Potential of entomopathogenic fungi for the control of *Macrotermes subhyalinus* (Isoptera: Termitidae)

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Abstract

In Ethiopia, particularly in Eastern and Western Wellega, termites pose a serious threat to agricultural and horticultural crops, forestry trees, rangeland and wooden structures. *Macrotermes subhyalinus* Rambur (Isoptera: Termitidae) is the dominant termite species of these areas. The potential of the two entomogenous fungi *Metarhizium anisopliae* (Metsch.) Sorok. and *Beauveria bassiana* (Bals.) (both Hyphomycetes) for the control of *M. subhyalinus* is evaluated.

A total of 120 samples of soil, insect cadavers that might have died because of a pathogen infection and termite-associated materials from outer and inner parts of termite mounds were collected in Zeway Eastern Shoa zone, Mendi and Gimbi in the Western Wellega zone in the Oromia regional state. Only a single *M. anisopliae* and one *B. bassiana* isolates were obtained from termite cadavers and termite mound material. These two isolates, two other *M. anisopliae* and three *B. bassiana* isolates from Ethiopia, nine *M. anisopliae* isolates from Benin and additionally two standard *M. anisopliae* isolates were screened for relative pathogenicity against *M. subhyalinus* workers in Petri dishes. The median survival time (MST) and the percentage sporulating cadavers were compared in laboratory trials both in Ethiopia and at the Benin station of the International Institute of Tropical Agriculture (IITA), Cotonou, Benin. All isolates were found to be pathogenic to *M. subhyalinus* workers and caused mycosis. In general, the Ethiopian *M. anisopliae* isolates were more virulent and produced a higher proportion of mycosised cadavers than the *B. bassiana* isolates. The Ethiopian *M. anisopliae* isolates were as virulent as the standard isolates. In laboratory screening tests using the Ethiopian isolates, the MSTs of treated termites ranged from 2.03 to 2.38 days in Ethiopia. In Benin, the MSTs of termites treated with both the Ethiopian and IITA isolates ranged from 3.63 to 8.67 days. The Ethiopian *M. anisopliae* isolate MM caused the shortest MST in termites (2.03 and 3.63 days), followed by *M. anisopliae* isolates TT (3.95), EE (2.24 and 4.41 days), and *B. bassiana* isolate FF (2.29 and 4.88 days) both in Ethiopia and Benin. Percentage sporulating cadavers of all termites treated ranged from 70 to 100% in the experiments in Ethiopia and from 1.7 to 75% during the trials in Benin. Some of the *M. anisopliae* and *B. bassiana* isolates, including the two standard isolates were further assessed at different dose rates. When assessing the time-dose relationship, MSTs of termites treated with different dose rates of all the *M. anisopliae* and *B. bassiana* isolates were generally lower than in non-treated termites. Modelling of time-dose-mortality data for the different *M. anisopliae* and *B. bassiana* isolates tested was carried out using Cox regression models. Cox regression models were found to be very useful in describing termite mortality as a function of time and dose rates. Plots of baseline cumulative hazard and LD_{50} values over time, plots of modelled and observed cumulative survival values of the different dose rates over time, the B parameter estimates, and MSTs in termites at average dose rates were used to compare the effectiveness between the fungal isolates. In general, Cox regression models provided a rather poor fit for the fungal isolates in the bioassays where the control survival was very low. Good fits of the time-, and dose-survival relationship were obtained for the different isolates in the bioassays where the control survival was high. Across all experiments Cox regression fits for the isolates which caused shorter MST at average dose in termites (ICIPE30, MM, TT, FF and EE), resulted in moderately steep ($h_{(0)}(t)$) baseline cumulative hazard curves and moderately high B values. Therefore, *M. anisopliae* isolates ICIPE30, MM were found to be the most virulent isolates tested against *M. subhyalinus*, followed by *B. bassiana* isolate FF and *M. anisopliae* isolate EE. Isolates that had B-values ranging from 0.362 to 0.461, i.e. isolates ICIPE30, FI23, MM and EE, had relatively lower

LD_{50} values at the beginning and in the following days of the experiments. The proportion of termite cadavers sporulating positively correlated with dose rate when data were pooled across isolates. In those bioassays where treated termites showed a clear dose response in survival, termites treated with 10^7 spores/ml percentage sporulating cadavers was significantly higher compared to the 10^6, 10^5 and 10^4 spores/ml application. The isolates were tested for their cross contamination potential under laboratory conditions. Termites contaminated with spore dust or spore suspension (10^7 spores/ml) were capable of transmitting the pathogen to other colony members. Comparatively larger groups of healthy termites exposed to termites contaminated with spore dust died significantly faster than groups of termites consisting of smaller numbers of individuals exposed to termites contaminated with spore suspension. In the cross contamination tests, the *M. anisopliae* isolates killed termites faster than the *B. bassiana* isolate. The *M. anisopliae* isolate MM caused the shortest MSTs in termites when used both as suspension (4.34 days) or as spore dust (2.50 days) against larger groups. This was also the case when spore dust was used against smaller groups of termites (3.6 days). The percentage sporulating cadavers ranged from 16.9 to 64.7% across all cross contamination tests. In the laboratory repellence tests using spore paste and dust of all tested *M. anisopliae* and *B. bassiana* isolates, there were no significant interactions between the different isolates and the observation period in all the repellency tests. Generally the variability of the distribution between the repetitions was very high, resulting in large standard errors. Using spore paste and dust of all tested *M. anisopliae* and *B. bassiana* isolates, significantly more termites visited Petri dishes containing the spore paste (only up to 75 minutes after the start of the experiment) or dust (200 and 225 minutes after the start of the experiment) of isolate ICIPE30 than Petri dishes containing the spore paste or dust of isolate MM and the Petri dishes without spore paste or dust. However, when spore paste of isolates ICIPE30 and FF was compared no significant difference in termites visiting treated and control Petri dishes through out the observation period was observed. On the contrary, in the choice tests where MM and FF spore paste were compared, termites were avoiding spore paste of MM and FF and stayed at significantly larger numbers in the control Petri dish. As a result termite behaviour towards spores of different fungal isolates in different formulations, or the mound material, used as substrate, or the fungal comb, provided as a potential attractant, is not clear. The Ethiopian *M. anisopliae* isolate MM produced rather large amounts of conidia both on host cadavers (0.95×10^9spores/ cadaver) and on the two different artificial media tested, i.e. Sabouraud dextrose agar (SDA) (5.7×10^9 spores/Petri dish) and rice (2.8×10^9 spores/g). However, no differences to the standard isolate ICIPE30 (0.88×10^9 spores/cadaver, 2.15×10^9 spores/Petri dish on SDA, 3.0×10^9 spores/g on rice) and the *M. anisopliae* isolate EE (1.30×10^9 spores/cadaver, 2.10×10^9 spores/Petri dish on SDA, 1.30×10^9 spores/g ON RICE) were recorded. The *M. anisopliae* isolate MM was selected for further field studies based on its overall performance in the preliminary pathogenicity test, in the dose-mortality relationship assessment study and the cross contamination test. In addition, MM sporulated well on the two artificial media and on termite cadavers.

In field tests, dressing of Eucalyptus pickets with oil suspension of the *M. anisopliae* isolate MM yielded no protection or repellent effect on *M. subhyalinus* workers. Termites attacked treated pickets that were placed on termite mounds as much as non-treated pickets. Spores of *M. anisopliae* isolate MM triggered epizootics when introduced into field colonies of *M. subhyalinus* at Lekemt Zuria and Sasiga, in the Eastern Wellega zone, Oromia regional state. Disease impact was quantified in terms of a possible reduction in termite activity, which was monitored by measuring the efficiency of termite colonies in sealing artificial holes in the termite mounds and termite foraging activity on Eucalyptus

pickets placed on treated mounds. Neither hole sealing nor foraging activity in three termite colonies at each spore dose starting 15 days after application were observed. These mounds were found dead 75 days after the application. Thus, it was possible to cause a reduction in termite activity, indicating ongoing epizootics in the remaining treated colonies. The percentage termite mound soil volume replaced by *M. subhyalinus* workers in mounds treated with different doses of isolate MM was significantly different from untreated mounds when comparing data over the whole observation period in Lekemt Zuria. In Lekemt Zuria the time effect was significant, too ($df=5$; $F=27.7$; $p<0.001$). Additionally, a significant interaction between time and treatment was observed ($df=15$; $F=3.9$; $p<0.05$). Results in Sasiga were similar, (Time effect: $df=3$; $F=21.5$; $p<0.001$; Time - treatment - interaction: $df=6$; $F=5.7$; $p<0.01$). In general, artificial hole sealing activity of termites in the treated colonies was reduced, starting from 15-30 days after the *M. anisopliae* application. The effect of the disease persisted and affected termites' foraging activity, starting from day 60 after application. There was no difference between the two application methods. Mounds treated through ventilation holes using a bicycle pump did not respond differently to spore doses (4.6, 9.3, and 20g spores/mound). However, in colonies that received manual treatment, mounds treated with 20g spores/mound, the volume of the replaced soil was significantly smaller compared to mounds treated with 4.6g spores/mound and the control mound at day 45 and 75 after treatment while comparing the data on daily basis. The percentage termite mound soil volume replaced by *M. subhyalinus* workers in mounds treated with different doses of isolate MM was significantly different from untreated mounds, with the high dose being significantly different from the low and medium dose, when comparing data over the whole observation period. The time effect was significant, too ($df=4$; $F=6.4$; $p<0.01$). There was no significant time-treatment interaction ($df=12$; $F=1$; $p>0.05$) There was no significant difference between percentage weight losses in pickets placed on mounds treated with different doses of isolate MM using bicycle pump or non treated when comparing data over the whole observation period. Changes over time, however, were significant ($df=4$; $F=20.1$; $p<0.001$). The time-treatment interaction was significant ($df=12$; $F=2.7$; $p<0.01$) Foraging activity of termites was significantly lower in mounds treated with 20g spores/mound compared to mounds treated with 4.6g spores/mound and the control mound starting from day 60 after treatment. The evaluation of the efficacy of *M. anisopliae* against termites in the field by monitoring its effect on the different activities of termites proved to be feasible for quantifying the impact of the pathogen on its host. Results from these studies indicate that both *M. anisopliae* and *B. bassiana* have a high potential as biocontrol agents for the control of *M. subhyalinus* under field conditions.

Key words: Termite, entomopathogenic fungi, biocontrol

Zusammenfassung

Termiten verursachen in Äthiopien, besonders in Ost- und West-Wellega, große Schäden an landwirtschaftlichen, gartenbaulichen und forstlichen Kulturen, im Weideland und an Holzkonstruktionen wie z.B. Gebäuden. Die dominierende Art ist *Macrotermes subhyalinus* Rambur (Isoptera: Termitidae), eine der pilzzüchtenden und hügelbauenden höheren Termitenarten.

Bisher wurden Termiten in Äthiopien hauptsächlich mit synthetischen Insektiziden bekämpft. Die vorliegende Arbeit befasst sich mit der Selektion und Prüfung von Stämmen der beiden entomopathogenen Pilze *Metarhizium anisopliae* (Metsch.) Sorok. und *Beauveria bassiana* (Bals.) (beides Hyphomycetes) zur Bekämpfung von *M. subhyalinus* in Labor- und Felduntersuchungen.

Insgesamt 120 Proben von Bodenmaterial, Insektenkadavern, sowie Material aus dem inneren und äußeren Bereichen von Termitenbauten wurden in Äthiopien gesammelt. Von allen Proben wurden nur zwei, ein *M. anisopliae*- und ein *B. bassiana*-Stamm isoliert. Die beiden Stämme wurden mit zwei anderen *M. anisopliae*- und drei *B. bassiana*-Stämmen aus Äthiopien und neun *M. anisopliae*-Stämmen aus Benin verglichen. Die Mediane der Zeiten, die 50% der Termiten nach der Behandlung mit unterschiedlichen Dosen der verschiedenen Stämme überlebten, wurde in Laboruntersuchungen verglichen. Einige äthiopische Stämme zeichneten sich durch eine besonders hohe Virulenz aus und resultierten in einem höheren Anteil von verpilzten Kadavern als die Beniner Stämme. Allerdings erwies sich keiner der untersuchten Stämme als virulenter oder verursachte einen höheren Anteil an verpilzten Kadavern als der *M. anisopliae*-Stamm ICIPE30, der als Standardisolat mitverglichen wurde.

Im Labor zeigte sich des weiteren, daß die virulenteren Stämme auch ein größeres Potential zur Verbreitung durch infizierte Termiten haben. Laborversuche, in denen eine mögliche abschreckende (repellente) Wirkung der verschiedenen Stämme untersucht und verglichen wurde, verliefen nicht sehr erfolgreich. Höchstwahrscheinlich waren methodische Probleme in der Versuchsanordnung ursächlich für das Scheitern dieser Untersuchungen verantwortlich. In einem Feldversuch in Äthiopien konnte die Wirksamkeit von *M. anisopliae*-Sporen zur Bekämpfung von Termitenhügeln demonstriert werden. Nach einer Applikation von 20 g *M. anisopliae*-Sporen pro Termitenhügel durch Einblasen in die Belüftungslöcher der Hügel konnte eine deutliche Reduktion der Fraß- und Reparaturaktivität der behandelten Termitenkolonien beobachtet werden. Einige der behandelten Termitenkolonien starben innerhalb von 75 Tagen. Die Behandlung von Eukalyptusstöcken mit verschiedenen Formulierungen von Sporen eines *M. anisopliae*-Stammes führte nicht zu den erwarteten geringeren Fraßschäden durch Termiten, nachdem die Stöcke für drei Monate in das Erdreich gesteckt worden waren. Demnach konnte weder eine Schutz- noch eine abschreckende (repellente) Wirkung der Pilzbehandlung nachgewiesen werden. Ergebnisse der vorliegenden Arbeit belegen, daß entomopathogene Pilze erfolgreich zur Bekämpfung von Termiten eingesetzt werden können. Bis zur vollständigen Entwicklung eines Myko-Insektizids auf der Basis eines *M. anisopliae*-Stammes bedarf es aber noch weitergehender Untersuchungen. Eine Schlüsselrolle spielt vor allem die wirtschaftliche Rentabilität einer solchen Behandlung, die hauptsächlich von den Produktionskosten des Myko-Insektizids abhängt.

Schlagwörter: Termiten, entomopathogenen Pilze, Biokontrolle

AAU	Addis Ababa University
ANOVA	Analysis of variance
ATSDR	Agency for Toxic Substance and Disease Registry
CABI	Commonwealth Agricultural Bureaux
CSIRO	Commonwealth Scientific and Industrial Research Organization
EFRANET	East African Loop of BioNet-International
EPN	Entomopathogenic nematodes
FAO	Food and Agriculture Organization of the United Nations
ICRA	International Cooperation on Agricultural Research
ICIPE	International Centre for Insect Physiology and Ecology
IITA	International Institute of Tropical Agriculture
LUBILOSA	Lutte Biologique contre les Locustes et Sauteriaux
MST	Median Survival Time
MoA	Ministry Of Agriculture
NMSA	National Meteorology Service Authority
OADB	Oromia Agricultural Development Bureau
RUP	Restricted Use Products
USEPA	United States Environmental Protection Agency

1 General Introduction

Termites are social insects that belong to the order Isoptera, which means equal winged. Winged individuals (alates) have two pairs of wings, which are similar in size and shape. Termites live in colonies consisting of a few thousands to several million individuals.

1.1 Termites

1.1.1 Termite taxonomy

Termites can be separated taxonomically using different features: external morphology, internal features, food and nest type, chemical and behavioural differences (Pearce, 1997). There are over 2,500 species of termites known today (Pearce, 1997). Wood and Johnson (1986) revised earlier works on classification of termites and classified known termite species into seven families, namely: Mastotermitidae, Kolotermitidae, Termopsidae, Hodotermitidae, Rhinotermitidae, Serritermitidae, and Temitidae. These seven families may be divided into two main groups, the ‘lower’ and 'higher termites' depending on their mode of digestion. The first six families are collectively known as ‘lower termites’ and rely on protozoan symbionts for digestion. The seventh family, Termitidae, represents over 80% of all termite genera and 74% of all termite species (Wood & Johnson, 1986). Termitidae are often referred to as ‘higher termites’ because of their advanced social behaviour (Bignell *et al.*, 1983). Their major characteristics are lack of symbiotic intestinal protozoa, large colonies (100,000 to millions) and the presence of a worker caste that has completed its development (Sands, 1977a), i.e. workers do not further develop into reproductive individuals or soldiers.

Termites can be also divided into wood and/or litter feeding, fungus growing, humus and soil feeding types (Pearce, 1997). Depending where their nests are found they are also divided into dry wood, damp wood and subterranean termites (Pearce, 1997). Dry wood termites are always found inside wood, but damp wood and subterranean termites can also be found in the soil. The shape and position of the nest can vary from species to species and also between the same species in different habitats. Different species of the same genus have different swarming periods, which prevents interbreeding, and can be used to distinguish these species (Wood, 1981).

1.1.2 Caste system

Termite colony consists of workers, pseudoworkers, soldiers, reproductive individuals, larvae and eggs. In certain Termitidae there are two types of workers, major (large) and minor (small). Size may also be linked to sex. One example is *Macrotermes michaelseni* (Sjöstedt) (Isoptera: Termitidae), where minor workers are female, and major workers are male (Pearce, 1997). In higher termites the workers are sterile and incapable of further development. However, in lower termites the worker cast has not reached its final stage of development and can further develop into reproductive individuals or soldiers. In lower termites these immature individuals, known as pseudogates or pseudoworkers, are playing the role of the worker cast in higher termites.

The function of the worker cast includes foraging for food, feeding and cleaning individuals of other casts, or building and repairing the nests. In the Macrotermitinae the workers are also responsible for growing fungus gardens (Wood & Johnson, 1986) and these tunnelling and food collecting activities of termite workers render some of these species to pests.

Soldiers are the second numerous caste in many termite species. Like workers they are sterile, wingless and in most species blind. They possess a hard large and solid head capsule to defend the colony against predators and parasitoids. Soldiers of differing size are found in some species, e.g. major and minor soldiers are common in some Termitinae. In most Macrotermitinae, the soldiers are female. Some termite species may have no soldiers at all (Wood & Johnson, 1986). Alates may vary from 9-90 mm in body length (Harris, 1971). There are two types of reproductives, i.e. primary and secondary reproductives. The primary reproductives are the queen and the king. They develop from winged alates after having left a nest paired as male and female, shed their wings and having established a colony. Generally a single pair of primary reproductives is found in a colony. However, in certain termite species multiple primary reproductives can be found (polygony) (Darlington, 1985). In termite species that have large colonies, the queen's abdomen grows to accumulate large numbers of developing eggs. Such growth reaches its maximum in the genus *Macrotermes*, where a single queen can produce 30,000-40,000 eggs per day and up to 10 million a year (Harris, 1971). The size of the king generally is about a fifth of the length of the queen, with an abdomen a hundred times smaller.

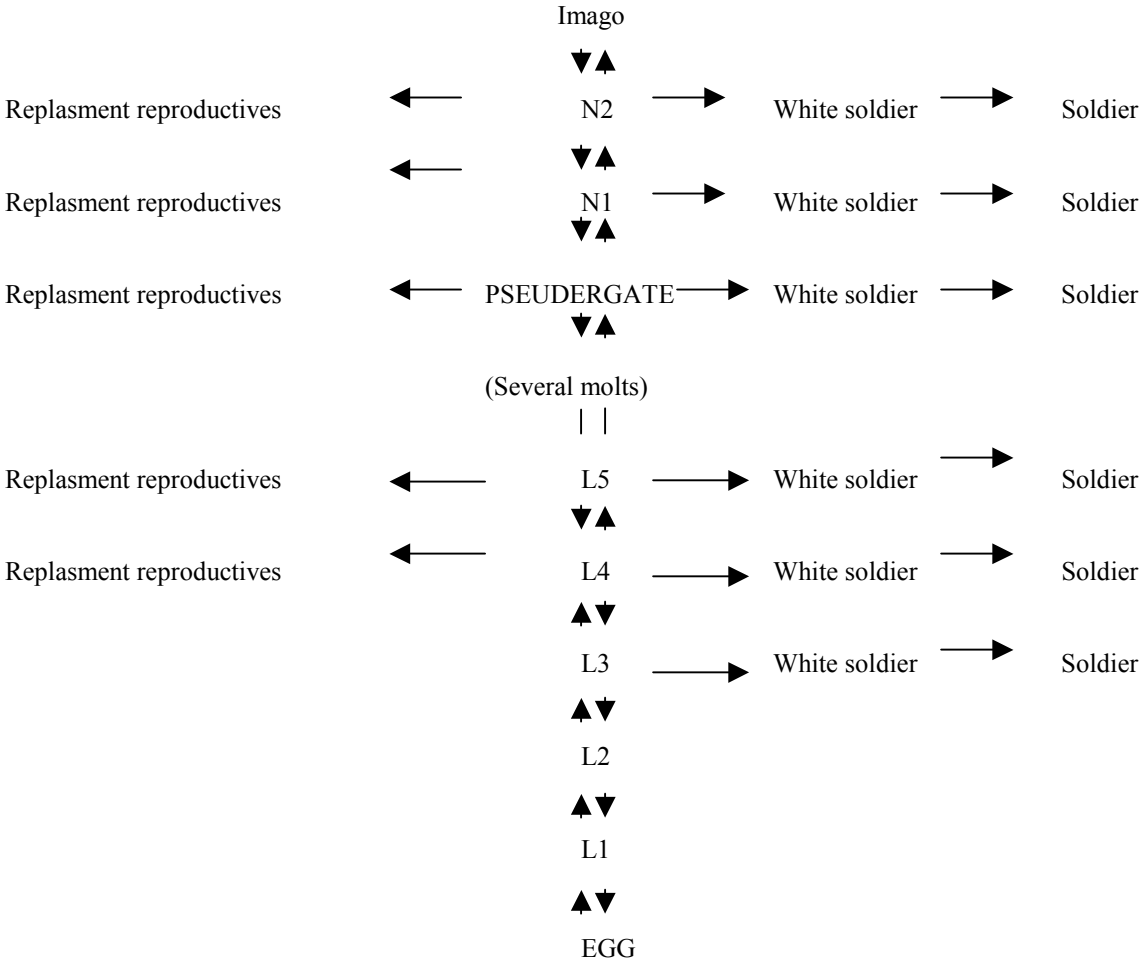


Figure 1 Diagramatic representation of the cast development of the 'lower termite' *Kalotermes flavicollis*. L=larva; N= nymph (Wood & Johnson; 1986).

An additional primary reproductive develops when either the king or the queen dies or the egg laying capacity of the queen drops (Harris, 1971). The immature individuals can develop into any of the castes depending upon the requirements of the colony (Okot-Kotber, 1985). Whether an immature develops into a worker, soldier, or into a primary reproductive is not determined by genetic factors but rather by pheromones and hormones (Okot-Kotber, 1985).

The proportion of the different castes varies depending on the species and the age of the colony. The workers are the most numerous castes in all colonies. For instance the mean percentage of different castes recorded in mature *Macrotermes bellicosus* (Smeathman) (Isoptera: Termitidae) colonies is 1.6% major soldiers, 2.4% minor soldiers, 35.6% major workers and 60.5% minor workers (Bagine *et al*., 1989).

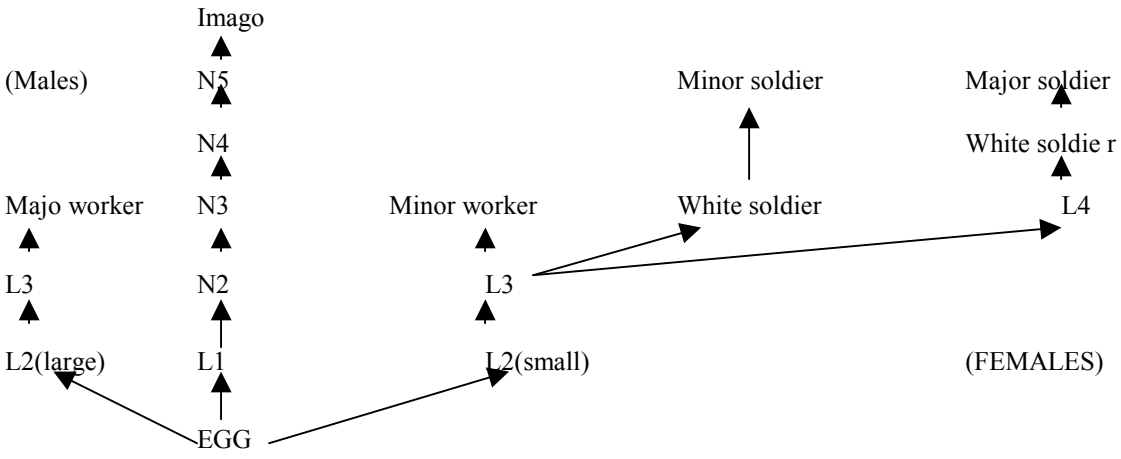


Figure 2 Diagramatic representation of the cast development of the 'higher termite' *Macrotermes michaelseni*. L=larva; N= nymph (Wood & Johnson; 1986).

1.1.3 Geographical distribution

Termite density and the number of termite species increase as one moves towards the equator. Termite distribution can be related to temperature and rainfall. Their distribution limit lies between a latitude of 45º and 50º in the north and south, respectively. Termites have been found in all types of soils except in semi-permanently water logged areas and in certain deeply cracking vertisols (Wood, 1988). The diversity of termites increases close to the equator and at lower altitudes, except in Australia where an equal numbers of termite species are found both north and south of the Tropic of Capricorn (Wood & Sands, 1977). The Macrotermitinae are found mainly in the Middle East and Asia and include several important pest genera (Pearce, 1997). They are absent in Central America and Australia (Pearce, 1997).

