs after their isolation from their hosts in different natural habitats. Detection of fungi from soil and plant tissues using immunological techniques have been elucidated by Chakraborty and Chakraborty (2002).

The present investigation on evaluation of *M. anisopliae* and *B. bassiana* as microbial insecticides to control termites provided positive results. *In vitro* and field study support the conclusion that the entomopathogenic fungi - *M. anisopliae* and *B. bassiana* may form effective biological agents for the management of termites in agro forest eco-systems.

SUMMARY

1. A review of literature pertaining to this investigation has been presented which deals mainly on termites and entomopathogenic fungi

2. Materials used in this investigation and experimental procedures followed have been discussed in detail.

3. *B. bassiana* strains 135,984, 2028, 1216 and *M. anisopliae* strains 140 and 892 were grown initially on four different culture media : yeast extract-glucose-agar (YGA) , potato-dextrose agar (PDA), potato-carrot-agar (PCA), and beef extract-agar (BA). Since maximum mycelial growth and sporulation took place in PDA and YGA media, these two media were selected for further experiments. Experimental results showed that 28-day-old cultures of *B. bassiana* isolate 2028 produced highest conidial concentration while *M. anisopliae* isolate 140 registered lowest conidial concentration. To determine the compatibility between the isolates, they were grown together in the same media. Excellent growth was observed between *B. bassiana* isolates 2028 and 984; 1216 and 984 and between *B. bassiana* isolate 135 and *M. anisopliae* isolate 140. In this study no antibiosis effect was observed between the isolates.

4. Four isolates of *B.bassiana* and two isolates of *M.anisopliae* were used for haemocytometer counts and transmission measurements from 28-day- old cultures for a period of 12 months.

5. Bioassays were performed with the isolates of *B.bassiana* and *M.anisopliae* to test against the worker termites (*Odontotermes obesus*) under laboratory conditions. All the isolates proved to be pathogenic to the termites at different degrees. *B.bassiana* isolate 2028 gave the lowest

lethal concentration (LC_{50}) of 1.334 x 10^4, 2.818 x 10^4 and 5.012 x 10^4 conidia / ml at bioassay - I, II and III respectively.. The Lethal time for 50% mortality (LT_{50}) was also recorded to be lowest (56.23 hrs, 50.12 hrs and 53.09 hrs respectively) when treated with isolate 2028 of *B.bassiana*.

6. The cumulative percent mortality among workers of *O.obesus* at different intervals was studied using two isolates (135 and 984) of *B.bassiana*. The isolate 135 required 5, 4, 4, 3 and 5 days exposure period to kill 50% of the termite population by 1 x 10^6 conidia / ml concentration from 10, 14, 20, 28 and 34 days old cultures respectively ; while isolate 984 required 5, 4.5, 4, 3 and 5 days exposure period to kill 50% of the termite population with the same conidial concentration and ages of culture.

7. Seven different substrates, such as, bajra, maize, wheat, wheat bran, groundnut, barley and rice bran were used for production of conidia. Different percents of water and sunflower oil were combined with the substrates to increase the conidial production. The highest yield of conidia varied between 6.55 x 10^8 and 1.15 x 10^9 conidia / ml of harvest from the bajra substrate on addition of 8% of oil and maintaining 70-80% moisture. The spore concentration of different isolates was tested for its bioefficiency against termite workers and the result showed that the highest mortality (88.8%) was achieved with *B.bassiana* isolate 2028. Molasses yeast broth was selected as a synthetic medium for mass scale production for all the isolates. Production was highest with *B.bassiana* isolate 2028 followed by isolate 1216. Their bioefficiency were determined against worker termites (*O.obesus*). The mortality was highest (89.6%) with *B.bassiana* isolate 2028.

8. Food preference tests on termites were conducted with different substrates; maize, bajra, Glyricidia powder, rice bran, filter paper and

wheat bran, following two different methods such as ' glass trough test ' and ' four arm glass chamber test '. The test results showed that bajra and Glyricidia powder were the most preferred substrates by the termites. Food deterrence tests were also conducted by the methods of ' glass trough test ' and 'four arm glass chamber test' . The isolates of *B.bassiana* and *M.anisopliae* mixed with substrates deterred the termites. The highest and the lowest deterrence were provided by the *B. bassiana* isolates 984 and 135 respectively.