1.1.4 Biology and ecology of *M. subhyalinus* Rambur (Isoptera:Temitidea)

Marotermes subhyalinus belongs to the higher termite family Termitidae and to the subfamily Macrotermitinae. *Marotermes subhyalinus* is morphologically very similar to *M. michaelseni* (Bagine *et al*., 1989). According to Bagine *et al*. (1994) the taxonomic status of *M. subhyalinus* and *M. michaelseni* has not yet been fully resolved, with open mounds being attributed to *M. subhyalinus* and closed mounds to *M. michaelseni.* Attempts have been made to discriminate between individuals from the two species by analysing epicuticular hydrocarbons from workers and through electrophoretic analysis of macerated fresh soldiers (Bagine *et al*., 1989, 1994).

The Macrotermitinae cultivate fungus gardens of the Basidiomycete *Termitomyces* spp. in a comb like structure in the centre of their nest (Sands, 1956). The food consumed by the workers passes through the gut relatively unchanged and is used for the construction of a fungus comb on which *Termitomyces* spp. are grown. It is suspected that the first foraging workers of a colony in most of the Macrotermitinae, including *M. subhyalinus*, carry basidiospores of *Termitomyces* spp. into the nest (Thomas, 1981). The fungus breaks down lignin and cellulose and also produces spores, which are ingested by the termites. Thomas (1981) has shown that *Termitomyces* spp. degrades nitrogen-poor substrates and consequently provides termites with a relatively nitrogen rich food. According to Abo-Khatwa (1977) the fungi provide termites with vitamins and nitrogen. The fungus comb consists of previously only partly digested food and when ingested by termites again, further digestion occurs in the gut by enzymes such as cellulase and β-glucosidase produced partly by the ingested fungus and partly by the midget epithelium and salivary glands of the termites themselves (Wood & Thomas, 1989).

As in most termite species of the Macrotermitinae, the *M. subhyalinus* caste system consists of major and minor workers, and soldiers. Young nests of *M. subhyalinus* contain more than 80% larvae, and out of the 20% adults, 55-89% are workers and 11-44% are soldiers, while mature nests have more than 74% adults which consist of 74-96% workers and 3-25% soldiers (Darlington, 1984b). The weight of the queen and the fungus comb in mature nests is almost 8 and 12 times higher, respectively, compared to young nests (Darlington, 1984b). Minor (female) workers are responsible for feeding and tending the immatures and reproductives, while foraging is done by major (male) workers (Darlington, 1984b; Pearce, 1997).

In western Ethiopia, swarming of alates of *M. subhyalinus* occurs at the beginning of the rainy season between March and early June, always from 7:00 to 7:30 pm (Abdurahman, 1990). According to Abdurahman (1990), *M. subhyalinus* forages throughout the year and after rains close to the nest. Only during the dry season, between December and March, their activity extends to areas further away from the nest.

Marotermes subhyalinus builds dome-shaped epigeous mounds with a density ranging from 10-12 mounds per ha in western Ethiopia (Abdurahman, 1990). The density of *Macrotermes* spp. mounds reported per ha in the region ranged between 2-3 (Sands,

1976b) and 305 (Sanna, 1973). In Kajiado, Kenya, 8.5 and 9 mounds per ha were recorded for *M. subhyalinus* and *M. michaelseni*, respectively (Pomeroy, 1983).

1.1.5 Economic significance

1.1.5.1 The benefit of termites

Termites are eaten in many parts of the world. Termites are rich in calories and a very good source of proteins (Pearce, 1997). The mushrooms growing out of termite mounds are also consumed by men. Termites affect the soil profile by disturbing it and by redistributing the organic matter (Lee & Wood, 1971; Wood & Sands, 1977). As there are few earthworms in Africa, termites are very important in recycling and regenerating the soil matter (Pearce, 1997). Kooyman and Onck (1987) studied the interactions between termite activity and soil characteristics in the Kisii district in Kenya and concluded that, 30-90% of the litter on the fields is removed by termites. However, return of the nutrients to the soil is obstructed since most of the litter removed is used by the termites themselves for constructing the fungus combs. The part of the nutrients which are added to the soil in the form of saliva and faeces during the construction of mounds and sheetings, and which ultimately enriches the topsoil, is small in comparison with the average uptake of nutrients by annual crops. Kooyman and Onck (1987) further stated that the excavation of tunnels by termites mainly enhances the aeration and rootability of the soil; the formation of the soil matrix composed of pellets appears to determine its moisture characteristics and its aggregated structure. Termites may be important in reclamation of landfill sites, and areas where large amount of rubbish have been deposited (Pearce, 1997).

1.1.5.2 Termites as pests

Of the few thousands species of termites that exist only a few hundreds cause economic damage. Among them around 50 species are considered to be serious pests (Pearce, 1997). This proportion is considered high compared to other social insects (Sands, 1977b). The Rhinotermitidae and Macrotemitinae contain most of the important pest species. The Rhinotermitidae (especially *Reticulitermes* spp. and *Coptotermes* spp.) are major pests in America, Europe and Asia (Pearce, 1997). The subfamily Macrotermitinae (especially the genera *Mocrotermes*, *Odontotermes* and *Microtermes*) contains the most important pests that can cause considerable damage to agricultural crops, forest trees, buildings and wooden structures in Africa and Asia (Pearce, 1997). In general, termites become a pest when humans alter their natural habitat (Pearce, 1997).

Harris (1966, 1969, 1971), Bigger (1966) and Wood *et al*. (1980) reviewed extensively crop damage by termites for various countries, including several countries in Africa. Losses in annual crops ranged from 5 to 50% in maize (Bigger, 1966; Johnson & Wood, 1979; Wood, 1986a), 6 to 25% in wheat (SenSerma, 1974; Johnson & Wood, 1979), 20 to 51% in groundnut (Johnson *et al*., 1981; Sudhakar & Veersh, 1985; Johnson & Wood, 1979;Wood, 1986a) and 10 to 31% in pepper, okra and tomato (Badawi *et al.,* 1984; Wood *et al*., 1987). In Ibadan, Nigeria, 29% loss of stands with 20-51% lodging was reported in maize (IITA, 1972). Yams and cassava are fairly consistently attacked as cuttings in West Africa, with losses of 70% and 40%, respectively, of stands recorded (Johnson & Wood, 1979).

Overgrazing increases the population density of grass feeding termites in South Africa, and damage ranged from 20% to 100% in a dry year (Coaton, 1950). Also Sands (1977a) considers termites as an economic pest of grazing land during drought periods. However, in areas where recommended animal stocking is followed, termites feed on grass litter and competition with livestock is insignificant (Collins, 1984).

Termites are important pests in tropical forestry, especially in exotic forestry tree plantations. In India, Nigeria and Guinea damage is particularly serious on Eucalyptus seedlings during the first three years after transplanting (Nair & Verma, 1981; FAO, 1985; Cowie *et al*., 1989; Harris, 1971; Thaker, 1977). In Kenya, tree seedling losses of 40% have been reported in *Callitris preissii* Miq (Cupressaceae) and *Eucalyptus camadulensi*s Dehn (Anonymous, 1985). In Ethiopia, serious damage, with losses of up to 100% in some localities, occurred on Eucalyptus one to two years after transplanting (Abdurahman, 1990).

Several species of termites are responsible for the destruction of buildings in the tropics and subtropics. They have started spreading to warmer temperate regions because of changes in building designs that create favourable environmental conditions for termites (Johnson, 1981b). In the rural areas of developing countries houses are built from grasses and wood without a proper foundation. Such structures are susceptible to attack because termites can dig through the foundation and mud brick walls. In Africa significant damage on buildings is caused by various species of the genera *Cryptotermes* (Isoptera: Kalotermitidae), *Coptotermes* (Isoptera: Rhinotermitidae), *Macrotermes*, *Odontotermes* and *Microtermes* (both Isoptera: Termitidae) (Harris, 1971). In the US, costs associated

with subterranean termite damage and controls are estimated to approach US$ 2 billion annually (Cullinery & Grace, 2000).

Valuable books, as well as cotton materials can be damaged by termites (Pearce, 1997). Termites often burrow through non-cellulose materials such as buried electrical power cables, railroad signal systems and telephones or telegraph communication circuits (Sternlicht, 1977). They also attack plastics causing leakage in plastic pipes. Termites also build nests in dams and dikes.

1.1.6 The termite problem in Western and Eastern Wellega

In Ethiopia, termites pose a serious threat to agricultural, horticultural crops, forestry trees, rangeland and wooden structures, particularly in Eastern and Western Wellega zone in the Oromia regional state. (Abdurahman, 1990). In these zones farmers and agricultural institutions consider the infestation and spread of termites as a high priority problem (Gauchan *et al*., 1998; Abdurahman, 1990; OADB, 2001). The problem has an impact on the over all socio economics conditions of the area and has caused farmers to abandon their land and migrate to low land areas. The migration of farmers was initially reported by Sanna in 1973 and has continued up to today to the extent of hampering the implementation of extension package programs in the affected areas (Gauchan *et al*., 1998).

In Ethiopia, 61 species of termites belonging to 25 genera and four families, namely, Kalotermitidae, Hodotermitidae, Rhinotermitidae and Termitidae, have been recorded (Cowie *et al*., 1990), out of which 10 species are endemic. About 25% of these species are pests of agricultural crops, forestry seedlings and grazing land (Abdurahman, 1990). Surveys conducted by Abdurahman (1990) indicated, that all the termite species causing crop damage in the region belong to the subfamily Macrotermitinae and the genera *Macrotermes, Microtermes, Odontotermes* and *Pseudacanthotermes*. The survey report further indicated *M. subhyalinus* to be the dominant species in several parts of the region at altitudes below 2,000 m and the only important pest on farmers' fields (Abdurahman, 1990). Other termite species were of minor economic importance. Sands (1976b) suggested that in Ethiopia *Macrotermes* spp. might have been able to spread into new areas following the conversion of a forest ecosystem to grassland.

The crops severely attacked by *M. subhyalinus* include maize, teff (*Eragrostis tef* Zuccagni [Poaceae]), finger millet, pepper, and sugar cane. Losses due to termites range from 45-50%, 50-100% and 25% in maize, teff and sorghum, respectively, in one of the highly termite infested districts of Western Wellega zone (OADB, 2001). Wood (1986b, in OADB, 2001) reported of termite-caused losses of 20% in maize, sorghum and groundnut and 25% losses in pepper.

Termite damage on indigenous trees is insignificant (Abdurahman, 1990; Wood, 1986b). In contrast, serious termite damage is common in exotic forestry trees, especially in Eucalyptus, particularly one to two years after transplanting, reaching up to 100% in some localities (Wood, 1986b; Abdurahman, 1990). Farmers and extension workers attribute the cause of wide spread denudation on grassland in the Eastern and Western Wellega zone to termites. However, Sands (1976b), Cowie and Wood (1989) and the ICRA (International Cooperation on Agriculture) termite team (Gauchan *et al*., 1998) believe that over-grazing by heavy cattle populations to be the primary cause of the termite problem. Moreover, according to the ICAR termite team (Gauchan *et al*., 1998) high population pressure, ecological change and termite infestation are inextricably inter-linked.

Termites also destroy fences, farm buildings, wooden bridge crosses and implements made of wood. Thatched grass roof huts are destroyed after about five years, while corrugated iron sheet roof houses might last about eight years (Abdurahman, 1990).

1.1.7 Control

1.1.7.1 Chemical control

In the past, termite control depended on the use of cyclodiene organochlorine insecticides. Increasing concern over human health and environmental hazards has now resulted in the banning of, or severe restrictions on the use of cyclodienes in many countries (Logan *et al.,* 1991). Research has concentrated on the development of less persistent insecticide alternatives. Pearce (1997) summarized some of the chemical control methods used for termite control (Table 1).

Field experiments have indicated the non-efficacy of pyrethroids against termites attacking maize (Gethi *et al*., 1995; Ochiel *et al*., 1995), cotton (Tiben *et al*., 1990) and tea bushes (Rattan, 1993). Logan *et al*. (1992) considered termite control through releases of insecticide granules to be too expensive for small-scale agricultural field applications for

subsistence farming communities of Africa. Some of the more recently developed chemicals for termite control in agricultural fields are imidacloprid (Storey, 1995) and fipronil (Pearce, 1997). Imidacloprid has been successfully used in combination with entomopathogenic fungi on the eastern subterranean termite *Reticulitermes flavipes* Kollar (Isoptera: Rhinotermitidae) (Zeck, 1992; Almeida & Alves, 1996). Colin and Andrew (1998) studied short-term impact of emergency locust control in Madagascar on non-target organisms and showed devastating negative impact of fipronil on termites. Tingle and McWilliam (1998) believe the ecological implication of this negative impact could be serious, given the importance of termites in nutrient cycling, soil structure and water infiltration, and also as a food source for a range of higher animals. They further suggested that environmentally fipronil could not be considered as the insecticide of choice for locust control, until the ecological implications of its adverse impact on termites' colonies have been precisely determined. An important property of fipronil is its long persistence. Consequently, its long-term impact on non-target termites species after locust control operations was a major reason for restricting its wide spread use.

Doki *et al*. (1984) and Jones (1994) reported 50-80% moulting and non-functional intercaste development in *R. flavipes* and in the formosan subterranean termite *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae) in the laboratory with baits containing insect growth regulators such as fenoxycarb Ro 16-1295. Other growth regulators are hydroprene and methoprene. In laboratory tests in Germany with the chitin-synthesis inhibitors diflubenzuron and triflumuron, termites died 2 to 15 days after ingesting the chemicals; however, diflubenzuron did not pass a screen test, mainly because of lack of efficacy (Edwards & Mill, 1986). Sulfuramid and subterfuge are respiratory inhibitor bait chemicals used for termite control (Ogg, 1998). Schoknecht *et al*. (1994) examined the possibility of using micro-capsulated permethrin in baits for the control of termites exploiting their grooming behaviour and trophallaxis. According to Duncan (1997), a detailed knowledge of the foraging and feeding behaviour of termites is needed before chemical baits can be successfully designed. Pearce (1997) considered baits safer to handle and more 'environmentally friendly' than many liquid insecticides. However, one disadvantage of baits is that they may require several months to take effect, and, hence, there will be some degree of damage before the bait works (Ogg, 1998). The control of termites by destroying fungus combs using fungicide impregnated baits has been shown to work in the laboratory but not in the field (El Bakri *et al*., 1989)

Health and environment concerns have recently been highlighted where chemical pesticides have been used for termite control. Cases of exposure by office workers and applicators in the United States suggest that pesticides will remain a continuous health and environmental hazard even in developed countries (Lessenger, 1992; Wright *et al*., 1991). The United States Environmental Protection Agency (EPA) has started reviewing pesticides for safety and has removed those chemicals that pose an unreasonable threat to human health. Hence, EPA is presently promoting the use of newer, safer alternatives; consequently the use of chloropyrifos as a termiticide for new home and building constructions will be eliminated by the end of 2004 (Browner, 2000).

1.1.7.2 Non-chemical control

Non-chemical techniques include a range of cultural practices, biological control, use of resistant crop/tree varieties and a number of other measures (Logan *et al*., 1990). Gold *et al*. (1991) consider non-chemical control methods as environmentally sound options for farmers.

1.1.7.2.1. Physical and cultural controls

1.1.7.2.1.1 Barriers

Sands, basalt, granite glass splinters or globule and fossilised coral of a special size can be used as barriers to prevent termites' entry to buildings (Tamashiro *et al*., 1991; Su & Scheffrahm, 1992; French, 1993; Grace & Yamamoto, 1993). In Australia, stainless steel is placed under new buildings (Lenz & Runko, 1994). Mixing insecticides with a synthetic polymer and placing one layer of the sheet on the soil under the foundation is used in Japan (Yoshimura & Tsunoda, 1994). However, such types of barriers have yet to be tested in the tropics (Pearce, 1997).

1.1.7.2.1.2 Cultural control

Cultural control practices include the use of good quality seeds, crop rotation, intercropping, early planting on fertile soil, regular irrigation, proper timing of crop maturation, the removal of damaged plants, timely weeding and quick harvesting and the removal of crop residues after harvest (Pearce, 1997). Some of the good silviculture practices include the use of polythene tubes filled with insecticide treated soil or planting

forestry seedlings and planting seedlings with insecticide treated soil around the stems (Pearce, 1997).

Application of nitrogen, phosphor and potassium (NPK) in wheat, barley and yam has been shown to reduce termite incidence both in India and Nigeria (Stapathy *et al*., 1990; Bhanot & Verma, 1990; Atu, 1993). On the contrary, the use of improved varieties and application of fertilizer did not reduce *Microtermes* spp. attack in maize in western Ethiopia (Abdurahman, 1990). Mulches may either increase or decrease termite incidence, depending on whether they have any repellent properties (Gold *et al*., 1989, 1991; Delate & Grace, 1995). Satter *et al*. (1993) in Pakistan concluded that intercropping with alternative food sources for termites might reduce damage in agricultural crops. In forestry, it can also prevent specific termite species from building up to pest status through maintaining high species diversity (Sands, 1977b; Tho, 1974). Finally an early harvest of groundnuts, i.e. between 70 and 90 days after sowing, reduced damage due to termites to less than 40% in Burkina Faso (Lynch *et al*., 1991).

1.1.7.2.2 Plants and plant extracts

Logan *et al*. (1990) and Ochiel (1995) listed 21-75 plant species from 10-37 families that can be used in different ways as botanical insecticides. Mangosan, a commercial preparation of extracts of the neem tree *Azadirachta indica* A. Juss. (Meliaceae), caused significant mortality in *Coptotermes* spp. but lasted only a short time (Grace & Yates, 1992). According to Gold *et al*. (1989), the use of neem as mulches can reduce termite attack in groundnuts. Yet Roa *et al*. (1991) reported no significant effect. Delate and Grace (1995) observed that neem mulches were somewhat deterrent to *Coptotermes* spp. but not to other termite species.

1.1.7.2.3 Plant resistance

In general crops showing a certain termite resistance or tolerance are indigenous, while the susceptible crops are often of exotic origin (Logan *et al*., 1990; Wood, 1986b). For instances in Africa, sorghum, millet, cowpea, and bambara nut are more resistant to termites than maize, groundnut, sugarcane, cotton, and wheat (Cowie & Wood, 1989; Johnson *et al*., 1981). Lee and Wood (1971) and Wood (1978) concluded that it is rarely possible to declare a tree species as generally termite resistant because host plant resistance can vary for different termite species. Out of 41 tree species, several species of the genera

Acacia (Mimosaceae), *Enterlobium* (Mimosaceae) and *Senna* (Capparaceae)showed some resistance to termite attack in Zimbabwe (Mitchell, 1989). Cowie *et al*. (1989) suggested the use of resistant timber as probably the only satisfactory strategy for dry wood termite control.

Table 1 A summary of chemical and non-chemical termite control

Chemical	Uses	Possible disadvantages	Registration status
Organochlorines	SB.SC	Slow breakdown, especially in temperate regions. Harmful to humans and the environment.	Withdrawn for use in most countries, especially as crop protectants.
Aldrin, dieldrin, DDT	W,ST		
Lindane, heptachlor, (endosulfan)			[1]Heptachlor banned in the US, [2]Lindane banned in many countries from all use.
Organophosphates	SB,SC	Low soil persistence, some phytotoxic at soil surface.	
Chlorfenvinphos, chlorpyrifos, fenitrothion, fenthion, isofenphos, phoxim; chlorpyrifos applied as slow release capsules or microcapsules in baits; phoxim is less stable in wet soil.		[5]chlorfenvinphose-dermal mammalian toxicity, [5]chloropyrifose – aquatic and avian toxicity.	[5]chlorophyrifose-will be eliminated as a termiticide for new home- and building constructions by the end of 2004 in the US.

SB = soil treatment building; SC = soil treatment crops and trees; ST = seed treatment; W = wood protection; BT = bait or infective treatment; [1] ATSDR, 1993; [2] Schafer, 2000; [3] USEPA, 2002;[4] Browner, 2000; [5] USEPA, 2001a; [6] USEPA, 2001b

Table 1. contd

Chemical	Uses	Possible disadvantages	Registration status
Pyrethroids	SB,SC	Easily broken down, can be toxic to fish and bees but low mammalian toxicity.	
Cypermethrin, fenvalerate, permethrin, silafluofen.			
Carbamates	SB,SC	Some need moisture for dispersal.	
Aldicarb, bendicarb, carbofuran, carbaryl carbosulfan, propoxur. Carbofuron and carbosulfan produced as slow release compounds for forestry and crops. Carbaryl used in baits for control of grass feeding termites.		[5]carbofuran high avian toxicity.	[3]bendicarb-registration cancelled in the US in 1999,
Toxic gases	Fumigants for nest and timber treatment	High human toxicity. Chloropicrin phytotoxic.	

SB = soil treatment building; SC = soil treatment crops and trees; ST = seed treatment; W = wood protection; BT = bait or infective treatment; [1] ATSDR, 1993; [2] Schafer, 2000; [3] USEPA, 2002;[4] Browner, 2000; [5] USEPA, 2001a; [6] USEPA, 2001b

Table 1. contd.

Chemical	Uses	Possible disadvantages	Registration status
Methyl bromide, phosphine, sulphuryl fluoride, chloropicrin, used to protect grain.		Chloropicrin phytotoxic; methyl bromide has negative effect on ozone layer	[6]Methyl bromide-will phase out in the US in 2005.
Others			
Arsenic compounds.	W	Very toxic.	
Borax dust and borates.	W,BT	Very soluble - leaches out, phytotoxic.	
Flurosilicates	BT		
Mirex	BT		Mirex-use in US cancelled.
Imidachloprid	SB, SC		
Fipronil	SB, SC		
Wood ash	SB, SC		
Plant extracts	W,SC	Unknown effects.	
Hydrophilic grease	Tree wounds		

SB = soil treatment building; SC = soil treatment crops and trees; ST = seed treatment; W = wood protection; BT = bait or infective treatment; [1] ATSDR, 1993; [2] Schafer, 2000; [3] USEPA, 2002;[4] Browner, 2000; [5] USEPA, 2001a; [6] USEPA, 2001b

Table 1. contd.

Chemical	Uses	Possible disadvantages	Registration status
Coal tar, pitch	Cement joins, W	Not always long term.	
Diflubenzuron (chitin inhibitor)	BT		
Hexaflumuron (chitin inhibitor)	BT, SB		
Fenoxycarb, methoprene, hydroprene - increase soldier ratio, also stomach poisons.	BT, SB		
Shields, netting, Steel mesh, particle sizes:	Barriers for new buildings	Often expensive	

SB = soil treatment building; SC = soil treatment crops and trees; ST = seed treatment; W = wood protection; BT = bait or infective treatment; [1] ATSDR, 1993; [2] Schafer, 2000; [3] USEPA, 2002;[4] Browner, 2000; [5] USEPA, 2001a; [6] USEPA, 2001b

1. 1.7.2.4 Biological control

In recent years, the shortcomings of conventional control methods have promoted policy makers and scientists to evaluate the potential of biological control of termites, that is, to determine the potential of natural enemies (predators, parasitoids, pathogens) to suppress termite populations (Culliney & Grace, 2000).

Although termites are an important part of the food chain for many animals such as insects, birds, reptiles and mammals (including man) (Rajagopal, 1984), the impact of natural enemies on termite population dynamics is only partially understood (Wood & Johnson, 1986; Culliney & Grace, 2000). In a review by Ochiel (1995), at least 10 vertebrate and 17 invertebrate natural enemies of termites in Africa were listed. In Kenya, two species of ants prey on *M. michaelseni* (Lepage & Darlington, 1984). Kenn *et al*. (2000) concluded, that under certain conditions the predatory ant *Myrmicaria opaciventris* Emery (Hymenoptera: Formicidae) can be used as a biological control agent against termites. While natural control of termites exists in nature and numerous studies have shown the importance of natural enemies, few have been used so far as biological control agents. This is partly due to the lack of specificity and difficulties in rearing of many natural enemies of termites (Beard, 1973)

For various reasons, pathogenic organisms such as viruses, bacteria, protozoa, nematodes and most fungi have shown little promise for use in biological termite control (Culliney & Grace, 2000). Reports of viruses and protozoa in termites are few and they appear to offer no serious prospects for an effective product development (Milner & Staples, 1995). *Bacillus thuringiensis* Berliner has been shown to cause 75-100% mortality in *R. flavipes, R. virginicus* (Banks), and *R. hesperus* (Banks) (all Isoptera: Rhinotermitidae) in the laboratory (Symthe & Coppel 1965; Stadykov, 1970; Khan *et al*., 1985, Grace & Ewart 1996). However, no field data on *B. thuringiensis* efficacy for termite control is yet available (Milner & Steples, 1995).

Most research on biological control of termites has been focussed on the potential use of entompathogenic nematodes (EPNs) and fungi (Milner & Staples, 1995). A review by Gitonga (1996) on biological control of termites indicates that five EPN species from three families caused 4.8 to 100% mortality in *Reticulitermes* spp. (Isoptera: Rhinotermitidae), *Cryptotermes* spp., (Isoptera: Kalotermitidae) and *Microcerotermes* spp. (Isoptera: Termitidae) in the laboratory (Mauldin & Beal, 1989; Epsky & Capinera, 1988; Wu *et al*.,

1991; Stadykov *et al*., 1973; Khan *et al.,* 1994). In field tests *Steinernema* spp. and *Heterohabditis* spp. provided some limited control of subterranean termites, but have generally not proved successful for long-term suppression (Mauldin & Beal, 1989). Therefore, Maudlin and Beal (1989) concluded that EPNs are not very effective for termite control. Pearce (1997) states that though many hundreds of infective juveniles were released from termite cadavers after infection by a single nematode, chances for re-infestation are generally rather low because of the high mobility of all growth stages of termites. In addition, EPNs need free water to survive (Pearce, 1997). Wang *et al*. (2002) studied the potential of EPNs for control of eastern subterranean termite *R. flavipes* and observed that although nematodes were able to reproduce in termites, EPNs were rarely seen emerging from dead termites. The reason was that mites, associated with *R. flavipes*, often consumed the nematode-killed termites. Moreover, healthy termites frequently ate dead termites, thus preventing the EPNs to reproduce in their hosts. Therefore, the authors concluded that due to the limited mobility of EPNs in termite mounds, mainly because of the walling-off behaviour of termites (Fujii, 1975), and the low rate of reproduction in dead termites, it is rather unlikely that EPNs will reach and maintain a large enough density to eliminate a termite colony in the field.

Fungi are the most promising entomopathogens for the development of a microbiological termiticide (Milner & Staples, 1995). Wide laboratory and field studies have been conducted using entomopathogenic fungi as biological control agents against termites. In his review Gitonga (1996) reports, that fungal species of at least 11 genera from four fungi families, i.e. Deuteromycetes, Zygomyceytes, Ascomyceytes and Mitosporic, have been tested against termites. The tested fungal species belonged to the genera *Metarhizium*, *Beauveria*, *Paecelomyces*, *Aspergillus*, *Verticillium*, *Nomuraea*, *Conidiobolus*, *Basidiobolus*, *Absidia* and *Cordycepioideus*.

Although the natural occurrence of *Cordycepioideus bisoporus* Stifler (Ascomycetes) on *M. subhyalinus* de-alates was reported by Ochiel *et al*., (1995), the fungus proved to be not pathogenic when tested against *M. michaelseni* workers and soldiers. Among the Zygomycetes, *Conidiobolus coronatus* (Costantin) Batko and *C. obscurus* (Hall & Dunn) Remaudiere & Keller have been shown to be pathogenic to *C. formosanus* and *Nasutitermes exitiosus* Hill (Isoptera: Termitidae) (Hanel, 1982b; Ko *et al*., 1982; Yendol & Paschke, 1965; Wells *et al*., 1995). Milner and Staples (1996) suggested that *C. coronatus* is unlikely to be considered for commercialisation because of safety and mass production problems.

Among the Deutromycetes tested against termites, *Verticillium indicum* (Petch) Gams and *V. lecanii* (Zimmermann) Viegas were more virulent to *Cryptotermes brevis* Walke (Isoptera: Rhinotermitidae) and *Odontotermes brunneus* Hagen (Isoptera: Termitidae) than *Metarhizium anisopliae* (Metsch) Sorok (Hyphomycetes) (Nasr & Moein, 1997; Khan, 1991). *Aspergillus niger* Tiegh. and *Paecilomyces furmosoroseus* (Wize) Brown & Smith (both Hyphomycetes) caused 100% mortality to both *C. formosanus* and *Reticulitermes speratus* (Kolbe) (Isoptera: Rhinotermitidae) (Khan *et al*., 1992a; Suzuki, 1991). Khan *et al*. (1992a) reported that *Beauveria bassiana* (Bals) (Hyphomycetes) was the most effective pathogen against *O. obesus* Rambur (Isoptera: Termitidae), followed by *M. anisopliae, P. fuormosoroseus* and *P. lilacinus* (Thom) Samson. Three other fungal pathogens, i.e. *V. lecanii*, *Paecilomyces farinosus* (Holm EX s.f Gray) Brown & Smith and *Nomuraea rileyi* (Farlow) Samson (Hyphomycetes) were not pathogenic. Wells *et al*. (1995) found one isolate of *M. flavoviride* Gams & Roszypal (Hyphomycetes) that was not pathogenic to *C. formosanus.*

Although quite a number of entomopathogenic fungi have been shown to be effective in laboratory tests, *Aspergillus* and *Conidiobolus* spp. are opportunistic fungi of man and higher animals (Milner & Staples, 1995). In addition, many fungi are not as easily amenable to mass production on specialized or simple liquid, semisolid and solid media, as *B. bassiana* and *M. anisopliae*. As a result, only these two species have been successfully used in the field (Fernandes & Alves, 1991; Hanel & Watson, 1983; Milner & Staples, 1995; Gitonga, 1996). Both fungi are commercially produced for the control of various insect pests in a number of countries, and isolates of both species have been registered by EPA in the US (Cook *et al*., 1996 in Rath, 2000).

Apart from Gitonga's (1996) work on the *M. anisopliae* and *B. bassiana* as potential biological control agent against *M. michaelseni*, there are no published records of application of entomopathogenic fungi for termite control in Africa.

1.2 Termite control using the entomopathogenic fungi *Metarhizium anisopliae* (Metschnikoff) Sorokin and *Beauveria bassiana* (Balsamo) Vuillemin

The entomopathogenic fungi genera *Beauveria* and *Metarhizium* belong to the class Hyphomycetes (CABI, 2001). Among the two genera, *M. anisopliae* and *B. bassiana* are the most widely studied and important species, known to infect more than 200 (Veen, 1968) and 700 (Li, 1988 in Rath, 2000) insect species, respectively.

Five authors have recently reviewed the biological control of termites, i.e. Grace (1997), Milner (2000a), Culliney & Grace (2000) and Rath (2000). All authors stressed that the greatest potential for biological control of termites appears to lie on insect pathogens, particularly on the two well-studied entomopathogenic fungi, *B. bassiana* and *M. anisopliae. Metarhizium anisopliae* and *B. bassiana* are the most widely tested entomopathogenic fungi against termites. In table No. 2 results of several laboratory experiments on the pathogenicity and virulence of isolates of *B. bassiana* and *M. anisopliae* to termites are listed. Some of the cited studies had been reviewed by Gitonga (1996). These tests were conducted against 15 species of termites from three families of lower termites, i.e. Kalotermitidae, Hodotermitidae, and Rhinotermitidae, and one higher termite family, i.e. Termitidae. Among the 15 species, 11 species belong to the lower termite families and four species to the higher termites.

Quite a number of *M. anisopliae* and *B. bassiana* strains isolated from the soil, termites and other insect cadavers have been found to be potentially virulent against termites. Next to pathogenicity potential or virulence, some workers considered high sporulation capacity *in vitro* and *in vivo* as an important attribute of a good fungal isolate for use against termites, since sporulation on cadavers relates to transmission of the pathogens (Hanel, 1982a; Milner & Staples, 1995; Milner *et al*., 1998b; Almeida *et al*., 1997). Ebret (1994) postulated that parasites maximize the number of successfully transmitted offspring by trading off propagule production against host survival. Emphasis of disease transmission from living termites that had been exposed to entomopathogenic fungi to unexposed healthy termites has also been given. Disease transmission in termite control is considered to be very important because in many species major parts of the colony and the nest are not accessible to direct treatments (Rath, 2000).

Although good control results of termites using *M. anisopliae* and *B. bassiana* have been obtained, further laboratory and field experiments have also confirmed that treatment results in exclusion of termites by walling-off or repellency, or treatment reduces the vigour of a colony only for a limited time (Milner, 2000b). Termites can detect and avoid spores of entomopathogenic fungi. Termites effectively clean each other by grooming and this is often believed to enhance the spread of pathogens such as *M. anisopliae* and *B. bassiana*. However, according to Milner (2000b), this is probably an effective defence strategy of termites because the groomed conidia end up in the gut of the termites where they are unable to infect the

insect. Moreover, termites respond to the presence of diseased conspecifics by walling-off contaminated workers once the fungus starts to kill termites (Milner, 2000b).

Despite these limitations, a number of strategies have been developed to especially use *Metarhizium* spp. for termite control (Milner & Staples, 1995; Milner, 2000b). The strategies include the use of less repellent strains, formulations that mask and inhibit repellency, and blowing large quantities of pure conidia directly into the nursery region of a mound (Milner, 2000b).

1.2.1 Description

1.2.1.1 Description of *B. bassiana*

Agostino Bassi first described the fungus in 1835. Collections of *Beauveria* spp. infected insects from tropical ecosystems, have revealed considerable genetic variation between isolates, rendering species identification sometimes difficult (Mugnai *et al*., 1989). There are four described entomopathogenic species in the genus *Beauveria*, i.e. *B. bassiana*, *B. brongniartii* (Saccardo) Petch, *B. amorpha* (Honel*)* Samson & Evans and *B. velata* (Honel) Samson & Evans (Mugnai *et al*., 1989). Only *B. bassiana* has ever been isolated from termites (Zoberi & Grace, 1990b). In addition, three other non-pathogenic *Beauveria* spp. have been recorded (Mugnai *et al*., 1989).

Beauveria spp. colonies are woolly, floccose and often appear powdery due to the abundant conidia production. After 14 days on potato dextrose agar (PDA) or malt agar (MA) colonies look velvet to powdery, rarely forming synnemata. At the beginning these colonies have white mycelial margins which become pale yellow or sometimes reddish. The underneath surface of the colonies is colourless, or yellow to reddish (Brady, 1979b).

Other distinguishing features are the conidiogenous apparatus which forms dense clusters of swollen cells which consists of a subglobose to flask shaped venture, 3-6 x 2.5-3.5 microns and a zigzag shaped denticulate rachis 2-3 microns long and 1 micron wide (Brady, 1979b). Conidia are round to ovoid (globose), smooth walled and 2-3 microns in diameter. *Beauveria bassiana* reproduces by production of dry spores conidiophores that grow sympodially.

Conidiogenesis starts after six days on agar medium, and after three to four days in liquid culture. Blastophors, which are thin walled and less resistant than conidia, develop from liquid

cultures (Samsinakova, 1966). The blastophores germinate more quickly than conidia but lose viability faster.

1.2.1.2 Description of *M. anisopliae*

A recent taxonomic revision of the genus *Metarhizium* by Driver *et al*. (2000), using internally transcribed spacer ribosomal DNA (rDNA) sequence data from 123 isolates, resulted in the description of ten distinct clades of *Metarhizium*. The authors recognised four clades within *M. anisopliae*. Two of the four correspond with *M. anisopliae* var. *anisopliae* and *M. anisopliae* var. *majus*, and Driver *et al*. (2000) described the other two as new varieties, i.e. *M. anisopliae* var. *lepidiotum* Driver & Milner and *M. anisopliae* var. *acridum* Driver & Milner. In publications prior to 1999 *M. anisopliae* var. *acridum* was referred to as *M. flavoviride*. *Metarhizium album, M. flavoviride* var. *flavoviride* and *M. flavoviride* var. *minus* are recognised as three clades. The remaining three clades are considered to represent new varieties, i.e. *M. flavoviride* var. *nova-zealandicum* Driver & Milner and *M. flavoviride* var. *pemphigum* Driver & Milne. The third clade has not been named, pending further data on other similar isolates. The telomorph (perfect or sexual) stage of *M. anisopliae* has not yet been identified (Lomer *et al*., 2001).

Metarhizium anisopliae forms colonies on PDA after 40 days. Such colonies have a white mycelial margin with clumps of conidiophors, which become coloured with the development of spores. The colour of the spores ranges from yellow green, dark herbage green, to sometimes pink. Spores are produced in columnar stands. Conidiophors are abundant, and arise from vegetative hyphae branching irregularly, usually with 2-3 branches at each node. Conidia are formed in chains, narrow cylindrical smooth, aseptae, 3.5-9 microns (mostly 5-8) long by 1.5-3.5 micron wide in *M.* var. *anisopliae* and , 9-16 micron (mostly 10-14) long by 1.8-4.5 microns wide in *M.* var. *major* (Brady, 1979a).

Metarhizium anisopliae can attack a wide range of insect hosts, including Orthoptera, Coleoptera, Lepidoptera, Hemiptera and Hymenoptera, and also soil-living Arachnida. In the genus *Metarhizium*, only *M. anisopliae* has ever been isolated from termites (Milner & Steples, 1995; Zoberi, 1995; Gitonga, 1996).

1.3 Conclusion

In the study region of Western and Eastern Wellega, small-scale farmers practice traditional termite control methods, like flooding and digging of mounds and removal of the queen. Between September 1987 and September 1988, in a government co-ordinated control campaign, 23,000 *Macrotermes* spp. queens were removed during one year (Abdurahman, 1990). Mound poisoning is the most commonly used control method against termites (Abdurahman, 1990). In the past, many mound-poisoning campaigns have been conducted in the region, but control attempts proved to be not sustainable since the termite populations recovered two to three years after the control campaign. In addition, mound poisoning had tremendous negative impact on the fauna and flora of the region (Gauchan *et al*., 1998).

Despite such extensive and expensive mound poisoning campaigns, termites continue to be a serious problem in Western and Eastern Wellega, affecting the livelihood of farmers in the region. The termite problem in the region is very complex. According to Gauchan *et al*. (1998), the natural and man made factors, such as poor management of farm land, livestock and communal grazing areas, forest clearing, and the use of mound poisoning as the only control option, have largely contributed to the present termite problems in the region. Based on the complexity of the problem, the ICRA termite team has given research recommendations on termite management. Studies on identification, promotion and use of biological control options are among the recommendations (Gauchan *et al.,* 1998).

The ICRA team indicated that natural enemies of termites, including predators, parasitoids and entomopathogens could provide foresters and farmers with free, self-renewing and ecologically sound means of control. They recommended this research to be specific for different locations. However, so far no work has been done on the biological control of termites in Ethiopia.

Therefore, based on the forgoing review and the recommendations made by the ICRA termite team (Gauchan *et al.,* 1998), this research program was initiated to study the possibilities for biological control of *M. subhyalinus* using the entomopathogenic fungi *B. bassiana* and *M. anisopliae*.

1.4 Objectives of the study

1. To review the literature on the control of termites using the entomopathogenic fungi *M. anisopliae* and *B. bassiana.*

2. To isolate *M. anisopliae* and *B. bassiana* from two termite prone regions in Ethiopia.

3. To determine the pathogenicity of selected *M. anisopliae* and *B. bassiana* isolates to *M. subhyalinus* under laboratory conditions.

4. To determine the sporulation capacity of Ethiopian *M. anisopliae* and *B. bassiana* isolates selected for the control of *M. subhyalinus.*

5. To determine the pathogenicity of selected *M. anisopliae* and/or *B. bassiana* isolates to *M. subhyalinus* under field conditions.

Table 2 Laboratory bioassays against termites using *Metarhizium anisopliae* and *Beauveria bassiana*

Fungus	Termite species	Objective	No. of isolates	Inoculation method	LC/LD_{50}	Effectiveness	Reference
M. anisopliae	*Coptotermes acinaciformis* Froggot (Isoptera: Rhinotermitidae)	Screening	49	A single termite rolled in 1:10 conidia: talc (10^7conidia/ml), considered as high dose and 1:1000 conidia: talc considered as low dose and placed among 50 untreated workers (grooming assay) tested in 70 ml plastic specimen jar. Termites get infested mainly though grooming of the treated termite worker for 24 hrs.	-	Low dose with 19 strains 40% mortality after 7 days High dose with 18 strains 80% mortality	Milner *et al.*, 1998b
	Coptotermes. frenchi Hill (Isoptera: Rhinotermitidae)	Screening	26	Methodology same as above	-	Low dose-7 strains 40% mortality after 7 days High dose-24 strains 80% mortality after 7 days	Milner *et al.*, 1998b

Table 2. contd.

Fungus	Termite species	Objective	No. of isolates	Inoculation method	LC/LD_{50}	Effectiveness	Reference
	N. exitiosus	Screening	49	Methodology same as above.	-	Low dose-16 strains 40%mortality High dose-33 strains 80% mortality	Milner *et al.*, 1998b
	C. acinaciformis	Quantitative assay	-	Methodology same as above.	LC_{50}, 10^3-10^4 conidia/termite after 7 days	-	Milner & Staples, 1995
	N. exitiosus	Quantitative assay	-	Methodology same as above	LC_{50}, 10^4 conidia/termites after 7 days	-	Milner & Staples, 1995
	N. extiosus	Quantitative assay	1	1ml conidial suspension sprayed from 20 cm distance onto 25 workers on a 10 cm diam Petri dish	LC_{50}, $3.6x10^4$& $5.6x10^5$ conidia/ml after 8 & 11 days, respectively	6-99% mortality depending upon the dose level	Hänel, 1981
	N. exitiosus	Screening	49	Methodology same as above.	-	Low dose-16 strains 40%mortality High dose-33 strains 80% mortality	Milner *et al.*, 1998b

Table 2. contd.

Fungus	Termite species	Objective	No. of isolates	Inoculation method	LC/LD_{50}	Effectiveness	Reference
	Nasutitermes. Spp	Quantitative assay	3	20 worker and soldier termites kept in a 4 cm dim & 7 cm container sprayed with conidia suspension of $10^{6,}$ 10^{7},10^{8},10^{9} & 10^{10} conidia/ml	LC_{50}, 0.7&1.8x 10^{7} conidia/ml after 4 days & 6 days, respectively.	15-100% mortality after 5 days depending upon dose level	Malagodi & Veiga, 1995
	C. formosanus, *Inscisitermes immigran* (Isoptera: Kalotermitidae, *Cryptotermes brevis* (Isoptera: Kalotermitidae), *Neotermes conenexus* (Isoptera: Kalotermitidae)	-	-	-	-	100% mortality in lab 100% mortality in lab 100% mortality in lab 100% mortality in lab	Leong, 1966

Table 2. contd.

Fungus	Termite species	Objective	No. of isolates	Inoculation method	LC/LD_{50}	Effectiveness	Reference
	C. formosanus	Bait choice test	-	Filter paper saturated with culture was rolled with the culture on the inside of the cylindrical rolled paper and placed in one of two plastic jars, connected by a glass tube. 180 worker and 20 soldier termites were placed in the other jar and allowed to forage through both jars for 14 days.	-	100% mortality after 14 days	Delate *et al.*, 1995a
	C. formosanus	Post-exposure mortality	-	Methodology same as above but stayed up to 6 days and surviving termites were transferred to a jar containing a moistened paper and stayed for 7 days.	-	100% mortality before post-exposure (after 6 days)	Delate *et al.*, 1995a

Table 2. contd.

Fungus	Termite species	Objective	No. of isolates	Inoculation method	LC/LD_{50}	Effectiveness	Reference
	C. formosanus	Quantitative assay	-	0.5 micron droplet of conidial suspension onto the prothorax of termite workers.	LC_{50}, 14.4-253.3 spores/mg of termite after 12 days.	100% mortality	Lai *et al.,* 1982
	C. formosanus	Compare isolates from phylogene-tically different hosts	3	1 micron droplet application of conidia suspension on to the ventral surface of each termites	LD_{50},0.4-$4.2x10^4$conidia/ml after 8 days	-	Wells *et al.,* 1995
	C. formosanus	Termite bait effect	3	30 worker termites were made to walk on culture for 10 min, removed and placed in plastic Petri dish of 5.5cm diam.	-	100%mortality after 8 days	Jones *et al.,* 1996
	C. formosanus	Quantitative assay	-	Application of 4 micron conidial suspension onto the dorsal surface of each at the base of the head.	LC_{50}, 36-40 micron conidia/termite	52.8-91.7% mortality after 8 days depending upon the dose level	Jones *et al.,* 1996

Table 2. contd.

Fungus	Termite species	Objective	No. of isolates	Inoculation method	LC/LD_{50}	Effectiveness	Reference
	C. formosanus	Survival of fungi on termite nest material	-	Termite cadavers were placed on carton material in 5.5 cm Petri dish for 14 days. 30 termites were made to walk on the culture for 3 h and removed to clean Petri dish. Observed for 8 days.	-	30-57% mortality after 8 days	Jones *et al.,* 1996
	C. formosanus	Termite bait effect	-	10, 20, and 100 worker termites exposed to conidia and added to untreated termites at the ratio of 1:10, 2:10 and 10:10 (treated: untreated). Each assay contained a total of 100 termites.	-	38-89%, 61-100% and 100% mort in 1: 2: and 10:10 treated - untreated termites ratio , respectively after 16 days	Jones *et al.,* 1996
	C. formosanus	Preliminary pathogenicity test	-	-	-	100% mortality	Chai *et al.,* 1995
	R. flavipes	Termite as bait	-	-	-	100% mortality after 3 days	Suzuki, 1991

Table 2. contd.

Fungus	Termite species	Objective	No. of isolates	Inoculation method	LC/LD_{50}	Effectiveness	Reference
	Coptotermes curvignathus Holmgren (Isoptera: Rhinotermitidae	Quantitative assay	-	0.005 ml droplet application conidial suspension onto the surface of worker termites	LD_{50}, $1.6x10^3$ and $3.5x10^4$ conidia/ml after 7 and 10 days, respectively	-	Sajap & Jan, 1990
	Odonotermes. wallonensis Wasmann (Isoptera: Termitidae)	Quantitative assay	-	3ml of conidia suspension sprayed on to major termites	LC_{50},10^1x10^4 conidia /ml after 7 days	-	Khan *et al.,* 1992a
	O. obesus	Quantitative assay	-	3ml of conidia suspension sprayed onto major, minor workers and soldiers	LC_{50}, 148.7, 225.3 & $780.4x\ 10^4$ conidial/ml for major workers, minor workers & soldiers, respectively, after 7day days	-	Khan *et al.,* 1993
	O. obesus	Pathogenicity test	1	Methodology same as above	-	33.7-47.3% mortality after 7 days	Khan *et al.,* 1993

Table 2. contd.

Fungus	Termite species	Objective	No. of isolates	Inoculation method	LC/LD_{50}	Effectiveness	Reference
	Reticulitermes spp.	Termite bait	-	20 dead termites exposed to fungal culture for 4, 8 and 12 hr and added to 8 untreated termites.	-	100% mortality	Kramm *et al.*, 1982
	Reticulitermes spp,	Cadaver for transmission of infection	1	-	-	100% mortality	Kramm *et al.*, 1982
	M. michaelseni	Preliminary pathogenicity test	8	Spraying of 50 workers in 9 cm diam Petri dish with 1 ml of conidial suspension	-	84.4-100% mortality after 5 days	Gitonga, 1996
	M. michaelseni	Quantitative assay	8	Methodology same as above. conidial concentration of $10^{7,}$ $3*10^6$, 10^6, $3*10^{5,}$ 10^5, $3*10^4$ and 10^4 conidia/ml	LD_{50}, $1.8x10^4$ conidia/ml after 2.2 and 5 days	Gitong, 1996	Gitonga, 1996

Table 2. contd.

Fungus	Termite species	Objective	No. of isolates	Inoculation method	LC/LD_{50}	Effectiveness	Reference
	M. michaelseni	Transmission of infection	1	A single dead and treated worker termite was placed in 9 cm Petri dish containing 50 untreated termites and removed after 15 sec, 1,4 &, 24 min and continuously left	-	30.6-42.7% and 63.2-97.6% mortality after 5 and 10 days, respectively	Gitonga, 1996
	Hodontotermes. tenuis Hagen (Isoptera:Rhinotermitidae)	Quantitative assay	2	25 workers and 5 soldier termites sprayed with 1 ml conidial suspension of $5x10^{6,}$ $1x10^{7}$, $1x10^{8}$and $1x10^{9}$ conidia/ml of suspension and kept in plastic Petri dish	-	6.7-67.3% mortality depending upon the dose level	Almeid *et al.,* 1997
B. bassiana	*R. flavipes*	Pathogenicity test	-	-	-	33.3% mortality	Zoberi & Grace, 1990b
	R. flavipes	Pathogenicity	-	-	-	100% mortality	Same
	R. flavipes	Pathogenicity	-	-	-	80-100% mortality	Grace & Zoberi, 1992
	R. flavipes	Pathogenicity	-	-	-	100% mortality	Kramm *et al.,* 1982

Table 2. contd.

Fungus	Termite species	Objective	No. of isolates	Inoculation method	LC/LD_{50}	Effectiveness	Reference
	C. formosanus & *Reticulitermes. speratus* Kolbe (Isoptera:Rhinotermitidae)	Pathogenicity	-	-	-	100% mortality	Suzuki, 1991
	Cornitermes cumulanus Kollar (Isoptera:Termitidae)	Choice test	1	Filter paper saturated with culture was rolled with the culture on the inside of the rolled paper and placed in one of two plastic jars, connected by a glass tube. 180 worker and 20 soldier termites were placed in the other jar and allowed to forage through both jars for 14 days.	-	70.4-100% mortality in	Delate *et al.*, 1995a
	Cornitermes cumulanus	-	-	Methodology same as above but stayed up to 6 days and surviving termites were transferred to a jar containing a moistened paper and stayed for 7 days.	-	31.1 and 69.2% mortality in treatment and post-treatment tests, respectively	Delate *et al.*, 1995a

Table 2. contd.

Fungus	Termite species	Objective	No. of isolates	Inoculation method	LC/LD_{50}	Effectiveness	Reference
	Cornitermes cumulanus	-	-	0.5 micron droplet of conidial suspension onto the prothorax of termite workers	LD_{50}, 59.3-116 spores/mg of termites after 12 days	-	Lai *et al.*, 1982
	Cornitermes cumulanus	Compare isolates from phlogenetically different hosts	-	1 micron droplet application of conidia suspension on to the ventral surface of each 30 CO_2 anaesthetized termites	LD_{50}, 1.6-7.1 x 10^4 conidia/ml after 8 days	-	Wells *et al.*, 1995
	C. curvignathus	Quantitative assay	-	droplets application of 0.005 ml conidial suspension onto the surface of worker termites	LD_{50}, 9x10^8 & 1.6x10^{11} conidia/ml after 7& 10 days, respectively	-	Sajap & Jan, 1990
	Nasutitermes spp	Quantitative assay	-	-	LD_{50}, 3.18x10^7 & 0.18x10^7 conidia/ml after 4 and 6 days, respectively	-	Malagodi & Veiga, 1995
	O. wallonensis	Quantitative assay	-	3 ml of conidial suspension sprayed onto major workers	LD_{50}, 5.1-179.1 x10^4 conidi/ml after 7 days	-	Khan *et al.*, 1992b

Table 2. contd.

Fungus	Termite species	Objective	No. of isolates	Inoculation method	LC/LD_{50}	Effectiveness	Reference
	O. obesus	Quantitative assay	-	3ml of conidia suspension sprayed onto major, minor workers and soldiers	LC_{50}, 10.0-330.6, 5.7-114.9 and 52.5-949.7x10^4 conidia/ml for major, minor workers & soldiers, respectively after 7 days	-	Khan *et al.*, 1993
	O. obesus	Quantitative assay with culture of different age	1	Spraying of 1 ml of 10^4, 10^5 & 10^6 conidia/ml of suspension prepared from culture of different age (10, 14, 20, 28 and 34 days) on soil and sawdust placed in polythene cups containing termites	-	21-73, 19-82,19-94,21-100 and 19-78% mortality with 10, 14, 20, 28 and 34 days old culture, respectively after 7 days	Gurusubramanian *et al.*, 1999

Table 2. contd.