9. A study on the effect of soil factors on the pathogenicity of *B. bassiana* and *M. anisopliae* isolates showed that there was no significant difference in the mortality of the termites (*O. obesus*) on acid and alkaline soil treated with the isolates. Under higher (60 and 90%) and lower (15 and 30%) moisture content, 57.67 - 86.33% and 10-29.67 % mortality was observed respectively.

10. Different types of formulations such as dust, wettable powder and liquid solutions of the entomopathogenic fungi were prepared and their efficacy against the termites were determined. Among dust formulations, clay formulation gave the highest percentage of mortality (75.3%). Among the wettable powder formulations, Dedenol recorded highest mortality (80%). Whereas in liquid formulations, Tween 80 showed highest percentage of mortality (90.7%). Certain adjuvants and UV protectants were also applied in the formulations to enhance their efficacy. With the addition of 0.2% sunflower oil in the formulations and their application, mortality of termites increased up to 92.7 percentage. A field trial on tea bushes was conducted to evaluate the efficacy of the liquid formulation alone and in different combinations with chlorpyriphos against live wood termites. Chlorpyriphos alone could reduce the termite infestations on the tea bushes within 3 weeks while the liquid formulation could do the same within 6-7 weeks time.

11. For the suppression of subterranean termites population in field, bait application was undertaken which showed that the population of *O. obesus* in colony-I reduced from 742495 to 102528 after 9 months, that of colony-II from 1266894 to 71880 and that of colony-III from 674025 to 73782 after 7 months of baiting.

12. The formulated mycoinsecticide was also tested for its efficacy against the termites colonies in the plantation area of *Dalbergia sisso, Albizia lebbeck, Michelia champaca* and *Lagerstroemia speciosa*. After the application of the formulated mycoinsecticide mortality of the plants due to termite infestation was not observed.

13. The storage life for the formulated strains of *B. bassiana* and *M. anisopliae* was measured by comparing the number of conidia sporulated after 6, 12 and 18 months of storage at $4^{o}C$. With the increase of storage period, the conidial concentration decreased gradually from 1.8×10^{8} to 1.9×10^{5} conidia / ml within 18 months of storage of the formulated isolate 2028 of *B.bassiana*. Isolate 140 of *M.anisopliae* showed the highest reduction of conidial concentration within 18 months of storage which is about 1.5×10^{8} to 1.1×10^{4} conidia / ml. Pathogenicity of fresh and stored formulated isolates of *B. bassiana* and *M. anisopliae* against *O. obesus* showed that percentage mortality was higher with the fresh formulated strains compared to stored ones.

14. The effectiveness of antigen preparation from the mycelia of *M.anisopliae* (isolate 892) and *B. bassiana* (isolate 2028) in raising

antibodies was checked following agar gel diffusion technique. Strong precipitin bands were observed when PAbs of *M.anisophiae* (892) and *B. bassiana* (2028) were reacted against its own antigens. However, weak or no precipitin bands could be observed in cross reactions between PAbs and antigens of other isolates.

15. Optimization of antigen and antibody concentrations were done using DAC-ELISA formats. Cross reactivity of PAbs of *M.anisopliae* (892) and *B.bassiana* (2028) was tested against mycelial antigens prepared from isolates of entomopathogenic fungi as well as other soil fungi . Maximum absorbance values were observed in homologous reactions.

16. Immunofluorescence tests were performed with the mycelia and spores of *M. anisopliae* (892) and *B. bassiana* (2028). Bright fluorescence was evident on young hyphae and conidia of both the isolates.

17. *M. anisopliae* (892) and *B. bassiana* (2028) from soil were also detected using dot-blot technique.

18. Molecular probing of different antigens with PAb raised against mycelial antigen of *M. anisopliae* isolate 892 was performed through Western blotting technique. Homologous antigen of *M. anisopliae* (892) exhibited 6 bands with molecular weights between 55-16 KDa. Amended soil antigen of *M. anisopliae* (892) was also probed with PAb of *M. anisopliae* (892) where four bands similar to homologous antigen were found.

Based on our present study it is apparent that the entomopathogenic fungi *M. anisopliae* and *B. bassiana* can form effective biological agents for the control of termites.

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