Fungus	Termite species	Objective	No. of isolates	Inoculation method	LC/LD_{50}	Effectiveness	Reference
	H. tenuis	Synergetic effect of mycilia and imidacloprid	-	-	-	3.1, 70.2, 69.2, 71 and 48.4% mortality with imidacloprid, conidia, conidia+ imidacloprid, respectively after 8 days	Almeida & Alves, 1996
	H. tenuis	-	-	25 worker and 5 soldier termites sprayed with .1 ml conidial suspension of $10x10^{6,}$ $1x10^{7,}$ $1x10^{8}$and $1x10^{9}$ conidia/ml of suspension & kept in plastic petri dish	-	23.3-100% mortality depending upon dose level in 8 days	Almeida *et al.*, 1997
	H. tenuis	Screening	142	Methodology is the same as above except $5x10^{8}$ conidia/ml was used	-	113 isolates caused 50-100% mortality	Almeida *et al.*, 1997

Table 2. contd.

Fungus	Termite species	Objective	No. of isolates	Inoculation method	LC/LD_{50}	Effectiveness	Reference
	M. michaelseni	Preliminary pathogenicity test	8	spraying of 50 worker in termites 9 cm diam. petri dish with 1 ml of conidial suspension	-	78,5-100% mortality after 5 days	Gitonga, 1996
	M. michaelseni	Quantitative assay		Methodology same as above. conidial concentration of $10^{7,}$ $3x10^6$, 10^6, $3x10^{5,}$ 10^5, $3x10^4$ and 10^4 conidia/ml conidia/ml	LC_{50}, $3.1x10^5$ conidia/ml after 5 days	-	Gitonga, 1996

2 Isolation of *M. anisopliae* and *B. bassiana* from soil and insect cadavers collected from Ethiopia.

2.1 Introduction

Metarhizium anisopliae and *B. bassiana* have been isolated either from insect cadavers or soil samples (Zoberi & Grace, 1990b; Grace & Zoberi, 1992; Zoberi, 1995; Milner, 1992; Milner *et al.,* 1998a). Soil is the main reservoir of infective propagules of many entomopathogenic fungi (Vänninen, 1995, Milner *et al.*, 1998a). Thus, the most effective isolates originate from this medium. Entomopathogenic fungi may be isolated from the soil through the use of the insect bait method and via selective media. In general, entomopathogenic Hyphomycetes are considered to be weak saprotrophs, but since they possess the ability to infect living insects, they can gain access to a living insect relatively free of competitors (Goettel & Inglis, 1997). Live larvae of *Galleria mellonella* L. (Lepidoptera: Pyralidae), *Acanthocinus aedilis* (L.) (Coleoptera: Bruchidae) and *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) have been successfully used for the detection of entemopathogenic fungi in the soil (Zimmermann, 1986). Fungi in the genera *Trichoderma* (Hyphomycetes), *Mucor* (Bacidomycetes) and *Rhizopus* (Zygomycetes) are fast growing and can rapidly obscure colonies of entomopathogenic Hyphomycetes, making isolation difficult or impossible (Goettel & Inglis, 1997). A variety of fungicides and antibiotics have been used in selective media to isolate *Metarhizium* spp. and *Beauveria* spp. from contaminated environments such as soil samples (Milner *et al*., 1993). The first selective media contained chloramphenicol and cycloheximide and was based on media developed for the isolation of fungi for clinical specimens (Veen & Ferron, 1966). This medium was simplified by Milner and Lutton (1976) and is referred to as Veen's medium. Other selective media include the dodine (n-dodecyt-guanidine-acetate referred to as Dodine medium) (Beilharz *et al*., 1982) and copper-based medium (referred to as Baath medium) (Baath, 1991), respectively.

Ecological studies on the distribution of *M. anisopliae* and *B. bassiana* have been undertaken, attempting to link the occurrence of the fungi to soil types, habitats and/or geographical locations (Brownbridge *et al*., 1993; Rath *et al*., 1992; Vanninen, 1995).

The most virulent isolates are usually those, which cause natural epizootics in a particular host (Milner, 2000a). However, it is best to search in the environment of the target pest for suitable

strains, while at the same time testing isolates from other hosts and soil samples, since specific termite-associated isolates do not exit and most isolates are virulent for termites (Milner *et al*., 1998ab). Brownbridge *et al*. (1993) believes that indigenous strains should be preferably used because they are already well adapted to survive and multiply in their native environment; moreover, using indigenous strains avoids the introduction of exotic species that might disrupt an ecosystem.

Milner and Staples (1995) and Milner *et al*. (1998a) isolated *M. anisopliae* and *B. bassiana* from *Mastotermes darwiniensis* Froggatt (Isoptera: Mastotermitidae), *Coptotermes lacteus* Froggatt (Isoptera: Rhinotermitidae), *Porotermes adamsoni* Froggatt (Isoptera: Termopsidae) (Isoptera: Hodotermitidae), *Kalotermes* spp. and *Neotermes insularis* Walker (the latter two Isoptera: Kalotermitidae) from live termite samples, collected mostly in eastern Australia. Zoberi and Grace (1990a) and Zoberi (1995) isolated *M. anisopliae* and *B. bassiana* strains from a *R. flavipes* cadaver and from collections of live termites in Toronto, Canada. Finally, Gitonga (1996) isolated a single strain of each *B. bassiana* and *M. anisopliae* from *Macrotermes* spp. in Kenya.

Only few indigenous *Beauveria* and *Metarhizium* isolates have been obtained in Ethiopia from insect cadavers, collected mainly in locust and tsetse flies breeding areas (Senshaw, 1998; Mamuye, 1999). The objective of this study was, therefore, to isolate *M. anisopliae* and *B. bassiana* strains from some of the termite infested zones of Ethiopia, i.e. Wellega and Zeway zone in the Oromia regional state. Attempt has also been made to include soil samples from the Menagesha Suba forest, one of the indigenous forests in the country to diversify the habitat where the samples are taken.

2.2 Materials and methods

2.2.1 Study sites

Sample collection was carried out in Zeway, Mendi and Gimbi, all highly termite infested areas. Soil samples were additionally collected in the Menagesh Suba forest (fig. 1)

2.2.1.1 Zeway

Zeway is found in the Eastern Shoa zone of the Oromia regional state 165 km east of Addis Ababa at an altitude of 1,650 m. It is located within the rift valley that runs the whole length

of Ethiopia, stretching from the border of Eritrea in the North East to the lake Turkana in the South, and in the South Western towards the border with Kenya. The area has bimodal rainfall patterns. The mean annual rainfall is 742.4 mm (taken from the 30 years of data) (Mamuye, 2001). Highest precipitation occurs between July and September. There is some additional rainfall between February and the end of April, but this usually varies. Rain-fed crops are most often not planted until mid June because of the unpredictable rainfall pattern in the preceding period. The mean annual temperature is 20.7ºC. The annual means of daily maximum and minimum temperatures are 27.2ºC and 14ºC, respectively.

The soils are variable in morphology and many parts of the study area are influenced by recent sediment deposition. These geographical activities largely influenced the soil types of the area (Mamuye, 2001). Lake Zeway terraces have coarse textured soils and pumice sand and gravel, and medium textured soils on ash. The pumice sands have weakly developed profiles and are low in organic matter.

The majority of the farmers are engaged in rain-fed agriculture (Mamuye, 2001). The long cycle stalk crops are maize and sorghum, and are also the most dominant crops. Among the other cereals wheat and teff are also grown. The farmers who are situated along the water sources have traditionally practiced irrigation. In recent years irrigation has substantially increased, mainly because of moisture stress during the growing seasons. Farmers produce crops under irrigation that are suitable (mostly row crops) and which have higher market prices. Common crops grown under irrigation are fruits (mostly papayas), vegetables (onion, tomato, cabbage etc.) and also cycle cereals like maize.

2.2.1.2 Mendi and Gimbi

Mendi and Gimbi are situated in the Western Wellega zone of the Oromia regional state, 500 and 450 km west of Addis Ababa at an altitude of 1,680 and 1,870 m above sea level, respectively. These areas have a single rainy season from April to October. Generally rainfall in Western Ethiopia is higher than in other parts of the country. The mean annual rainfall in these areas varies from 1,649.7 to 1,947.7 mm (Abdurahman, 1990). The mean minimum and maximum monthly temperatures range from 13.5 to 15.7°C and from 25.6 to 28.0°C, respectively.

The soils of Mendi and Gimbi area are predominantly Nitosols (Abdurahman, 1990). The most important diagnostic features of Nitosols are the reddish brown to red colour and clay in texture with an agrillic B-horizon without abrupt textural changes. Nitosols have good physical properties, a uniform profile, are porous, well drained, have a stable structure, high moisture holding capacity and a deep rooting volume (Ethiopian Mapping Authority, 1988). However, important limitations of these soils are that they are strongly acidic with pH values of less than 5.4 (Murphy, 1968; Aduga, 1984), and that they are highly deficient in available phosphorous (Murphy, 1968; Desta, 1982; Aduga, 1984). Hence, these soils require heavy fertilization, especially with phosphorus and liberal addition of lime.

Cereals such as teff, maize, sorghum, finger millet, barley, wheat and oats are the most important food crops grown in the area. The next widely grown commodities are oil crops such as Niger seed, sesame, groundnuts, rapeseed and sunflower. Various pulse crops, such as field peas, horse beans, chickpeas, lentils and haricot beans are also grown. Vegetable crops are less common in the area, except in some homestead and state farms. Ploughing is done either by oxen or hoe, and does not destroy mound-building termites.

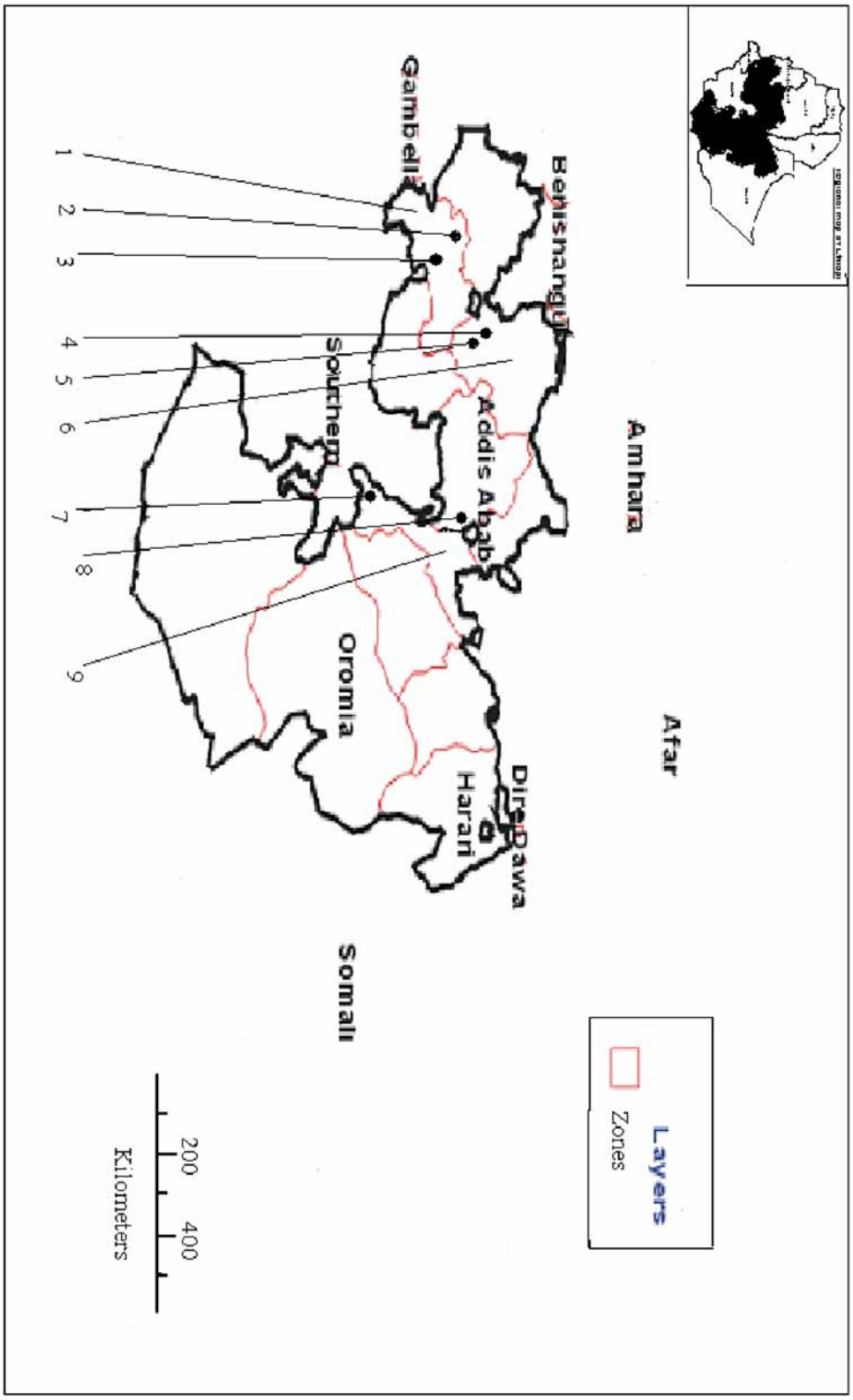
2.2.1.3 The Menagesh Suba forest

The Menagesh Suba State Forest is located approximately 45 km west of Addis Ababa in the Wechecha Mountain range. The area is made up of rolling terrain with an elevation ranging from 2,200-3,385 m above sea level (MoA, 1998). The mean annual rainfall is estimated to be 1,156 mm and the mean annual temperature of the entire central area averages from 11-22ºC (MoA, 1998). Arising as a consequence of the lava substratum, there are soils of different nature and properties in the area. Upper slope soils constitute shallow, yellowish to reddish brown and stony clay while the lower slopes are occupied by heavy dark red silt-loam soils (MoA, 1998). The reddish brown to red clay soils are most abundant.

The most important indigenous forest trees are *Juniperus procera* Hochst (Cupressaceae), *Olea africana* Mill (Oleaceae), *Podocarpus gracilior* Pilg (Podocarpaceae), *Pygeum africanum* Hook. f (Rosaceae), *Dovyalis abyssinica* (A. Rich) Warburg (Flacourtiaceae), and *Hagenia abyssinica* (Bruce) J. F Gmel (Rosaceae). Besides the natural forests there are man-made plantations of mainly exotic species such as *Eucalyptus globulus* Labill, *E. saligna* Sm (both Myrtaceae), *Cuprerssus lusitanica* Mill (Apiaceae) and *Pinus patula* Schlecht & Cham (Pinaceae).

2.2.2 Sample collection

A total of 120 samples of soil, cadavers of insects that might have died because of pathogen infection and termite associated materials from the outer and inner parts of termite mounds were collected in Zeway, Mendi, Gimbi and Menagesh Suba. About 1 kg of soil per sample was collected in 30 cm x 20 cm plastic bags from cultivated and fallow land, forest floor, lake sides, pasture, orchards and other plantations by removing the soil at depths of 0-15 cm. Insect cadavers were collected using forceps and stored in sterile 2.5 x 8 cm glass vials, equipped with screw tops. Live termites were collected along with termite combs by breaking up and distracting active mounds and were stored in insect rearing boxes (25 x 15 cm in diameter) with leads which had opening at the centre covered by wire mesh. The collections were carried out between September 1998 and September 1999. All samples were taken to the Addis Ababa University (AAU) mycology laboratory. Samples of soil, mound material and insect cadaver were stored in a fridge at 4°C.



1-Western Wellega zone
2-Mendi
3-Nejo
4-Sasiga
5-Lekemt Zuria
6-Eastern Wellega zone
7-Zeway
8-Menagesh Suba
9-Estern Shoa zone

Figure 3 Map showing the Oromia regional state and the research sites

2.2.3 Preparation of media

2.2.3.1 Sabouraud dextrose agar (SDA)

Twenty grams of mycological peptone, 200 g of glucose and 20 g of pure mycological agar (Merck, Darmstadt, Germany) were dissolved in 1 l of boiling tap water. After cooling down, the antibiotic Chloramphenicol (1 g/l) (Karnataka Antibiotics & Pharmaceuticals Limited, Bangalore, India) was added to the suspension in order to suppress bacterial growth. Then the final suspension was autoclaved at 121°C and 115 psi for 15-20 minutes.

2.2.3.2 SDA selective media (Veen's medium)

Twenty grams of mycological peptone, 200 g of glucose and 20 g of mycological agar were dissolved in 1 l of boiling tap water. Chloramphenicol (1 g/l) and and cyclohexamide (1.4 g/l) (Aldrich, Chemical Company Inc, Milwaukee, USA) were added after cooling the media suspension in order to suppress the growth of bacteria and fast growing contaminant fungi (Veen & Ferron, 1966). Then the final suspension was autoclaved at 121°C and 115 psi for 15-20 minutes.

2.2.3.3 Malt extract agar (MEA)

Twenty grams of malt extract and 20 g of mycological agar were dissolved in 1 l of boiling tap water. Chloramphenicol (1 g/l) was added after cooling the suspension in order to suppress bacterial growth. Then the final suspension was autoclaved at 121°C and 115 psi for 15-20 minutes.

2.2.3.4 Potato dextrose agar (PDA)

Two hundred grams of potatoes were scrubbed and cleaned without peeling. The cleaned potatoes were cut into about 12 mm cubes, and 200 g of potatoes were weighed out and rinsed rapidly under running water. Thereafter the rinsed potatoes were cooked in 1 l of water for one hour, mashed and squeezed through gauze cloth. Twenty grams of mycological agar was added to the boiling pulp suspension. Additional 20 g of dextrose was added and the suspension was stirred until the dextrose dissolved. The mixture was diluted with tap water to a total volume of 1 l. Chloramphenicol (1 g/l) was added to the suspension after cooling the

suspension in order to suppress bacterial growth. Then the final suspension was autoclaved at 121°C and 115 psi for 15-20 minutes.

2.2.4. Isolation

2.2.4.1 Isolation from the soil

Soil samples collected from each site were manually ground, using a mortar and a pestle, and passed through a 0.63 μm mesh sieve. Sterile water (10 ml) was added to separate sterile test tubes, using a 20 ml sterile pipette, and 1 g of soil or termite material sample was added to each test tube. Each test tube was agitated in test tube shaker for 10-20 seconds. The suspension was further diluted twice; according to a ten fold dilution series.

Of each soil suspension 0.5 ml of the resultant preparation was transferred, using 1 ml sterile pipettes, onto separate Veen's media plates and spread using a sterile, L-shaped glass rod. The plates were then incubated at 26°C ± 2°C in a dark incubator and observed for fungal growth for 14 days. After growth was eminent, fungi that were suspected to be *Beauveria* spp. and/or *Metarhizium* spp. were sub-cultured to obtain pure cultures of the suspected entomopathogens.

2.2.4.1.1 Sub-culturing

Spores were taken from the initial cultures, using a sterile loop and streaked on SDA plates. The plates were then incubated at 26°C ± 2°C in a dark incubator for 14 days. Spores growing on these secondary cultures were sub-cultured again for 14 days to obtain clean cultures.

2.2.4.2 Isolation from insects

2.2.4.2.1 Dead insects

A part of the insect cadavers that showed signs of fungal growth was touched with a sterile needle and a small amount of the fungus material was transferred onto SDA plates. The plates were placed at 26°C ± 2°C in a dark incubator and were twice examined for fungal growth under a binocular microscope after seven and ten days. Fungal colonies suspected to be *Beauveria* spp. and/or *Metarhizium* spp. were sub-cultured to obtain pure cultures.

2.2.4.2.2 Live termites

Samples of 30 *M. subhyalinus* workers were kept in ten Petri dishes (9 cm in diameter) lined with moist Whatman No. 2 filter paper. The plates were incubated in a dark wooden box at 24°C ± 2°C and observations were made daily until all the termites died. Dead termites were removed with a sterile forcep and placed on separate sterile plates (9 cm in diameter) lined with sterile moistened Whatman No. 2 filter paper. The plates were placed at 26°C ± 2°C in a dark incubator and monitored daily for external sporulation. Spores were removed from sporulating cadavers, using a fine sterile needle, and placed on SDA plates. The plates were incubated at 26°C ± 2°C in a dark incubator for 14 days. Fungi, suspected to be *Beauveria* spp. and/or *Metarhizium* spp. were sub-cultured to obtain pure cultures, following the previously described procedure.

2.2.5 Identification

The identification of the isolates was done at Deutsche Sammlung von Microorganismen und Zellkulturen, Braunschweig, Germany, by sending the SDA slant culture of the isolates.

2.2.6 Storage of pathogens

Cadavers of insects were placed in sterile 2.5 x 8 cm glass vials with screw tops and maintained at 4°C in the fridge.

Stocks of pure cultures of each fungal isolate were grown on sterile test tube slopes with screw tops. For that, spores were removed from the sub-culture of each isolate that had been prepared after the first isolation, using a sterile loop. Spores were then streaked evenly on separate sterile test tube slopes of PDA. The test tubes were kept at 26°C ± 2°C in a dark incubator for 21 days with the screw top loosely closed to allow aeration until sporulation. The slope cultures were then maintained at 4°C in a fridge. Working cultures (on SDA plates) were prepared from these slopes for each isolate. Working cultures were rejected when older than 14 days.

2.3 Results

2.3.1 Survey and fungal isolates

The 120 samples collected comprised 110 soil samples from termite mounds, cultivated soil, natural grassing land and forest soil (table 3). The ten insect cadavers were obtained from different Coleoptera, Hemiptera, Diptera and Lepidoptera (table 3). The *M. anisopliae* isolate TT was isolated from a *M. subhyalinus* worker collected live, which subsequently died in the laboratory. The *B. bassiana* isolate S41 was isolated from mound material that was collected from Zeway, in the Oromia regional state (table 3). No isolates were found in Eastern and Western Wellega and in the high elevation Menagesha Suba forest. No *Metarhizium* or *Beauveria* spp. strains could be isolated from the insect cadavers collected in Zeway and Wellega.

Table 3 Summery of results from the surveys in Zeway, Eastern and Western Wellega and in the Menagesha Suba forest.

Locations	Sample types and numbers					
	Soil types				Insect cadavers	Live termites
	Mound	Cultivated land	Forest	Grass land		
Eastern and Western Wellega	10	-	-	6	3	-
Zeway	13	60	-	3	7	1
Menagesh Suba	-	-	18	-	-	-

2.4 Discussion

From the 120 samples collected from different soils, termite mounds and termite cadavers, only two fungal isolate were identified. One strain was isolated from a termite that had died in the laboratory and the second from termite mound material. No isolate was obtained from soil samples taken from cultivated and grazing land in Zeway (hot and dry), mound soil of the cooler and wetter area of Eastern and Western Wellega, and from soil samples taken in the high elevation Menagesha Suba forest. Milner *et al*. (1998a) in Australia collected 479 samples of termite-associated materials and obtained *M. anisopliae* from 20% of all the samples. Of 419 soil samples collected from pastoral zones in Tasmania, Australia, 28% (119) contained *M. anisopliae* (Rath *et al.,* 1992). Vänninen (1995) in Finland obtained from a total of 590 soil samples, 117 (19.8%) and 92 (15.6%) *M. anisopliae* and *B. bassiana* isolates,

respectively. Possible reasons for the low success in isolating entomopathogenic fungi during the here presented study might have been the use of old and thus expired selective media and/or the fact that most samples were collected from sandy soils and in dry zones. *Metarhizium anisopliae* is more often isolated from loamy soil, relative to other soil types (Rath *et al*., 1992; Steenberg, 1995). Its abundance is related with high rainfall (Milner *et al*., 1998a; Rath *et al*., 1992, Steenberg, 1995; Vänninen, 1995). From this study it is not possible to relate the distribution of entomopathogenic fungi with different soil types or different climatic zones, because of the small size of samples. *Metarhizium anisopliae* is considered to be thermophilic as it is rare in cold areas, while *B. bassiana* is tolerant to a wider range of climatic conditions but very sensitive to the disturbing effect of cultivation (Vänninen, 1995). Vänninen (1995) and Steenberg (1995) reported that *M. anisopliae* is less severely affected by cultivation. However, Milner (1992) indicated a scarcity of *M. anisopliae* in frequently cultivated fields in relatively dry and hot climates. Harny and Widden (1991) suggested forest soil as a natural reservoir of a broad spectrum of entomopathogenic fungi, a view that was later also supported by Brownbridge *et al*. (1993).

The two isolates were obtained from a termite, which died in the laboratory and mound material collected from a *M. subhyalinus* colony. According to Milner *et al*. (1998a), the evidence to date does not allow a definitive answer whether *M. anisopliae* is a natural pathogen of termites. Evidence which supports this hypothesis includes: the widespread occurrence of conidia in termite-associated material, disease development in termites recently collected from the field and the high susceptibility of termites to the isolates found in termite nest material. However, so far a naturally infected termite has never been found. The survey samples contained only very few conidia and most of the isolates from mound material could have originally derived from the surrounding soil and later incorporated into the termite mound material.

Prior (1992) discussed the problem of where to search for suitable strains of entomopathogenic fungi. He points out that with our present knowledge it is not possible to determine whether it is advantageous to use "old associations" or "new encounters". For practical reasons, however, it is often best to search in the environment of the target pest for suitable strains of entomopathogenic fungi, and simultaneously testing isolates from other hosts (Milner *et al*., 1998a). A very good example for the "old associations" approach is the *M. anisopliae* var. *acridum* strain that had been isolated from grasshoppers and is today very

effectively as myco-insecticide against many locust and grasshopper species in sub-Saharan Africa (Lomer *et al*., 2001).

More isolates might have been obtained in this study if the insect soil baiting isolation method would have been used. The detection limits for insect baits are known to be generally better than for selective media (Milner, 1992). The advantage of using selective media, as opposed to live bait, is that no laboratory rearing of insects is required and that soil-born organisms such as nematodes do not affect selective media. Thus in future, a combination of both insect baits and selective media should therefore be used, for isolating a maximum number of strains from the soil.

A broad range of fungal isolates makes the selection of efficiently virulent strains for the use in specific management programs more likely (Brownbridge *et al*., 1993). Therefore for future studies, country wide surveys that take into account different habitats and the influence of altitude, climatic conditions, soil type and vegetation, to collect and isolate entomopathogenic fungi that have different properties and could be of potential use against termites and other economically important pests should be conducted.

3 Pathogenicity of *Metarhizium anisopliae* (Metsch.) Sorok. and *Beauveria bassiana* (Bals.) to *Macrotermes subhyalinus* (Rambur) (Isoptera: Termitidae)

3.1 Introduction

In Ethiopia, termites pose a serious threat to agricultural, horticultural crops, forestry trees, rangeland and wooden structures, particularly in Eastern and Western Wellega (Abdurahman, 1990). *Macrotermes subhyalinus* is the dominant termite species in these areas at an altitude below 2,000 m (Abdurahman, 1990).

In the past conventional termite control largely depended on the use of persistent organochlorine cyclodiene insecticides. Several control campaigns against termite have been conducted in Ethiopia using these pesticides in the past and still today. However, the termite problem remains unchanged. Thus, due to the ineffectiveness of chemical control, and because of the inherent health and environmental hazards of chemical pesticides, search for less disruptive and environmentally more friendly control strategies has increased over the recent years (Gauchan *et al*., 1998).

One possible option for alternative termite control is biological control, using entomopathogenic fungi (Milner & Staples, 1995). Entomopathogenic fungi enter their hosts by direct penetration of the cuticle, that functions as a barrier against most microbial attack. Consequently, fungal entomopathogens have a particularly high potential for biological control of for instance sap-sucking insects that are difficult to combat with synthetic insecticides (Kang *et al*., 1999). In the case of social insects like termites, a small amount of inoculum might spread throughout a termite nest before being detected by the insects, resulting in an epizootic (Jones *et al*., 1996). Social activities like grooming and food sharing could additionally help to disperse the inoculum of the fungi (Kramm *et al*., 1982).

In previous studies the pathogenicity of several strains of *B. bassiana* and *M. anisopliae* for different termite species have been investigated in the laboratory (Hänel, 1981, 1982a; Lai *et al*., 1982; Sajap & Jan, 1990; Suzuki, 1991; Khan *et al*., 1992ab; Milner & Staples, 1996; Wells *et al*., 1995 Gitonga, 1996; Milner *et al*., 1998b). Although *M. anisopliae* and *B. bassiana* have a very wide host range (Veen, 1968; Brady, 1979ab), individual isolates can be considerably host-specific (Goettel *et al*., 1990, in Rath, 2000). In his review on the use of entomopathogenic fungi for termite control Rath (2000), however, concluded that

host specificity in termites is rather low since individual fungal isolates are virulent to a range of termite species, and a wide range of fungal isolates often infects individual species of termites. Within the same termite species, differences in virulence of different strains can occur depending on factors like conidial dosages (Jones *et al*., 1996; Milner, 2000b), formulation (Almeida *et al*., 1997; Delate *et al*., 1995a), or the termite caste targeted (Khan *et al*., 1993).

The purpose of this study was to determine the relative pathogenicity and virulence of *M. anisopliae* and *B. bassiana* isolates indigenous to Ethiopia against *M. subhyalinus.* The possibility of transmission of the disease from infected termites to healthy conspecifics, and behavioural responses of termites towards the entomopathogenic fungi were also investigated.

3.2 Materials and Methods

These studies were conducted both in the AAU mycology laboratory, Ethiopia, and in the laboratories of the Benin station of the International Institute of Tropical Agriculture (IITA). Although most of the protocols followed at both sites were similar, some minor adjustments had been made due to variation in weather conditions and resource availability.

3.2.1 *M. anisopliae* and *B. bassiana* production

3.2.1.1 Fungi

The *M. anisopliae* TT and the *B. bassiana* S41 strains were isolated in Ethiopia from *M. subhyalinus* workers and *M. subhyalinus* mound materials, using selective media (Veen and Ferron, 1966). The indigenous *M. anisopliae* isolates MM and EE and the *B. bassiana* isolates HH, GG and FF were obtained from the mycology unit at AAU. The other 12 *M. anisopliae* isolates, i.e. the Kenyan isolate ICIPE30, the Australian isolate FI23 and IITA isolates Z162, B82, B149, B122, ATL167, Z27, Z94, O91, O64 and B124 were obtained from IITA. A detailed description of the isolates and their origin is given in table 4.

3.2.1.2 Viability tests

Working cultures of each isolate were prepared on SDA slopes (or plates), using spores of the stock culture as described in section 2.2.6. Subsequently the stock cultures were returned to the fridge. The viability of the spores of each isolate was determined before conducting any bioassay. Spores of each isolate was taken from the 14 day old working slant cultures of each isolate, using a sterile loop and suspended in separate test tubes which contained sterile water and 0.05% Tween 80. The suspensions were made up to 10^4-10^5spore/ml concentrations. The conidial concentration in the suspension was determined by counting the number of spores using a Neubauer hemocytometer (Weber Scientific International Ltd. 40, Middex, UK) under a phase contrast microscope (Olympus System Microscopes, Olympus Optical Co. Ltd, Tokyo, Japan). Then two drops of each suspension was transferred onto separate SDA plates (5 cm diameter), using a sterile Pasteur pipette and spread with an L-shaped glass rod. The plates were placed in Ethiopia at 26°C ± 2°C in a dark incubator, and in Benin at 28°C ± 2°C under natural light in an incubation room. After 24 hours of incubation, germinating and non-germinating spores were counted by placing the plates under a microscope. Any budding spore was counted as germinating spore. The percentage germination was calculated.

Table 4 Description of *Metarhizium anisopliae* and *Beauveria bassiana* isolates tested against *Macrotermes subhyalinus*

Codes	IITA codes	Fungal spp.	Year of isolation	Source of isolate/habitat	Locality	Provider/country
TT	-	*M. anisopliae*	1998	*M. subhyalinus*	Zeway	Ethiopia
S41	-	*B. bassiana*	1998	Mound soil	Zeway	Ethiopia
EE	20	*M. anisopliae*	1997	Crustacea	Alamata	AAU/Ethiopia
FF	18	*B. bassiana*	1997	Coleoptera	Ashengie	AAU/Ethiopia
GG	17	*B. bassiana*	1997	Coleoptera	Ashengei	AAU/Ethiopia
HH	16	*B. bassiana*	1997	Coleoptera	Ashengei	AAU/Ethiopia
MM	-	*M. anisopliae*	1997	Soil	Arba Minch	AAU/Ethiopia
ICIPE30	-	*M. anisopliae*	-	-	-	ICIPE/ Kenya
FI23	-	*M. anisopliae*	-	-	Mexico	CSIRO
-	ALT167	*M. anisopliae*	-	Mound soil	Atlantic	IITA/Benin
-	B82	*M. anisopliae*	-	Mound soil	Borgou	IITA/Benin
-	B122	*M. anisopliae*	-	Mound soil	Borgou	IITA/Benin
-	B124	*M. anisopliae*	-	Mound soil	Borgou	IITA/Benin
-	B149	*M. anisopliae*	-	Termite mound	Borgou	IITA/Benin
-	O64	*M. anisopliae*	-	Soil	Oueme	IITA/Benin
-	O91	*M. anisopliae*	-	Soil	Oueme	IITA/Benin
-	Z94	*M. anisopliae*	-	Mound soil	Zou	IITA/Benin
-	Z162	*M. anisopliae*	-	Termite mound	Zou	IITA/Benin
-	Z27	*M. anisopliae*	-	Termite mound	Zou	IITA/Benin

3.2.1.3 Spore production and preparation of spore suspension

Each isolate was sub-cultured 14 days before the start of an experiment, using spores of 14-day-old test tube working slope cultures. Those working slope cultures were rejected when germination was below 80%. Spores of each isolate were taken from the working culture, using a sterile steel loop and streaked on fresh separate SDA plates. The plates were again kept for 14 days at 26°C ± 2° in a dark incubator in Ethiopia, and at 28°C ± 2°C under natural light in an incubation room in Benin. The working slope cultures were returned to a fridge and kept 4°C.

A spore suspension of each isolate was prepared by harvesting conidia from the SDA plates just before application. The conidia were harvested by adding 50 ml sterile water containing 0.05% Tween 80 onto the Petri dish that contained the 14 days old cultures. The conidia suspensions of each isolate were transferred to 150 ml conical flasks and agitated in a sonicator (Kerry Ultra Sonic Ltd., Hertfordshire, UK) at 1,500 rpm for three minutes to break up the spore clumps. Conidial concentration in the suspension was determined by counting the number of spores using a Neubauer hemocytometer under a phase contrast

microscope. For the dose mortality bioassays, conidial suspensions were prepared in a 10 fold dilution series into concentrations of $1x10^4$, $1x10^5$, $1x10^6$ and $1x10^7$ conidia/ ml, using sterile distilled water containing 0.05% Tween 80 (Roberts and Yendol, 1971b).

3.2.2 Sources of termites

All experiments were carried out with workers of *M. subhyalinus.*

3.2.2.1 Ethiopia

Workers of *M. subhyalinus* were collected in Zeway, 160 km east of Addis Ababa in the Oromia Region, one day before the start of the experiment. Three to four years old active mounds were carefully broken up and distracted. Termites were collected along with some termite combs and mound material, and were stored in 25 cm diameter and 15 cm deep black and round plastic buckets with a lid that had openings at the centre covered with wire mesh. Subsequently the buckets were placed in 100 x 40 x 50 cm plastic boxes. The boxes were covered with black plastic sheets in order to mimic light conditions inside a mound.

3.2.2.2 Benin

Workers of *M. subhyalinus* were collected near Abomey, about 120 km north of Cotonou one day before the start of the experiment. Mounds were dug up and distracted and termites along with mound material and combs were collected and placed in a 15 x 25 cm plastic bucket, filled with sand up to half and covered with a plastic lid.

3.2.3 Relative pathogenicity test

3.2.3.1 Direct contamination tests on fungal cultures (Ethiopian isolates)

The aim of this experiment was to demonstrate and compare the pathogenic effect of the *M. anisopliae* isolates MM, EE, and TT and the *B. bassiana* isolates FF, GG and S41 on *M. subhyalinus.* A total of 120 active worker termites were placed to walk on 14 days old SDA cultures of each fungal isolate for five minutes, following the protocol developed by Jones *et al.* (1996). After exposure, each group of the 120 termites were carefully transferred from the culture plates onto a second sterile Petri dish to minimize transfer loss of conidia. Out of the 120 termites, groups of 30 individuals (uncontaminated termites for the control treatment) were transferred onto four glass Petri dishes (9 cm diameter) lined

with sterile moistened Whatman No. 2 filter paper. Four replicates for each isolate, i.e. Petri dishes containing the contaminated termites, were incubated in 38 x 15 cm plastic bags, lined with sterile moistened towels, in order to maintain the humidity. The opening of each bag was lightly tied to allow aeration, and the bags were then kept in a dark wooden box at 24°C ± 2°C. Using sterile Pasteur pipettes, sterile water was added to the filter papers in the Petri dishes when they became dry. Mortality was recorded daily over a period of 15 days. The dead termites from each Petri dish were transferred to separate Petri dishes lined with moist Whatman No. 2 filter paper, using forceps, and were kept in the same dark wooden box at 24°C ± 2°C. Petri dishes containing the cadavers were observed for fungal growth at least for one week. The forceps were flamed each time before transferring a new dead termite.

3.2.3.2 Direct contamination tests using spore suspensions

In these experiments, the virulence of seven *M. anisopliae* and *B. bassiana* isolates from Ethiopia (MM, EE, TT, FF, GG, HH and S41) and nine *M. anisopliae* isolates from Benin (Z162, B82, B149, B122, ATL167, Z27, Z94, O91, O64 and B124) was compared separately to two *M. anisopliae* isolates (i.e. ICIPE30 and FI23) that had been previously described be virulent to termites (Gitonga, 1996; J. Langewald, IITA Benin, pers. comm.).

Ninety termites were placed on different Petri dishes that contained 2 ml spore suspension at a concentration of 10^7 conidia/ml for each fungal isolate (or 2 ml of control suspension solution which contained sterile water and 0.05% Tween 80). The termites were rolled in the suspension for 30 seconds. The treated termites were then transferred in groups of 30 individuals onto three sterile glass Petri dishes (9 cm in diameter), lined with sterile moistened Whatman No. 2 filter paper. Petri dishes were kept at 28°C ± 2°C room temperature under natural light conditions. Sterile water, using sterile Pasteur pipettes, was added to the filter papers in the Petri dishes when they became dry. Mortality was recorded daily over a period of 15 days. The dead termites from each Petri dish were transferred to separate Petri dishes lined with moist Whatman No. 2 filter paper, using forceps, and were kept at 28°C ± 2°C room temperature under natural light conditions. Petri dishes containing cadavers were observed for fungal growth at least for one week. The forceps were flamed each time before transferring a new dead termite.

3.2.3.3 Statistical analysis

For the relative pathogenicity test the comparison of Median Survival Time (MST) was carried out using Survival Analysis followed by Wilcoxon test (SPSS, 1999). Percentage sporulating cadavers, was first subjected to an arc sin transformation and analysed using one-way-ANOVA (analysis of variance) (SPSS, 1999). Comparison of the means was carried out using the Tukey T-test. The significance level was set at $p<0.05$.

3.2.4 Assessment of dose mortality relation

3.2.4.1 Ethiopia

Four bioassays to compare the dose-time-mortality relation between different isolates were conducted in Ethiopia. The *B. bassiana* isolate S41 was not used in the dose-response bioassays because it was the least virulent isolate in the previous pathogenicity tests. In the first bioassay, the *M. anisopliae* isolates MM and EE and the *B. bassiana* isolates FF, GG and HH were used. In the second bioassay the *M. anisopliae* isolates TT and FI23 were included, where FI23 served as a standard isolate and TT as a newly isolated strain. The *B. bassiana* isolates HH and GG were discarded after the second bioassay because of their poor performance.

Spore suspensions of each isolate at concentrations of 10^4, 10^5, 10^6 and 10^7 conidia/ml (or 2 ml of control suspension solution of sterile water which contained 0.05% Tween 80) were prepared as described in section 3.2.1.3. Two ml of stock suspension of different concentrations of each fungal isolate was placed on separate sterile Petri dishes (9 cm in diameter), using a sterile Pasteur pipette. Then, 120 worker termites were placed in each Petri dish. The termites were carefully rolled in the suspension for 30 seconds. The treated termites were then transferred in groups of 30 individuals onto four sterile glass Petri dishes (9 cm in diameter) lined with sterile moistened Whatman No. 2 filter paper. The four replicates (i.e. Petri dishes) for each isolate and dose, containing the contaminated termites, were incubated in 38 x 15 cm plastic bags, lined with sterile moistened towels, in order to maintain the humidity. The opening of each bag was lightly tied to allow aeration, and the bags were then kept in a dark wooden box at 24°C ± 2°C. There were four replications per treatment. Sterile water, using sterile Pasteur pipettes, was added to the filter papers in the Petri dishes when they became dry. Mortality was recorded daily over a period of 15 days. Dead termites from each Petri dish were transferred to separate Petri

dishes lined with moist Whatman No. 2 filter paper and were kept in the same dark wooden box at 24°C ±2ºC. The Petri dishes containing the cadavers were observed for fungal growth at least for one week. The forceps were flamed each time before transferring a new dead termite.

3.2.4.2 Benin

At IITA Benin the bioassay on assessment of dose-time-mortality relation was carried out only with the four Ethiopian isolates that had yielded the significantly shortest MSTs in the preliminary tests in Benin. Those were the *M. anisopliae* isolates MM, EE and TT, and the *B. bassiana* isolate FF. The *M. anisopliae* isolates ICIPE30 and FI23 were additionally used as standard isolates.

The same experimental protocol was used as in the bioassays in Ethiopia, except that at IITA Petri dishes were kept at 28ºC ± 2ºC room temperature under natural light conditions on laboratory benches. There were three replications per isolate and dose rate.

3.2.4.3 Statistical analysis and modelling

Comparison of MSTs (data pooled across dosages) of termites treated with the suspensions of six *M. anisopliae* and *B. bassiana* isolates and non-treated termites was carried out using Survival Analysis (SPSS, 1999). Analysis and modelling of time-dose-mortality data for the six *M. anisopliae* and *B. bassiana* isolates was carried out using the Cox regression model (SSPS, 1999). Cox regression models use the hazard function to estimate the relative risk of failure. The hazard function, h(t), is an estimate of the potential for death per unit time at a particular instant, given that the case has survived until that instant (Kleinbaum, 1996, in SPSS, 1999). Cox regression models are expressed in terms of the hazard function as:

$$h(t)=[h_0(t)]e^{(BX)} \quad (1),$$

where X stands for the log (dose), B is a regression coefficient, *e* is the base of a natural logarithm and $h_0(t)$ is the baseline hazard function when X is set to 0. The cumulative hazard function, H(t), is related to the survival function and can be derived from the survival function as:

$$H(t)= -\ln S(t). \quad (2)$$

The hazard function and the survival function are closely related, and both were calculated using the Cox regression procedure (SPSS, 1999).

Estimation of the LD_{50}

The LD_{50} is defined as the dose of an agent (chemical or biological) necessary to produce death in half of the test organisms at a given time after application (Maddox, 1982). The LD_{50} can be derived from equations 1 and 2 as:

$X=10\text{^}((\ln(\ln(0.5)-\ln(h_0(t))/B$ (3)

Confidence intervals for the LD_{50} over time after application were calculated based on the same equations, using the SPSS output information (SE of B and SE of $h_0(t)$).

For percentage of sporulating cadavers the data were first subjected to an arc sin transformation and then analysed using one-way-ANOVAs (SPSS, 1999). Comparison of the means was carried out using the Tukey test. The significance level was set at $p<0.05$.

Percentage sporulated cadavers was compared between isolates, across dosages for each experiment using one-way-ANOVAs (SPSS, 1999) followed by Tukey tests. The percent sporulation in response to dose was compared across isolates, again by using one-way-ANOVAs (SPSS, 1999) followed by Tukey tests. Data on percent cadavers sporulating were available for all experiments, except for the second experiment carried out in Ethiopia.

3.2.5 Transmission of infection

This experiment was carried out at IITA Benin in order to compare cross-contamination effects between isolates and two different types of spore formulations. Additionally cross-contamination was tested at two different densities of host insects.

For the first spore formulation treatment, two 14 days old SDA cultures of each of the *M. anisopliae* isolates ICIPE30, FI23, MM, EE, and TT and the *B. bassiana* isolate FF were prepared. For each strain, two groups of 15 *M. subhyalinus* workers were placed to walk on the four separate cultures for five minutes. After exposure, each group of the 15 termites were carefully transferred from the culture plates onto a second sterile Petri dish to minimize transfer loss of conidia. Then, each of the groups of 15 contaminated insects was

separated into three groups of five contaminated termites and diverted over six Petri dishes. Each of the six Petri dishes contained healthy clean termites at different densities. Three out of the six dishes contained 30 termites. The other three contained 50 termite workers.

For the second type of formulation the experiment was repeated by rolling termites for 30 seconds in 0.5 ml of spore suspension of 10^7 conidia/ml and 0.05% Tween 80 instead of SDA cultures to contaminate the termites. For the control a blank solution of sterile water and 0.05% Tween 80 was used. All further steps were identical with the spore dust treatment.

The Petri dishes were kept at 28°C ± 2°C in the laboratory under natural light conditions on the laboratory bench. Sterile water, using sterile Pasteur pipettes, was added to the filter papers in the Petri dishes when they became dry. Mortality was recorded daily over a period of 15 days. The dead termites from each Petri dish were transferred to separate Petri dishes lined with moist Whatman No. 2 filter paper, using forceps, and were kept at 28°C ± 2°C room temperature and under natural light conditions. The Petri dishes containing the cadavers were observed for fungal growth at least for one week. The forceps were flamed each time before transferring a new dead termite.

3.2.5.1 Statistical analysis

Comparison of MSTs was carried out using Survival Analysis (SPSS, 1999) followed by Wilcoxon test, first with group size of clean exposed termites across both sources of initial contamination and secondly with source of initial contamination as independent factors across both group sizes. For the percentage of sporulating cadavers, the data were first subjected to an arc sin transformation and then analysed using one-way-ANOVAs (SPSS, 1999). Comparison of means was carried out using the Tukey test. The significance level was set at $p<0.05$.

3.2.6 Repellency test

In this experiment the repellent effect of *M. anisopliae* isolates ICIPE30 and MM and the *B. bassiana* isolate FF on workers of *M. subhyalinus* was compared.

The experimental set-up consisted of three plastic Petri dishes (9 cm in diameter), arranged around a fourth central Petri dish of similar size. Each of the three outer dishes was connected with the central dish through 2 cm long transparent plastic tubes (0.5 cm in

diameter). Twenty grams of sterile mound material and 0.5 g of termite comb were placed in each of the three outer Petri dishes. Among the three outer Petri dishes, one served as the control compartment whereas the two others were treated with two different isolates. Spores of each isolate were mixed with sufficient vegetable oil in a 50 ml beaker using a spatula to produce a thin spore paste. Three drops of spore paste of the two isolates that were compared simultaneously were placed with a spatula on the sterile mound material in separate treatment Petri dishes. Finally, 20 *M. subhyalinus* workers were added to the central Petri dish. The termites could choose to move to any compartment of their preference. The number of termites in each compartment was recorded over a total observation time of 225 minutes at 25 minutes intervals. All three isolates (i.e. ICIPE30, MM and FF) were compared pair wise. The experiment was replicated three times.

In addition, the experiments were repeated with spore dust as the potentially repellent source. Only isolate ICIPE30 and MM, which caused higher virulence in the dose-response trials (for details see section 3.4.2), were compared. The same experimental design as described above was used, and 0.25 g of spore dust of each isolate was placed on the sterile mound material in both treatment Petri dishes. The experiment was replicated three times.

3.2.6.1 Statistical analysis

The percentage of termites that were counted in the different test compartments at different time intervals was first subjected to an arc sin transformation. Comparison of means across the whole observation time was carried out using repeated measure ANOVAs. Additionally percentage termite distribution was compared at single interval level using one-way-ANOVAs followed by the Tukey (SPSS, 1999). The significance level was set at $p<0.05$.

3.3 Results

Metarhizium anisopliae and *B. bassiana* strains that were used for the dose mortality and disease transmission tests were selected based on their virulence against *M. subhyalinus* in the relative pathogenicity tests. In the relative pathogenicity tests the virulence of each strain was measured using MST and percentage of sporulating cadavers.

3.3.1 Relative pathogenicity test

3.3.1.1 MST

3.3.1.1.1 Direct contamination test on fungal cultures (Ethiopian isolates)

In Ethiopia, exposed termites were quickly contaminated and died rapidly. All the *M. anisopliae* and *B. bassiana* isolates killed termites significantly faster than in the control treatment (table 5). MSTs for the *M. anisopliae* isolate MM were significantly shorter than for the three *B. bassiana* isolates HH, GG and S41 (table 5). The MSTs between the *M. anisopliae* isolates MM, EE and the *B. bassiana* isolate FF did not differ significantly. No significant differences were recorded between EE and the three *B. bassiana* isolates FF, HH and GG. The slowest mortality was observed in termites treated with the *B. bassiana* isolate S41, resulting in the significantly longest MST across all tested isolates.

Table 5 Differences in MSTs, in days, of *Macrotermes subhyalinus* workers exposed to 14 days old SDA cultures of different strains of *Metarhizium anisopliae* and *Beauveria bassiana* in Ethiopia.

	Isolate	Control	MM	EE	FF	GG	HH
Isolate	MST	>9.00	2.03	2.24	2.29	2.42	2.38
MM[1]	2.03	S					
EE[1]	2.24	S	NS				
FF[2]	2.29	S	NS	NS			
GG[2]	2.42	S	S	NS	NS		
HH[2]	2.38	S	S	NS	NS	NS	
S41[2]	5.65	S	S	S	S	S	S

1= *M. anisopliae* isolates; 2 = *B. bassiana* isolates; S = significant at $p<0.05$ using Survival Analysis followed by Wilcoxon test; NS = not significant at $p<0.05$ using Survival Analysis followed by Wilcoxon test.

3.3.1.1.2 Direct contamination test using spore suspension (Benin isolates)

In Benin, where *M. subhyalinus* workers were rolled in spore suspensions of 10^7 spores/ml of several *M. anisopliae* and *B. bassiana* isolates, all tested *M. anisopliae* isolates killed termite workers faster compared to the control treatment (table 6). Isolate FI23 killed termites the fastest. The MST of termites treated with the Benin isolates ranged from 5.29 to 8.67 days. However, termites treated with all the IITA *M. anisopliae* isolates showed significantly longer MSTs than the ones treated with the standard isolates ICIPE30 and FI23. Consequently, the IITA *M. anisopliae* isolates were discarded for the subsequent dose response bioassays.

Table 6 Differences in MSTs, in days, of *Macrotermes subhyalinus* workers treated with 10^7 spores/ml spore suspension of isolates of different strains of *Metarhizium anisopliae* collected in Benin and two reference isolates.

	Isolate	Control	IC30	FI23	Z162	B82	B149	B122	AT167	Z27	Z94	O91	O64
Isolate	MST	>9.00	4.56	4.39	5.29	6.78	5.63	6.82	5.68	5.30	7.07	5.29	5.94
IC30*	4.56	S											
FI23*	4.39	S	S										
Z162	5.29	S	S	S									
B82	6.78	S	S	S	S								
B149	5.63	S	S	S	NS	NS							
B122	6.82	S	S	S	S	NS	NS						
ATL167	5.68	S	S	S	NS	S	NS	NS					
Z27	5.30	S	S	S	NS	S	NS	S	NS				
Z94	7.07	S	S	S	S	NS	N	NS	NS	S			
O91	5.29	S	S	S	NS	S	S	S	S	NS	S		
O64	5.94	S	S	S	S	NS	NS	NS	NS	S	SN	S	
B124	8.67	S	S	S	S	S	S	S	S	S	S	S	S

*Standard isolates; S = significant at $p<0.05$ using Survival Analysis; NS = not significant at $p<0.05$ using Survival Analysis.

3.3.1.1.3 Direct contamination test using spore suspension (Ethiopian isolates)

In the second set of the relative pathogenicity tests conducted at IITA Benin, all Ethiopian *M. anisopliae* and *B. bassiana* isolates killed termites significantly faster than the control treatment (table 7). The two *M. anisopliae* isolates MM and TT did not differ significantly in terms of MSTs from the standard isolates ICIPE30 and FI23. All *M. anisopliae* strains (ICIPE30, FI23, MM, TT, EE) caused significantly shorter MSTs compared to the *B. bassiana* isolates (FF, GG, HH, S41). No significant difference was found between the three *B. bassiana* isolates FF, GG and HH. The longest MSTs were recorded for the *B. bassiana* isolate S41 (table 7).

Table 7 Difference in MSTs, in days, of *Macrotermes subhyalinus* workers treated with 10^7 spores/ml spore suspension of Ethiopian *Metarhizium anisopliae* and *Beauveria bassiana* and two standard isolates.

	Isolate	Control	IC30	FI23	MM	EE	TT	FF	GG	HH
Isolate	MST	>9.00	3.86	3.66	3.63	4.41	3.95	4.88	5.39	5.00
IC30*	3.86	S								
FI23*	3.66	S	NS							
MM[1]	3.63	S	NS	NS						
EE[1]	4.41	S	S	S	S					
TT[1]	3.95	S	NS	NS	NS	S				
FF[2]	4.88	S	S	S	S	S	S			
GG[2]	5.39	S	S	S	S	S	S	NS		
HH[2]	5.00	S	S	S	S	S	S	NS	NS	
S41[2]	5.93	S	S	S	S	S	S	S	S	S

*Standard *M. anisopliae* isolates; 1 = *M. anisopliae* isolates; 2 = *B. bassiana* isolates; S = significant at $p<0.05$ using Survival Analysis followed by Wilcoxon test; NS = not significant at $p<0.05$ using Survival Analysis followed by Wilcoxon test.

3.3.1.2 Percentage sporulating cadavers in all termites treated

3.3.1.2.1 Direct contamination test on fungal cultures (Ethiopian isolates)

In Ethiopia, after exposure of *M. subhyalinus* workers to SDA cultures of the *M. anisopliae* isolates MM and EE and the *B. bassiana* isolate GG, 100% of the cadavers sporulated (table 8a). The percentage of sporulating cadavers resulting from exposure to *B. bassiana* isolates FF, HH, and S41 was lower. However, these differences were not significant (table 8a).

3.3.1.2.2 Direct contamination test using spore suspension (Benin isolates)

Termites' cadavers resulting from treatments with the 11 Benin *M. anisopliae* isolates at IITA showed a significantly lower percentage sporulation than termite cadavers resulting

from the treatment with the standard *M. anisopliae* isolates ICIPE30. Only two Benin isolates (Z162 and B122) caused significantly similar sporulation as the reference isolate FI23 (table 8b). All treatments with *M. anisopliae* isolates from Benin did cause significantly the same proportion of sporulation on termite cadavers.

3.3.1.2.3 Direct contamination test using spore suspension (Ethiopian isolates)

Termite cadavers resulting from treatments with *M. anisopliae* isolates, including the two standard isolates and the *B. bassiana* isolates FF and GG sporulated at significantly similar proportions. The isolate S41 sporulated only on 1.67% of the cadavers and differed significantly from the two standard isolates and isolates MM and EE (table 8c). In general, termites in Benin inoculated with spore suspensions of *M. anisopliae* and *B. bassiana* isolates developed considerably less mycosis than termites in Ethiopia that were exposed to dry spores on Petri dish cultures of the same isolates.

3.3.2 Dose-mortality-time interaction assessments

3.3.2.1 MST across dose rates and Cox regression model fits

3.3.2.1.1 MST across doses rates

All the *M. anisopliae* and *B. bassiana* isolates caused significantly shorter MSTs across dosages than in the control treatments in all bioassays performed (table 9), except for isolate HH in the second bioassay carried out in Ethiopia (table 9b).

In Ethiopia, in the first bioassay the same isolate HH caused the significantly shortest MST across dosages in *M. subhyalinus* workers (table 9a). There was no significant difference in MSTs across dosages in termites treated with isolates MM and FF, and these isolates caused significantly shorter MSTs across dosages than isolates EE and GG. Termites treated with isolates EE and GG had significantly different MSTs across dosages, and termites exposed to isolate GG having the significantly longest MST across dosages of all tested isolates (table 9a).

Table 8 Percentage of sporulating cadavers of *Macrotermes subhyalinus* workers exposed to different strains of *Metarhizium anisopliae* and *Beauveria bassiana* in Ethiopia on 14 days old SDA cultures (a) and in Benin on 10^7 spores/ml spore suspension (b and c).

a.

Isolates	Percentage sporulating cadavers ± SE		
MM[1]	100.00	±	0.00a
EE[1]	100.00	±	0.00a
FF[2]	75.00	±	3.96a
HH[2]	100.00	±	0.00a
GG[2]	88.00	±	1.55a
S41[2]	70.00	±	2.19a

b.

Isolates	Percentage sporulating cadavers ± SE		
ICIPE30*	81.67	±	0.04a
FI23*	61.67	±	0.21ab
Z162[1]	23.33	±	1.60bc
B82[1]	8.33	±	0.33c
B149[1]	13.33	±	1.37c
B122[1]	21.67	±	0.61bc
ATL167[1]	11.67	±	1.00c
Z27[1]	11.67	±	1.00c
Z94[1]	5.00	±	1.00c
O91[1]	5.00	±	1.00c
O64[1]	5.00	±	1.00c
B124[1]	18.33	±	1.10c

c.

Isolates	Percentage sporulating cadavers ± SE		
ICIPE30*	75.00	±	2.00a
FI23*	63.33	±	1.15ab
MM[1]	56.67	±	1.23ab
EE[1]	36.67	±	2.49ab
TT[1]	61.67	±	0.03abc
FF[2]	38.33	±	2.47abc
HH[2]	15.00	±	3.6bc
GG[2]	30.00	±	2.14abc
S41[2]	1.67	±	2.32c

*Standard isolates; 1 = *M. anisopliae* isolates; 2 = *B. bassiana* isolates. Means followed by the same letter, in the same column are not significantly different at $p<0.05$, using the Tukey test. Data were subjected to arc sin transformation before analysis of variance.

In the second bioassay, isolates FI23 and FF caused significantly the shortest MSTs across all tested dosages than all other isolates and the control. There was no significant difference in MSTs across dosages in termites treated with isolates MM, TT and GG, and all these isolates had significantly shorter MSTs across dosages than isolates EE and HH. Termites treated with isolates EE and HH responded with significantly different MSTs across dosages, and termites exposed to isolate HH did not die significantly faster than in the control (table 9b).

In the third bioassay termites exposed to the *M. anisopliae* isolates EE, MM and TT did not differ significantly and caused significantly shorter MSTs across dosages than *B. bassiana* isolate FF (table 9c).

In the fourth bioassay, MST values across dosages in the MM and TT treatments did not differ significantly, and had the shortest MSTs across dosages compared to the other isolates tested. MSTs across dosages of isolates EE, FF and FI23 differed significantly, with isolate FI23 having the significantly longest MST across dosages (table 9d).

In the fifth experiment, carried out at IITA Benin, termites exposed to the average dose of isolates ICIPE30, MM, and EE and on the other hand isolates FI23 and TT did not differ significantly in MSTs across doses and had significantly shorter MSTs than isolate FI23 (table 9e). The *B. bassiana* isolate FF caused the significantly longest MSTs.

3.3.2.1.2 Cox regression model fits

Except for isolates FI23, MM, EE, TT, and GG in the second, and isolates FI23 and TT in the fourth and FF in the fifth experiment, Cox regression analysis indicated that different fungal dosage levels were a significant and independent predictor of termite survival ($p< 0.05$) (table 10) and that the model provided a rather good fit.

3.3.2.1.2.1 B-values

The B-value stands for differences in dose response, not absolute dose response. In dose response tests carried out in Ethiopia, isolates FI23, MM, EE and GG in the second bioassay isolates FI23 and TT in the fourth bioassay and FF in the fifth bioassay carried out at IITA Benin had non-significant B-values (table 10). Termites treated with these isolates, responded with MSTs that were not related with dose (fig. 5, 7 and 8). Termites treated with isolates with significant but comparatively low B-values such as HH in the first isolate, FF in the third and isolate MM and FF in the fourth bioassays also showed little dose response in survival (fig. 4, 6 and 7). There was a stronger dose response in termite survival in isolates that had higher B-values (isolate MM and FF in the first, isolates MM, EE and TT in the third and isolate EE in the fourth bioassays) (fig. 4, 6 and 7). The strongest dose responses were obtained with B-values of 0.461 and 0.522 with the isolates EE and GG in the first bioassay (fig. 4). In the dose response bioassay conducted at IITA, the *M. anisopliae* isolates ICIPE30, FI23, MM and EE had B-values that ranged

from 0.362 to 0.461 (table 10) (fig. 8). These isolates showed a stronger dose response. As in the first bioassay carried out in Ethiopia, isolate TT had the largest B-value of all isolates tested (0.743) and thus resulted in the strongest dose response (fig. 8).

3.3.2.1.2.2 Baseline cumulative hazard

In dose response tests carried out in Ethiopia, in the fourth bioassay isolates TT and MM caused very steep slope baseline cumulative hazard curves for termites (fig. 7). Termites exposed to isolates GG, MM, EE, FI23, TT and FF in the second, and isolates FI23 and FF in the fourth bioassays responded in less steep slope baseline cumulative hazard curves than termites exposed to isolates TT and MM in the fourth bioassay (fig. 5 and 7). Isolates that caused moderately steep slope baseline cumulative hazard curves were HH, MM, and FF in the first (fig. 4), HH in the second (fig. 5), FF, MM, EE and TT in the third (fig. 6), and EE in the fourth bioassays (fig. 7). Isolates EE and GG in the first bioassay caused almost flat baseline cumulative hazard curves for termites (fig. 4). In the fifth bioassay carried out at IITA Benin, *B. bassiana* isolate FF caused a steep slope and likewise a non-significant B-value (fig. 8). Isolates ICIPE30, EE, FI23 and MM caused moderately steeper slopes and isolate TT an almost flat baseline cumulative hazard curve for termite mortality.

3.3.2.1.2.3 LD_{50} over time

LD_{50} plots of *M. anisopliae* and *B. bassiana* isolates of the bioassays conducted in Ethiopia are presented in figures 4, 5, 6 and 7. The Cox regression model for the LD_{50} curves did not fit the data well for isolates with non-significant B-values, which is reflected by large confidence intervals (fig. 5 and 7). These isolates are isolates FI23, MM, EE and GG in the second (fig. 5) and isolates FI23 and TT in the fourth bioassays (figs 7). LD_{50} curves for isolates that had significant but comparatively small B-values ($B<0.218$), like isolates HH and FF in the first bioassay (fig. 4), isolate FF in the second (fig. 5), isolates FF in the third (fig. 6) and isolates FF and MM in the fourth bioassays (fig. 7), had relatively large intercept values and a steep slope. LD_{50} curves resulting from treatments with isolates that had comparatively median B-values, like isolate MM in the first and third (fig. 4 and 6), isolate TT in the third (fig. 6) and isolate EE in the third and fourth bioassay (fig. 7), had relatively lower LD_{50} values, both at the beginning (low intercept values) and at the end of the observation time (low negative rate). Isolates EE and GG in the first bioassay had the highest B-values (0.461 and 0.522), relatively lower LD_{50} values at the beginning (low

intercept values), with small change in LD_{50} over the observation period. These isolates had flatter LD_{50} curves (fig. 4).

LD_{50} plots of the *M. anisopliae* and *B. bassiana* isolates tested in the bioassay at IITA Benin are shown in figure 8. The Cox regression model did not fit the data well for isolate FF (fig. 8). The lack of dose response results in large confidence interval and a steep LD_{50} curve (fig. 8). Isolates that had B-values ranging from 0.362 to 0.461, i.e. isolates ICIPE30, FI23, MM and EE, had relatively lower LD_{50} values at the beginning and in the following days of the experiments (fig. 8). Isolate TT possessed a relatively large B-value (table 10). LD_{50} values were relatively lower for isolate TT at the beginning of the bioassay (low intercept), with small changes in LD_{50} levels throughout the observation period, resulting in a flat LD_{50} curve (fig 8).

Table 9 a-d Difference in MSTs (in days) of *Macrotermes subhyalinus* workers exposed to Ethiopian *Metarhizium anisopliae* and *Beauveria bassiana* isolates (data pooled across dosages) and non-treated termites.

a	Isolate	Control	MM	EE	FF	GG
Isolate	MST	15.80	6.48	7.36	6.76	7.85
MM[1]	6.48	S				
EE[1]	7.36	S	S			
FF[2]	6.76	S	NS	S		
GG[2]	7.85	S	S	S	S	
HH[2]	5.63	S	S	S	S	S

.

b	Isolate	Control	FI23*	MM	EE	TT	FF	GG
Isolate	MST	8.29	4.38	4.93	5.79	4.99	4.74	4.85
FI23*	4.38	S						
MM[1]	4.93	S	S					
EE[1]	5.79	S	S	S				
TT[1]	4.99	S	S	NS	S			
FF[2]	4.74	S	NS	S	S	S		
GG[2]	4.85	S	S	NS	S	NS	S	
HH[2]	7.09	NS	S	S	S	S	S	S

c	Isolate	Control	MM	EE	TT
Isolate	MST	9.79	5.47	5.19	4.92
MM[1]	5.47	S			
EE[1]	5.19	S	NS		
TT[1]	4.92	S	NS	NS	
FF[2]	5.84	S	S	S	S

d	Isolate	Control	FI23	MM	EE	TT
Isolate	MST	6.69	5.34	3.56	4.49	3.55
FI23*	5.34	S				
MM[1]	3.56	S	S			
EE[1]	4.49	S	S	S		
TT[1]	3.55	S	S	NS	S	
FF[2]	4.06	S	S	S	S	S

e	Isolate	Control	ICIPE30	FI23	MM	EE	TT
Isolate	MST	14.29	4.74	5.75	4.76	4.74	4.91
ICIPE30*	4.74	S					
FI23*	5.75	S	S				
MM[1]	4.76	S	NS	S			
EE[1]	4.74	S	NS	S	NS		
TT[1]	4.91	S	S	NS	S	S	
FF[2]	6.74	S	S	S	S	S	S

*Standard *M. anisopliae* isolates; 1 = *M. anisopliae* isolates; 2 = *B. bassiana* isolates; S = significant at $p<0.05$; NS = not significant at $p<0.05$, using Survival Analysis followed by Wilcoxon test; assays a-d = dose response bioassays conducted in Ethiopia; assay e = dose response bioassay conducted at IITA Benin.

Table 10 Model estimates for B-values resulting from Cox regressions for all different isolates of *Metarhizium anisopliae* and *Beauveria bassiana* tested in bioassays in Ethiopia (a-d) and Benin (e), including Wald's coefficient.

a					
Isolates	B	SE	Wald	df	Sig.
MM[1]	0.228	0.043	28.044	1	0.000.
EE[1]	0.458	0.048	90.839	1	0.000
FF[2]	0.249	0.046	28.044	1	0.000
GG[2]	0.522	0.049	114.238	1	0.000
HH[2]	0.170	0.045	14.018	1	0.000
b					
FI23*	0.084	0.044	3.630	1	0.057
MM[1]	0.057	0.042	1.819	1	0.178
EE[1]	0.071	0.048	2.230	1	0.135
TT[1]	0.089	0.044	4.059	1	0.044
FF[2]	0.111	0.043	6.730	1	0.009
GG[2]	0.021	0.047	0.191	1	0.662
HH[2]	0.188	0.049	14.844	1	0.000
c					
MM[1]	0.218	0.043	26.339	1	0.000
EE[1]	0.231	0.043	28.182	1	0.000
TT[1]	0.367	0.045	66.278	1	0.000
FF[2]	0.142	0.043	11.104	1	0.001
d					
FI23*	0.014	0.042	0.113	1	0.737
MM[1]	0.080	0.040	3.990	1	0.046
EE[1]	0.357	0.045	64.023	1	0.000
TT[1]	0.082	0.042	3.735	1	0.053
FF[2]	0.103	0.041	6.302	1	0.012
e					
ICIPE30*	0.362	0.065	32.687	1	0.000
FI23*	0.382	0.067	32.687	1	0.000
MM[1]	0.461	0.067	40.973	1	0.000
EE[1]	0.429	0.067	47.308	1	0.000
TT[1]	0.743	0.074	99.857	1	0.000
FF[2]	0.093	0.057	2.663	1	0.102

*Standard *M. anisopliae* isolates; 1 = *M. anisopliae* isolates; 2 = *B. bassiana* isolates; B = Cox regression B-values; SE = standard error; df = degrees of freedom; Sig. = significance level; assays a-d = dose response bioassays conducted in Ethiopia; assay e = dose response bioassay conducted at IITA Benin.

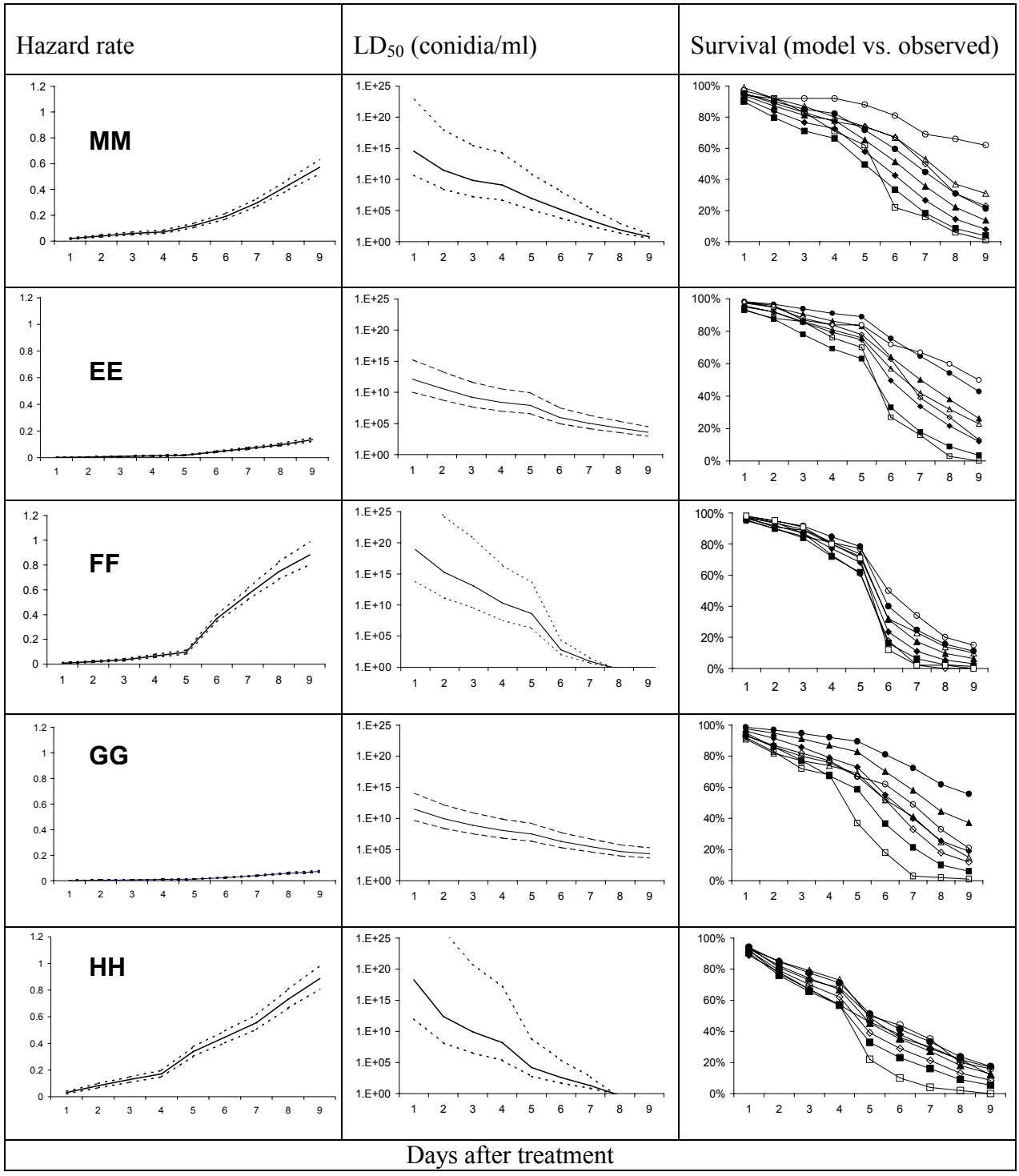


Figure 4 Plots of the baseline cumulative hazard values, LD_{50} values, and modelled versus observed values of the cumulative survival of *Macrotermes subhyalinus* workers after treatment with different dosages of entomopathogenic fungi in the first bioassay carried out in Ethiopia; o, Δ, ◊ and □ observed 10^4, 10^5, 10^6 and 10^7 dose response values respectively; ●; ▲; ♦ and ■ estimated dose response values respectively.

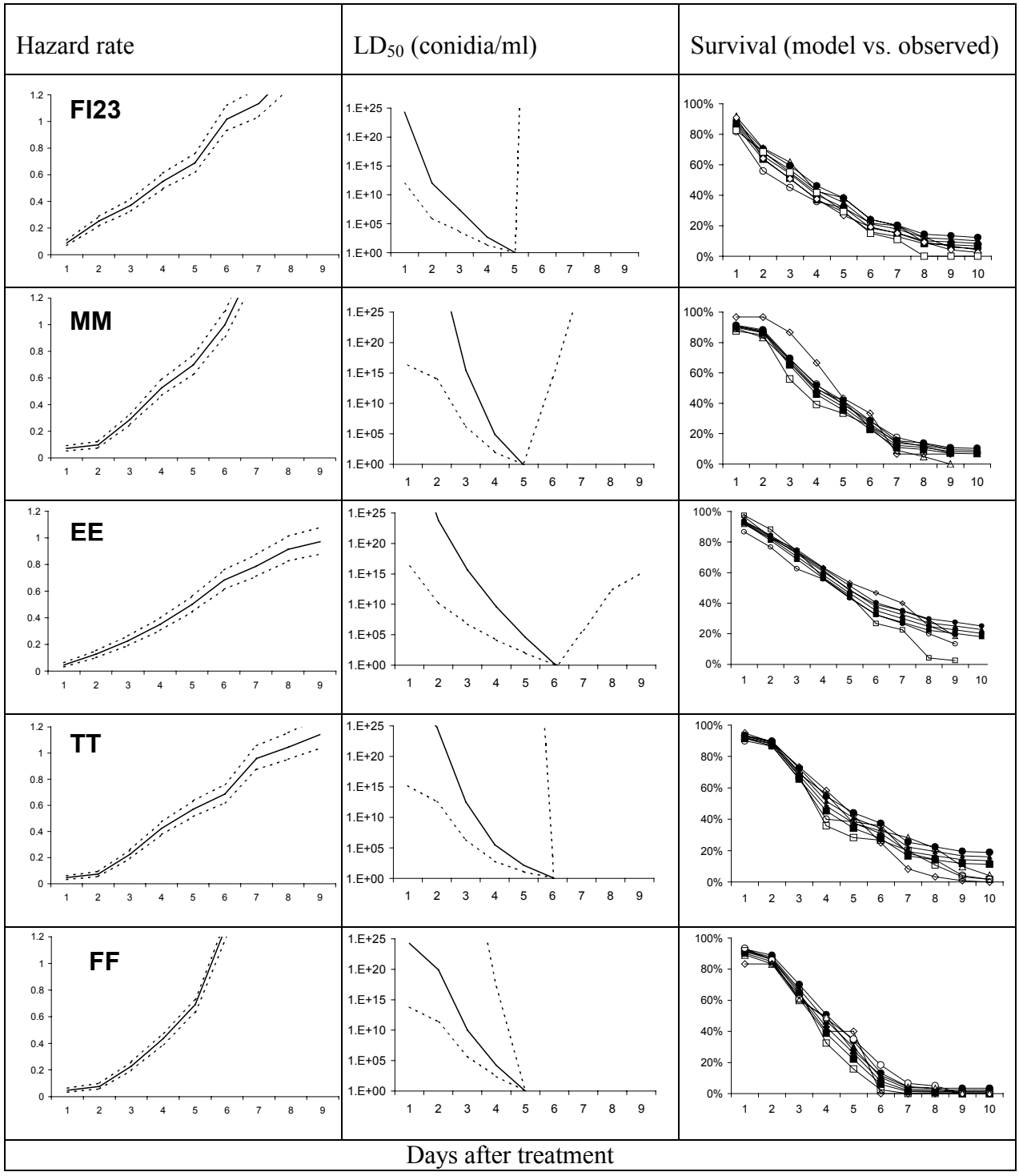


Figure 5 Plots of the baseline cumulative hazard values, LD_{50} values, and modelled versus observed values of the cumulative survival of *Macrotermes subhyalinus* workers after treatment with different dosages of entomopathogenic fungi in the second bioassay carried out in Ethiopia; o, Δ, ◊ and □ observed 10^4, 10^5, 10^6 and 10^7 dose response values respectively; ●; ▲; ♦ and ■ estimated dose response values respectively.

Figure 5 contd.

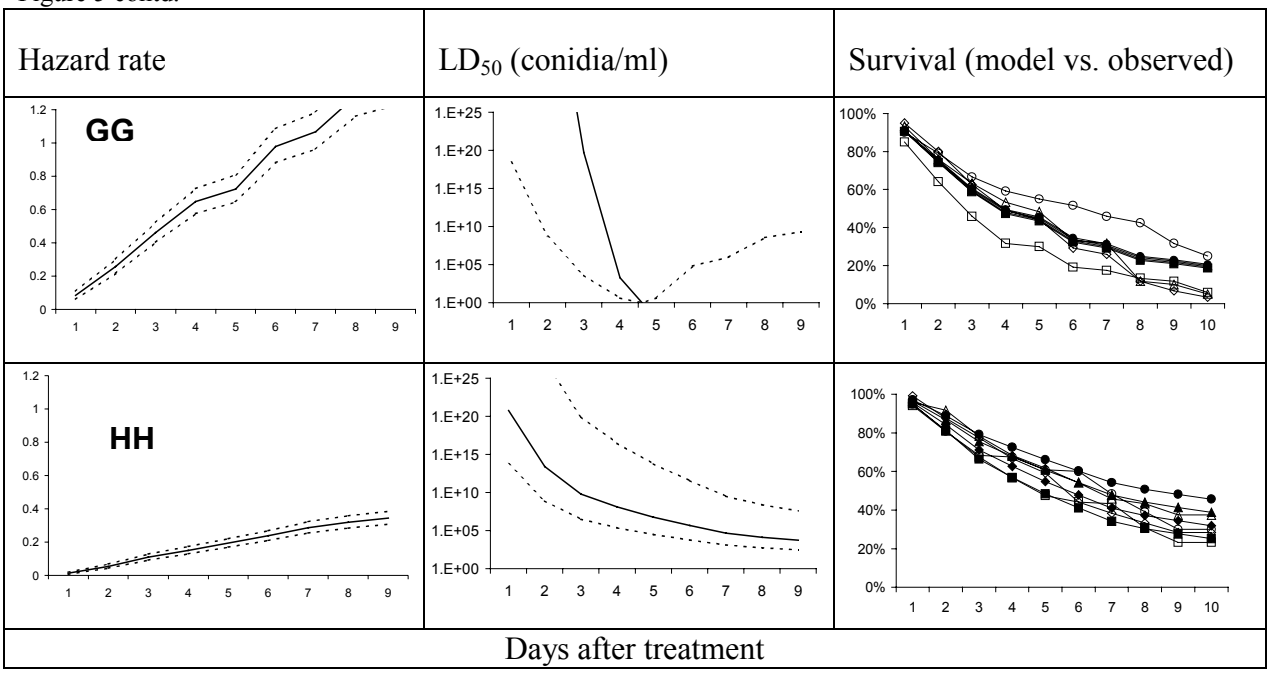


Figure 5 Plots of the baseline cumulative hazard values, LD_{50} values, and modelled versus observed values of the cumulative survival of *Macrotermes subhyalinus* workers after treatment with different dosages of entomopathogenic fungi in the second bioassay carried out in Ethiopia; o, Δ, ◊ and □ observed 10^4, 10^5, 10^6 and 10^7 dose response values respectively; ●; ▲; ♦ and ■ estimated dose response values respectively.

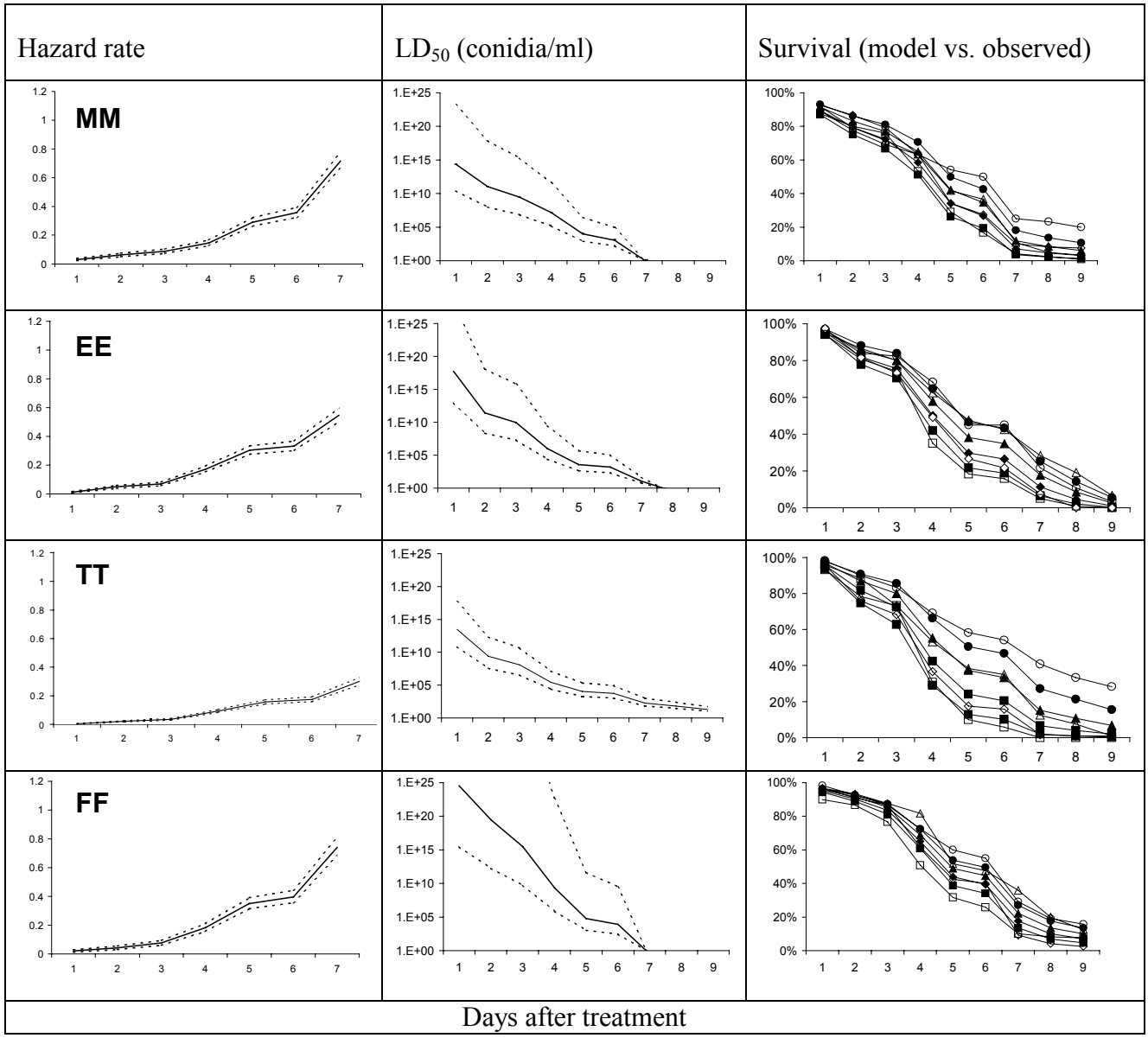


Figure 6 Plots of the baseline cumulative hazard values, LD_{50} values, and modelled versus observed values of the cumulative survival of *Macrotermes subhyalinus* workers after treatment with different dosages of entomopathogenic fungi in the third bioassay carried out in Ethiopia; o, Δ, ◊ and □ observed 10^4, 10^5, 10^6 and 10^7 dose response values respectively; ●; ▲; ♦ and ■ estimated dose response values respectively

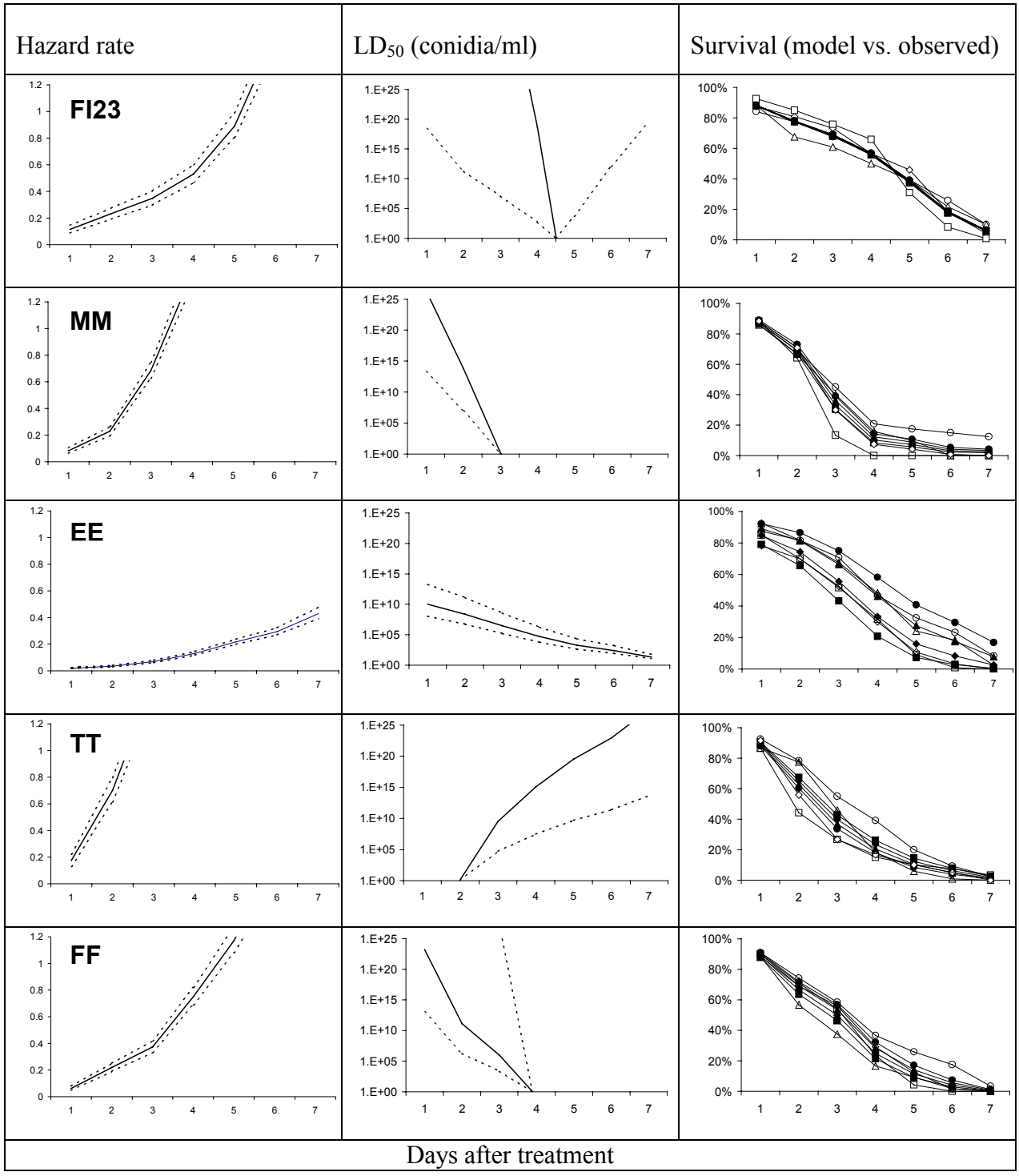


Figure 7 Plots of the baseline cumulative hazard values, LD_{50} values, and modelled versus observed values of the cumulative survival of *Macrotermes subhyalinus* workers after treatment with different dosages of entomopathogenic fungi in the fourth bioassay carried out in Ethiopia; o, Δ, ◊ and □ observed 10^4, 10^5, 10^6 and 10^7 dose response values respectively; ●; ▲; ♦ and ■ estimated dose response values respectively

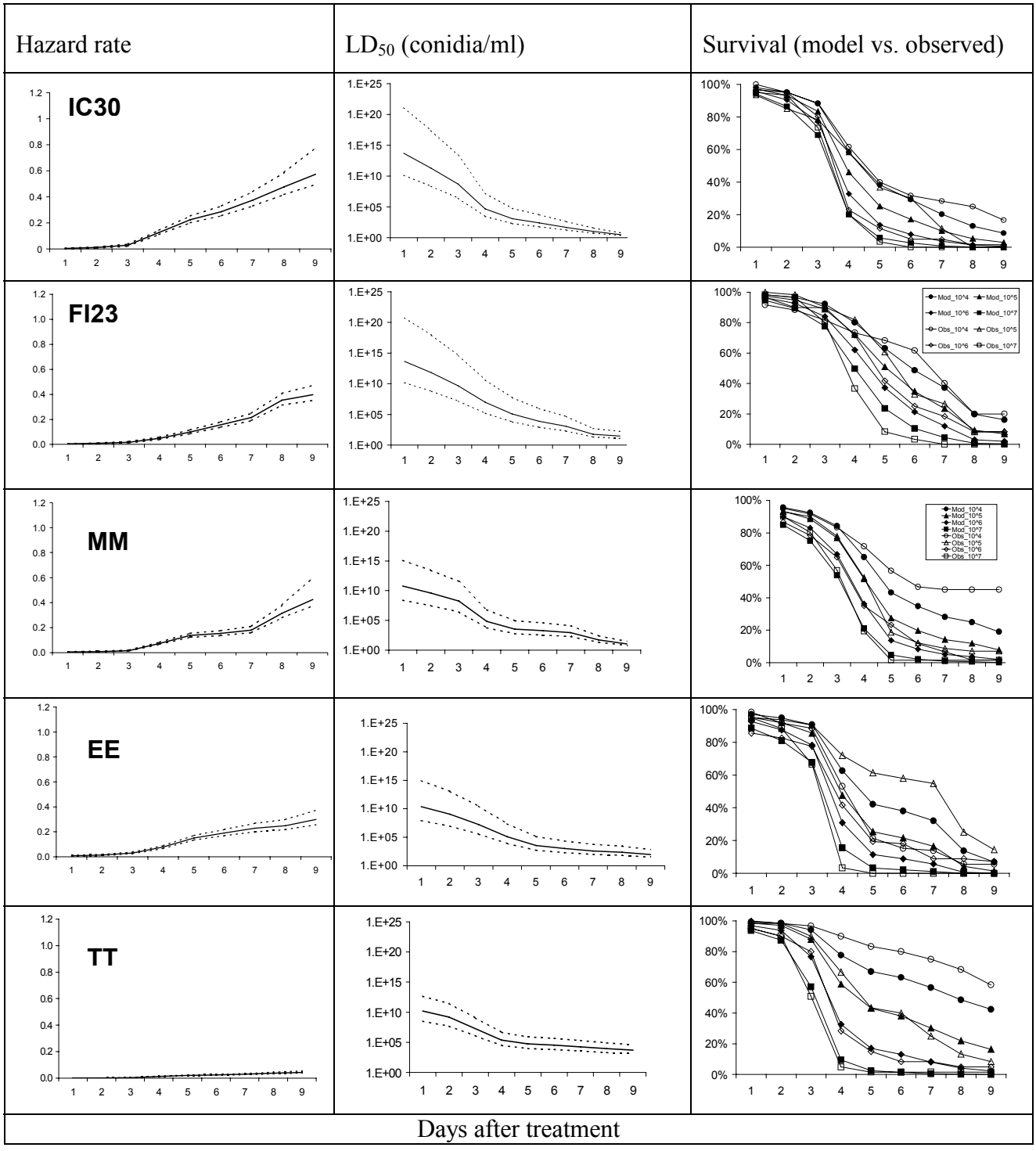


Figure 8 Plots of the baseline cumulative hazard values, LD_{50} values, and modelled versus observed values of the cumulative survival of *Macrotermes subhyalinus* workers after treatment with different dosages of entomopathogenic fungi in the bioassay carried out at IITA, Benin; o, Δ, ◊ and □ observed 10^4, 10^5, 10^6 and 10^7 dose response values respectively; ●; ▲; ♦ and ■ estimated dose response values respectively

Figure 8. contd.

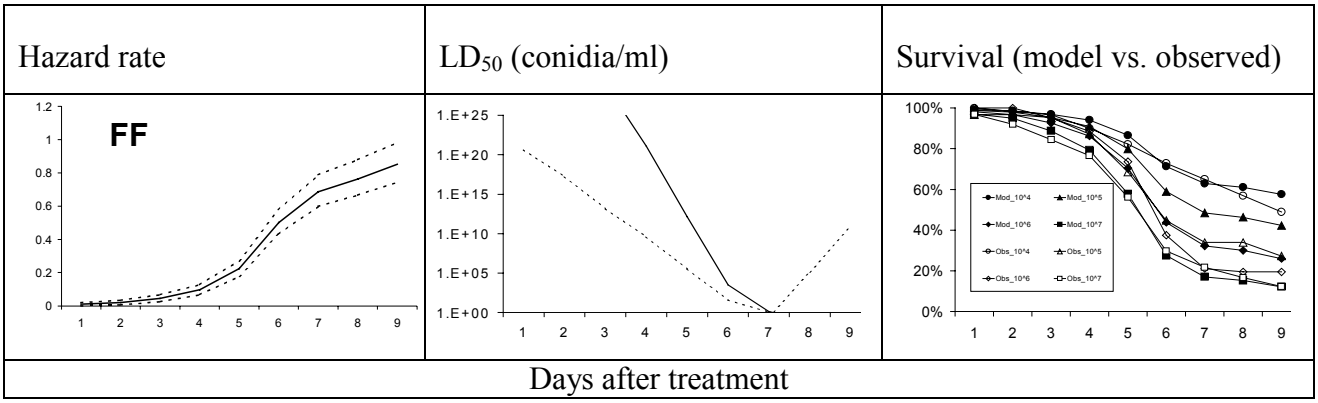


Figure 8 Plots of the baseline cumulative hazard values, LD_{50} values, and modelled versus observed values of the cumulative survival of *Macrotermes subhyalinus* workers after treatment with different dosages of entomopathogenic fungi in the bioassay carried out at IITA, Benin; o, Δ, ◊ and □ observed 10^4, 10^5, 10^6 and 10^7 dose response values respectively; ●; ▲; ♦ and ■ estimated dose response values respectively.

3.3.2.1.3 Percentage MSTs across dose rates and bioassays

When compared across all bioassays, the isolates MM and TT caused shortest MSTs across dosages followed by isolates FF and EE. However, the differences between most isolates were small. Termites treated with the *B. bassiana* isolate HH had the longest percentage MST (table 11).

Table 11 Percentage MSTs of termites treated with different isolates of *Metarhizium anisopliae* and *Beauveria bassiana* compared with non-treated termites, in five different bioassays (calculated from values in tables 10).

Isolate	assay a	assay b	assay c	assay d	assay e	Average
ICIPE30*	-	-	-	-	33.17	33.17**
MM[1]	41.09	59.47	55.87	53.20	33.31	48.59
TT[1]	-	60.19	50.26	53.06	34.36	49.47
EE[1]	46.67	69.84	53.01	67.10	33.17	53.96
FI23*	-	52.83	-	79.82	40.24	57.63
FF[2]	42.87	57.18	59.65	60.69	47.17	53.51
GG[2]	49.77	58.50	-	-	-	54.14
HH[2]	35.70	85.52	-	-	-	60.61

*Standard isolates; 1 = *M. anisopliae* isolates; 2 = *B. bassiana* isolates; assays a-d = dose response bioassays conducted in Ethiopia; assay e = dose response bioassay conducted at IITA Benin. **Only tested in one assay.

3.3.2.2 Percentage sporulation

3.3.2.2.1 Percentage sporulating cadavers of treated *Macrotermes subhyalinus* workers

In the first bioassay conducted in Ethiopia, the *M. anisopliae* isolate MM lead to a significant higher sporulation rate on termite cadavers compare to all tested *B. bassiana* isolates. No significant differences were recorded among the two *M. anisopliae* isolates MM and EE, and also between isolates EE and GG and the *B. bassiana* isolates GG, FF and HH (table 12a,a). No sporulation was observed on cadavers in the second bioassay (table 12a,b). Isolates did not differ significantly in bioassay three (table 12a,c). The significantly highest sporulation rate in bioassay four was obtained for the *M. anisopliae* isolate MM (table 12a,d). No significant differences in sporulation rate were recorded among the isolates TT, EE and FF. The isolates FI23, EE and FF caused the significant lowest percent sporulation in cadavers. The percentage of sporulating cadavers in the three bioassays ranged from 20.4-86.4%.

In the bioassay carried out at IITA Benin, the percentage sporulating cadavers of *M. subhyalinus* workers ranged from 35.4-59.9% (table 12a,e). There were no significant differences in percentage sporulating cadavers of *M. subhyalinus* among the tested isolates.

3.3.2.2.2 Sporulation across dosages

When the data was pooled across isolates, a significant difference in percentage sporulating cadavers was observed among different dose rates (table 12b). In the first bioassay, at a dose rate of 10^7 spores/ml, percentage sporulating cadavers was significantly higher compared to the 10^6, 10^5 and 10^4 spores/ml application (table 12b,a). The cadavers treated with the lowest dose (10^4 spores/ml) did produce significantly less mycoses compared to the two higher dosages. No cadavers sporulated during the second experiments (table 12b,b). In the third bioassay (table 12b,c), at a dose rate of 10^7 spores/ml, percentage sporulating cadavers were significantly higher than with lower dosages (table 12b,d). No significant differences in percent sporulating cadavers were recorded among dose rates in the fourth bioassay carried out in Ethiopia (table 12b,d).

In the bioassay carried out at IITA Benin, at a dose rate of 10^7 and 10^6 spores/ml percentage sporulating cadavers was significantly higher compared to 10^5 and 10^4 spores/ml spore concentration (table 12b,e).

3.3.2.2.3 Percentage sporulating cadavers of termites across all doses and bioassays

Termites treated with isolate MM yielded the highest percentage of sporulating cadavers when data were compared across four bioassays, excluding the second bioassay because of lack of sporulating cadavers (table 13). Isolate FI23 yielded a considerably lower percentage of sporulating cadavers.

Table 12 Percentage sporulating cadavers of *Macrotermes subhyalinus* workers (a) across all dosages (b) across all isolates of the *Metarhizium anisopliae* and *Beauveria bassiana* isolates.

a.

a Isolates	Percentage sporulating cadavers	± SE	
MM[1]	86.40	±	0.05a
EE[1]	58.75	±	0.30ab
FF[2]	31.64	±	1.60b
HH[2]	43.54	±	1.00b
GG[2]	35.42	±	1.00b
b Isolate	Percentage sporulating cadavers	± SE	
FI23*	0	±	0a
MM[1]	0	±	0a
EE[1]	0	±	0a
TT[1]	0	±	0a
FF[2]	0	±	0a
GG[2]	0	±	0a
HH[2]	0	±	0a
c Isolates	Percentage sporulating cadavers	± SE	
MM[1]	26.25	±	1.03a
EE[1]	20.83	±	0.70a
TT[1]	23.75	±	1.01a
FF[2]	3.13	±	0.06a
d Isolates	Percentage sporulating cadavers	± SE	
FI23*	20.42	±	0.40c
MM[1]	82.71	±	0.31a
EE[1]	37.29	±	0.48bc
TT[1]	47.08	±	0.51b
FF[2]	34.58	±	0.29bc
e Isolates	Percentage sporulating cadavers	± SE	
ICIPE30*	59.90	±	0.24a
FI23*	47.00	±	0.90a
MM[1]	57.00	±	0.50a
EE[1]	37.80	±	0.65a
TT[1]	48.00	±	1.22a
FF[2]	35.40	±	0.65a

*= standard isolates; 1 = *M. anisopliae* isolates; 2 = *B. bassiana* isolates; a-d = dose response bioassays conducted in Ethiopia; e = dose response bioassay conducted in Benin; means followed by the same letter, in the same column are not significantly different at $p<0.05$, using the Tukey test. Data were subjected to arc sin transformation before analysis of variance.

b. Table 12 contd.

a Dose spores/ml	Percentage sporulating cadavers	± SE
10^4	21.39	± 0.04c
10^5	37.67	± 1.00bc
10^6	52.67	± 1.00b
10^7	73.06	± 1.00a
b Dose spores/ml	Percentage sporulating cadavers	± SE
10^4	0	± 0a
10^5	0	± 0a
10^6	0	± 0a
10^7	0	± 0a
c Dose spores/ml	Percentage sporulating cadavers	± SE
10^4	4.58	± 0.31b
10^5	5.00	± 0.21b
10^6	18.96	± 1.00b
10^7	42.42	± 0.23a
d Dose spores/ml	Percentage sporulating cadavers	± SE
10^4	36.83	± 0.37a
10^5	39.83	± 0.42a
10^6	43.33	± 0.64a
10^7	59.17	± 0.95a
e Dose spores/ml	Percentage sporulating cadavers	± SE
10^4	24.27	± 0.13b
10^5	30.87	± 0.32b
10^6	59.59	± 0.20a
10^7	72.71	± 0.21a

*= standard isolates; 1 = *M. anisopliae* isolates; 2 = *B. bassiana* isolates; a-d = dose response bioassays conducted in Ethiopia; e = dose response bioassay conducted in Benin; means followed by the same letter, in the same column are not significantly different at $p<0.05$, using the Tukey test. Data were subjected to arc sin transformation before analysis of variance.

Table 13 Percentage sporulating cadavers of *Macrotermes subhyalinus* workers treated with four dose rates of *Metarhizium anisopliae* and *Beauveria bassiana*, data pooled across the bioassays and dose rates tested (calculated from values in tables 12a).

Isolate	assay a	assay c	assay d	assay e	Average
ICIPE30*	-		-	33.33	33.33[3]
MM[1]	86.40	26.25	82.71	39.72	58.77
EE[1]	58.75	20.83	37.29	26.11	35.60
TT[1]	31.64	23.75	47.08	32.22	33.67
FF[2]	-	3.13	34.58	24.72	30.14
FI23*	-		20.42	31.39	26.01
GG[2]	43.54		-	-	43.54[3]
HH[2]	35.42		-	-	35.42[3]

*Standard isolates; 1 = *M. anisopliae* isolate; 2 = *B. bassiana* isolate; assays a-d = dose response bioassay conducted in Ethiopia; assay e = dose response bioassay conducted in Benin; 3 = percentage sporulating cadaver of one bioassay.

3.3.3 Transmission of infection (cross contamination trials)

3.3.3.1 MST

The MSTs of groups of healthy *M. subhyalinus* workers exposed to termites inoculated with either spore dust or spore suspension of *M. anisopliae* and *B. bassiana* are presented in tables 14, 15 a, b, c and d.

Cross-contamination was generally significantly more effective using dry spores from SDA plates as source of initial contamination, as compared with spore suspensions when termites in smaller or lager groups were exposed (table 14). When comparing the MSTs between small (30 individuals) and large groups (50 individuals) of healthy termites exposed to inoculated termites across the two sources of initial contamination, larger groups died significantly faster than smaller groups (table 14).

Table 14 MSTs of larger and smaller groups of healthy *Macrotermes subhyalinus* workers exposed to termites contaminated with spores of SDA cultures or spore suspension of different strains of *Metarhizium anisopliae* and *Beauveria bassiana*; data pooled across all isolates.

Isolates	No. of termites/Petri dish	MST for different formulations (days)		Significance level between sources of contamination
		Spore suspension	Dry spores	
Control	50a	7.72	7.60	
	30a	7.78	7.60	NS
ICIPE30*	50a	5.21	2.94	
ICIPE30*	30b	6.57	3.65	S
FI23*	50a	4.73	3.84	
FI23*	30b	5.47	4.49	S
MM[1]	50a	4.22	2.50	
MM[1]	30b	6.00	3.60	S
EE[1]	50a	5.33	3.68	
EE[1]	30b	6.13	4.32	S
TT[1]	50a	5.07	3.15	
TT[1]	30b	5.88	3.92	S
FF[2]	50a	7.56	9.00	
FF[2]	30b	4.89	5.2	S

*Standard isolates; 1 = *M. anisopliae* isolates; 2 = *B. bassiana* isolates; MSTs following exposure to different formulations S = significant at $p<0.05$, NS = not significant at $p<0.05$ using Survival Analysis followed by Wilcoxon test; MSTs for group sizes followed by the same letter are not significantly different at $p<0.05$ using Survival Analysis followed by Wilcoxon test.

The results for the comparison of isolates at an individual level for all four treatments combinations are presented in table 15a-c. When using spore dust as source of contamination for initial infection, in the *M. anisopliae* isolates significantly lower MSTs

were recorded compared with the uncontaminated control groups (table 15a and c). When using spore suspension as source of contamination, MSTs in the treated groups differed significantly from the uncontaminated control groups only when large groups (n=50) of healthy termites were exposed (table 15b). For smaller groups of healthy termites exposed to termites contaminated with spore suspension, no significant differences in MSTs were obtained except for isolates FI23 and TT (table 15d).

Significant differences in MSTs in cross contamination tests were also observed among different isolates. Compared with other isolates, MM yielded significantly shorter MSTs, both as suspension and as spore dust, when larger groups (n=50) of healthy termites were tested (table 16a and b). When tested with small groups of termites (n=30) as spore dust, MSTs in MM were not significantly shorter compared to the standard isolate ICIPE30 (table 16c). With spore suspension as source of initial contamination, no significant differences in MSTs were obtained among all the *M. anisopliae* isolates including the two standard isolates (table 16d). In all four experiments isolate TT scored third in terms of MSTs. FF, the only *B. bassiana* isolate, caused the longest MSTs in contaminated termites in all four experiments, being significantly different from all other strains, except for FI23 at small groups and spore suspension as source of initial contamination (table 16 a-c). In MSTs isolate EE scored fourth, except when formulated as dust against small groups of termites (table a-c).

Table 15 Differences in MSTs of 50 *Macrotermes subhyalinus* non treated workers exposed for 15 days to five termites contaminated with spores of 14 days old SDA cultures and 10^7 spores/ml of suspension (a and b) and 30 *Macrotermes subhyalinus* non treated workers exposed for 15 days to five termites contaminated with spores of 14 days old SDA cultures and 10^7 spores/ml of suspension (c and d) of different strains of *Metarhizium anisopliae* and *Beauveria bassiana.*

a.	Isolate	Control	IC30*	FI23*	MM	EE	TT
Isolate	MST	7.78	2.94	3.84	2.50	3.68	3.15
ICIPE30*	2.94	S					
FI23	3.84	S	S				
MM[1]	2.50	S	S	S			
EE[1]	3.68	S	S	NS	S		
TT[1]	3.15	S	NS	S	S	S	
FF[2]	4.89	S	S	S	S	S	S
b.	Isolate	Control	IC30*	FI23*	MM	EE	TT
Isolate	MST	7.21	5.21	4.73	4.34	5.33	5.07
ICIPE30*	5.21	S					
FI23*	4.73	S	NS				
MM[1]	4.34	S	S	S			
EE[1]	5.33	S	NS	NS	S		
TT[1]	5.07	S	NS	NS	S	NS	
FF[2]	7.56	NS	S	S	S	S	S
c.	Isolate	Control	IC30*	FI23*	MM	EE	TT
Isolate	MST	7.60	3.65	4.49	3.60	4.32	3.92
ICIPE30*	3.65	S					
FI23*	4.49	S	S				
MM[1]	3.60	S	NS	S			
EE[1]	4.32	S	S	NS	S		
TT[1]	3.92	S	S	S	S	NS	
FF[2]	5.20	S	S	S	S	S	S
D.	Isolate	Control	IC30*	FI23*	MM	EE	TT
Isolate	MST	7.60	6.57	5.77	5.88	6.13	6.00
ICIPE30*	3.65	NS					
FI23*	4.49	S	S				
MM[1]	3.60	NS	NS	NS			
EE[1]	4.32	NS	NS	NS	NS		
TT[1]	3.92	S	NS	NS	NS	NS	
FF[2]	5.20	NS	S	NS	S	S	S

*Standard isolates; 1 = *M. anisopliae* isolate; 2 = *B. bassiana* isolate; S = significant at $p<0.05$ and NS = not significant at $p<0.05$, using Survival Analysis followed by Wilcoxon test.

3.3.3.2 Percentage sporulating cadavers of all termites treated.

In the cross contamination test, when spore dust was used against larger groups of uncontaminated termites, isolates ICIPE30 and MM sporulated at significantly higher rates than isolates FI23 and FF but not significantly at different rates compared to isolates TT and EE (table 16a). No significant differences between isolates were recoded for the three other treatment combinations. The percentage sporulating cadavers ranged from 16.9-64.67% across all the cross contamination tests.

Table 16 Percentage sporulating cadavers of 50 *Macrotermes subhyalinus* workers exposed for 15 days to five termites treated with spores of 14 days old SDA cultures and 10^7 spores/ml of suspension (a and b) and 30 *Macrotermes subhyalinus* non treated workers exposed for 15 days to five termites contaminated with spores of 14 days old SDA cultures and 10^7 spores/ml of suspension (c and d) of different strains of *Metarhizium anisopliae* and *Beauveria bassiana.*

a Isolate	Percentage sporulating cadavers ± SE		
ICIPE30*	64.67	±	0.58a
FI23*	10.67	±	1.81b
MM[1]	60.00	±	0.00a
EE[1]	36.67	±	5.13ab
TT[1]	38.00	±	0.30ab
FF[2]	16.95	±	1.21b
b Isolate	Percentage sporulating cadavers ± SE		
ICIPE30*	43.03	±	0.52a
FI23*	35.33	±	0.37a
MM[1]	20.67	±	2.34a
EE[1]	16.00	±	0.70a
TT[1]	50.67	±	0.58a
FF[2]	17.33	±	0.52a
c Isolate.	Percentage sporulating cadavers ± SE		
ICIPE30*	27.78	±	2.63a
FI23*	36.00	±	0.53a
MM[1]	52.22	±	3.08a
EE[1]	51.40	±	0.33a
TT[1]	48.24	±	4.00a
FF[2]	54.70	±	1.07a
d Isolate	Percentage sporulating cadavers ± SE		
ICIPE30*	27.78	±	2.63a
FI23*	60.00	±	1.61a
MM[1]	52.22	±	3.35a
EE[1]	51.11	±	4.86a
TT[1]	47.78	±	4.04a
FF[2]	54.44	±	1.00a

*Standard isolates; 1 = *M. anisopliae* isolate; 2 = *B. bassiana* isolate. Means followed by the same letter, in the same column are not significantly different at $p<0.05$, using the Tukey test. Data were first subjected to arcsine transformation before analysis of variance.

3.3.4 Repellency Test

There were no significant interactions between the distribution of termites visiting Petri dishes loaded with different isolates and the observation period in all the repellency tests. Only in the experiment where spore dust of the *M. anisopliae* isolates ICIPE30 and MM was used, a significant difference between the different isolates was observed across the whole observation period, with ICIPE30 being preferred to the control and strain MM (Tukey test).

When comparing the data on the basis of each time interval, the distributions of termites between the different Petri dishes that contained no fungal spores, spore dust of *M. anisopliae* isolates ICIPE30 and MM, is presented in table 17a. Generally the

variability of the distribution between the repetitions was very high, resulting in large standard errors. In the experiment using spore dust as odour source, comparing the standard isolate ICIPE30 with the isolate MM, at the end of the experiment termites preferred the Petri dish loaded with ICIPE30 (table 17a). In the second experiment, when using spore paste of the same isolates as odour source, termites did also prefer the Petri dish loaded with ICIPE30, but contrary to the previous results, at the beginning of the observation period (table 17b). In the third experiment comparing spore paste of the standard *M. anisopliae* isolate ICPE30 and the *B. bassiana* isolate FF, no significant differences in distribution were observed, at any time interval (table 17c). In the fourth experiment, comparing spore paste of *M. anisopliae* isolate MM with *B. bassiana* isolate FF, termites preferred to stay in the control Petri dish, starting from the forth observation interval (table17d).

Table 17 Distribution of *Macrotermes subhyalinus* workers in percent (± SE) in Petri dishes, comparing the repellency of (a) spore dust of *Metarhizium anisopliae* isolates ICIPE30 and MM, (b) spore paste of *M. anisopliae* isolates ICIPE30 and MM, (c) spore paste of *M. anisopliae* isolate ICIPE30 and *Beauveria bassiana* isolate FF, and (d) spore paste of *M. anisopliae* isolate MM and *B. bassiana* isolate FF, over a period of 225 minutes.

Time interval (min)	**a**. Distribution of termites (%)		
	Isolates (spore dust)		
	Control (± SE)	ICIPE30 (± SE)	MM (± SE)
25	13.3 ± 01.7a	28.3 ± 13.6a	28.3 ± 1.7a
50	18.3 ± 19.8a	31.7 ± 6.0a	18.8 ± 8.8a
75	25.0 ± 11.5a	33.3 ± 14.5a	25.0 ± 10.4a
100	15.3 ± 8.3a	30.0 ± 10.0a	20.0 ± 7.6a
125	10.0 ± 2.9a	30.0 ± 7.6a	11.7 ± 4.4a
150	12.5 ± 11.3a	35.0 ± 12.6a	3.8 ± 1.3a
175	20.0 ± 0.0ab	46.7 ± 16.4a	11.7 ± 1.7b
200	8.3 ± 1.7a	53.3 ± 15.3b	5.4 ± 2.5a
225	10.4 ± 7.8a	51.7 ± 17.4b	6.7 ± 1.7a
Time interval (min)	**b**. Distribution of termites (%)		
	Isolates (spore paste)		
	Control (± SE)	ICIPE30 (± SE)	MM (± SE)
25	5.4 ± 2.5a	63.3 ± 9.3b	13.3 ± 6.0a
50	5.8 ± 4.6a	71.7 ± 13.6b	12.1 ± 9.0a
75	15.0 ± 5.0a	60.0 ± 8.7b	10.4 ± 7.4a
100	12.1 ± 6.9a	45.0 ± 25.7a	20.4 ± 17.3a
125	23.3 ± 8.3a	25.4 ± 22.3a	23.3 ± 7.3a
150	41.7 ± 6.0a	31.7 ± 07.3a	13.3 ± 4.4a
175	28.3 ± 3.3a	33.3 ± 12.0a	13.3 ± 4.4a
200	20.0 ± 5.0a	13.3 ± 6.0a	40.0 ± 7.6a
225	30.0 ± 13.2a	11.7 ± 3.3a	25.0 ± 10.4a
Time interval (min)	**c.** Distribution of termites (%)		
	Isolates (spore paste)		
	Control (± SE)	ICIPE30 (± SE)	FF (± SE)
25	20.0 ± 10.0a	11.7 ± 3.3a	8.8 ± 4.0a
50	8.3 ± 5.7a	17.1 ± 14.0a	3.8 ± 1.3a
75	6.9 ± 6.3a	18.8 ± 15.7a	26.7 ± 14.2a
100	18.3 ± 23.4a	18.3 ± 15.7a	32.1 ± 24.4a
125	18.3 ± 10.9a	28.3 ± 11.7a	26.7 ± 21.7a
150	43.3 ± 6.7a	13.3 ± 6.0a	12.1 ± 9.0a
175	31.7 ± 6.0a	31.7 ± 4.4a	15.0 ± 5.8a
200	18.3 ± 8.8a	41.7 ± 11.7a	14.2 ± 12.9a
225	28.3 ± 17.1a	16.7 ± 9.3a	31.1 ± 24.3a

Means followed by the same letter, in the same row are not significantly different at $p<0.05$, using the Tukey test. Data were first subjected to arc sin transformation before analysis of variance.

Table 17 contd.

Time interval (min)	**d**. Distribution of termites (%)		
	Isolates (spore paste)		
	Control (± SE)	MM (± SE)	FF (± SE)
25	7.1 ± 4.1a	31.7 ± 19.6a	3.3 ± 1.3a
50	8.8 ± 4.0a	26.7 ± 21.7a	13.3 ± 10.7a
75	33.3 ± 8.3a	13.8 ± 6.9a	25.0 ± 11.5a
100	60.0 ± 18.1a	13.3 ± 6.0b	8.3 ± 1.7b
125	38.3 ± 26.2a	15.4 ± 10.1b	23.3 ± 18.3b
150	43.3 ± 10.1a	11.7 ± 6.7b	13.3 ± 10.7b
175	51.7 ± 19.6a	13.8 ± 6.3b	13.3 ± 6.3b
200	68.3 ± 13.6a	5.4 ± 2.5b	6.7 ± 4.1b
225	61.7 ± 13.0a	8.8 ± 5.7b	5.0 ± 2.5b

Means followed by the same letter, in the same row are not significantly different at $p<0.05$, using the Tukey test. Data were first subjected to arc sin transformation before analysis of variance.

3.4 Discussion

3.4.1 Relative pathogenicity test

Results of the preliminary pathogenicity test showed that entomopathogenic fungi that have potentials in controlling termites could be found in nature. All the *M. anisopliae* and *B. bassiana* isolates tested were pathogenic to *M. subhyalinus*. The term pathogenicity denotes the intrinsic capability of a macro-organism to overcome host defences (Siegel, 1997). Rath *et al*. (1995) linked spore surface antigens of *M. anisopliae* to pathogenicity. The tested isolates also sporulated well on termite cadaver. Hänel (1982b) considered isolates that sporulate well on termite cadavers as well adapted parasites that dominate over other saprophytic organisms due to their production of toxins.

Differences in virulence against *M. subhyalinus* were observed between the isolates. When termites were contaminated directly with dry spores or spore suspension of the Ethiopian isolates, *M. anisopliae* isolates generally caused faster mortality in *M. subhyalinus* than *B. bassiana* with the exception of isolate FF. This corroborates findings from previous studies with several termite species (e.g. Lai, 1977; Lai *et al*., 1982; Sajapa & Jan, 1990; Gitonga, 1996, Jones *et al*., 1996) where similar pattern of virulence for several isolates of *B. bassiana* and *M anisopliae* were observed. However, Leong (1966) recorded no significant difference in pathogenicity to *C. formosanus* between the two fungal species.

Moreover, in this study all termite cadavers contaminated directly with cultures of different *M. anisopliae* isolates sporulated well, whereas in the *B. bassiana* strains, except for isolate GG, a lower proportion of cadavers sporulated.

Metarhizium anisopliae isolates collected in Benin caused longer median survival times in *M. subhyalinus* and sporulated on less cadavers compared to the standard *M. anisopliae* isolates. In the relative pathogenicity tests, isolate MM showed the highest level of virulence against *M. subhyalinus*, both in Ethiopia and Benin, followed by isolates TT, EE, and FF. However, the performance of isolates MM and TT was not better than the standard isolates ICIPE30 and FI23.

Ebert (1994) generally relates speed of kill of a host organism and propagule production in host cadaver with virulence. According to him virulence is the consequence of balancing host mortality and propagule production. Wells *et al*. (1995) and Jones *et al*. (1996) proposed speed of kill as one criterion for selection of fungal strains. They argue, that slow death of an infected insect would help to spread the disease more widely to other individuals over a large period of time. The range of LT_{50} values cited in different studies for selection of fungal strains varies largely, even for the same termite species (Almeida & Alves, 1996; Malagodi & Veiga, 1995; Gurusubramanian *et al*., 1999). Jones *et al*. (1996) considered a LT_{50} value of 2.9 days for a particular *B. bassiana* strain as moderately low and selected the strain as the most promising one against *C. formosanus.* Although isolate MM killed termites the fastest of all tested isolates, its sporulation rate on termite cadavers was also very high. According to Wells *et al*. (1995) and Leong (1969), speed of kill is related with the rate of sporulation on cadavers. Isolates that kill quickly do not sporulate well on cadavers as for instance shown in tests with the biological termicide Bioblast™ EcoScience Corporation (New Jersey, USA) against *M. subhyalinus* in Benin (J. Langewald, IITA Benin, pers. comm.). Hence it is possible that in this study such fast killing strains were not encountered. The adaptation (virulence) of an entomopathogenic fungus to its host involves biochemical, morphological and tropic mechanisms to attach and adhere to the cuticle, solubilize, penetrate and utilize the cuticle, and overcome the resistance mechanisms in the haemocoel (Bidochka *et al*., 1997).

In this study emphasis has been placed on the development of those isolates that were found on termites, because they were demonstrated in many cases to be most virulent (Hänel, 1981; Grace & Zoberi, 1992; Wells *et al*., 1995). It is, however, apparent from the results of this study that high pathogenicity is not necessarily confined to isolates that originate directly from cadavers of the target insect. The *M. anisopliae* isolates MM and EE are soil and Crustacean isolates (Mamuye, 1999; Sneshaw, 1998). The *M. anisopliae*

isolate used in Bioblast™and the *M. anisopliae* isolate ICIPE30 are also no termite isolates but are highly virulent.

Termites collected in Benin and incubated with spore suspensions of Ethiopian isolates of *M. anisopliae* and *B. bassiana* died slower and developed less mycosis than Ethiopian termites that were treated in Ethiopia. Apart from the fact that both experiments were carried out at different places, the isolates being indigenous to Ethiopia might also be better adapted to local hosts and local climatic conditions. Virulence might be correlated with the initial host and the locality (Lomer *et al*., 2001). Increasing geographical distance between host and parasite origin was found to correlate with a decrease in spore production and virulence (Ebert, 1994).

Because of their long median survival times and low levels on mycosis in *M. subhyalinus*, the *M. anisopliae* isolates collected in Benin were discarded in further tests. Both *M. anisopliae* and *B. bassiana* isolates from Ethiopia were found to be sufficiently pathogenic and virulent to *M. subhyalinus* and therefore selected for the subsequent investigations.

3.4.2 Assessment of dose relations

Although all isolates caused significantly lower MSTs than in the control in the second and fourth bioassays (table 9), these results are not reliable because of the high control mortality. Consequently, most of the Cox regressions fitted to the survival data obtained from termites treated with different dosages of the different isolates, resulted in very steep baseline cumulative hazard curves and very small B-values. The B-value fits were not significant in most cases, indicating that the data show no or a confused dose response. For the same reason LD_{50} curves are very steep and their confidence limits are very large, the latter calculated on the basis of the Cox regression parameter estimates.

In bioassays where MSTs of termites in the control groups was relatively longer, the baseline cumulative hazard curve fits ($h_{(0)}(t)$) were less steep, compared with the previous experiments. The baseline cumulative hazard ($h_{(0)}(t)$) stands for the virulence of an isolate at the average of the applied dosages, but its value does also depend on the size of B. The meaning of the baseline cumulative hazard ($h_{(0)}(t)$) shall be explained using the following two examples:

Baseline cumulative hazard rates ($h_{(0)}(t)$) for two isolates with similar response to average dose (similar MSTs) but different in dose response (different fits for B) must according to equation 1 (for details refer to section 3.2.4.3) be different. In case of a rather strong dose response (comparatively large B-value) the ($h_{(0)}(t)$) values are rather low compared with a case of little dose response (small B-value) where the ($h_{(0)}(t)$) values are high. In Ethiopia, during the second bioassay isolate FF, and during the third bioassay isolate TT caused similar MSTs (i.e. 4.74 and 4.92 days) in termites treated with an average dose, but dose response (B) was different (table 10b,c). The baseline cumulative hazard rates ($h_{(0)}(t)$) were different The isolate with relatively large B-value, isolate TT (0.367) (table 10c), resulted in low baseline cumulative hazard rates ($h_{(0)}(t)$) (fig 6) while the isolate with comparatively small B-value, isolate FF (0.111) (table 10b), resulted in high baseline cumulative hazard rates ($h_{(0)}(t)$) (fig 5). Thus in this particular case, where virulence (MST) is nearly constant, the baseline cumulative hazard rates ($h_{(0)}(t)$) respond to the B-values.

In cases where the dose response is similar (similar B-values), but the response to the average dose is different (MST and h(t) are different) ($h_{(0)}(t)$) stands only for virulence, being low when the strain is less virulent, high when the strain is more virulent and similar when they are equally virulent. This can be demonstrated with the following example: In the second bioassay conducted in Ethiopia (table 9b) isolate HH, and in the third bioassay (table 9c) isolate MM caused different MSTs (i.e. 7.09 and 5.47 days) on termites but similar dose response (B: 0.188 and 0.218) (table 10b,c). The baseline cumulative hazard rates ($h_{(0)}(t)$) (fig 5 and 6) were high for the MM and low for the HH strain.

In the bioassays conducted in Ethiopia, isolates TT (B-value 0.367 and MST 4.92) and EE (B-value 0.357 and MST 4.49 days) in the third and fourth bioassay (table 9c,d and table 10c,d) with similar MSTs and B-value, respectively caused similar baseline cumulative hazard curves ($h_{(0)}(t)$). Similarly, in the IITA bioassays (tables 9e and 10e), isolates ICIPE30 (B-value 0.382 and MST 4.74 days) and FI23 (B-value 0.382 and MST 5.75 days) have similar MSTs and B-values, causing similar baseline cumulative hazard curves ($h_{(0)}(t)$) on termites.

Isolates that caused shorter MSTs in termites had steeper slopes in the baseline cumulative hazard curves ($h_{(0)}(t)$), depending on the size of B. Cox regression fits in termites treated with isolates that caused longer MSTs at average dose rate resulted in flat baseline cumulative hazard curves ($h_{(0)}(t)$). Across all experiments, Cox regression fits for the

isolates which caused shorter MSTs at average dose rate (ICIPE30, MM, TT, FF and EE), resulted in moderately steep baseline cumulative hazard curves and moderately high B-values, conforming the results of the preliminary pathogenicity tests.

The shape of the LD_{50} curves for the different isolates is determined by the level of dose response or by their B-values. Isolates that caused a significant but relatively weaker dose response in termite survival, resulting in small B-value estimates, yielded also LD_{50} curves with a large intercept and a large negative rate. Isolates that caused a relatively strong dose response in termite survival, resulting in large B-value estimates, had LD_{50} curves with a low intercept and a low negative rate. The LD_{50} curves for ICIPE30 in one bioassay, and for MM followed by EE in most of the bioassays, had rather low intercepts with a comparatively large negative rate, hence being rather efficient at both higher and lower dosages. The MST at average dose in termites following treatments with these comparatively virulent isolates is proportional to the initial LD_{50} estimate for day 1 after application, and even very low dose treatments eventually cause a high rate of mortality. This is because even a single spore of these isolates can penetrate the host cuticle rather successfully and can multiply quickly inside the host insect following penetration, and eventually kill it, unlike chemical insecticides where the insect dies or recovers (Hajek & Papierok, 1994).

However, in general the LD_{50} curves need to be interpreted carefully, since the parameter estimates are interpolated between a rather narrow dose ranges ($1x10^4$ - $1x10^7$ spores/ml). Particularly the LD_{50} estimates for the first two days after application are extrapolated and very large, biologically not making any sense. Similarly the LD_{50} values estimates extrapolated for dosages below the range that was applied during the experiments have to be taken with caution. More consideration should be given to LD_{50} values that lay within the range of doses that were used.

The proportion of termite cadavers sporulating correlated with dose, when data were pooled across isolates. In those bioassays where treated termites showed clear dose response in survival, there was also a dose response in the percentage cadavers sporulating. Ferron (1978) recorded a positive correlation between the number of infective spores and mortality by mycosis. However, Leong (1966) and Jones *et al*. (1996) observed that termites exposed to higher conidial doses exhibited symptoms of chemical intoxication and died before the infection could take place. Both tested fungal species are reported to

produce exoprotases with insecticidal activity, i.e. beauverin in *B. bassiana* (Hamill *et al*., 1969) and destruxin in *M. anisopliae* (Pais & Ferron, 1981). Destruxins cause immediate, muscular paralysis in insects, followed by flaccidity (Samuels *et al*., 1988a). Yet, Leong's (1966) and Jones *et al*. (1996) observations refer to dosages much higher than the highest dose used in this study. Termites were heavily coated with conidia. Thus, the sporulation rate in cadavers will increase with the applied dose up to a level, where fungal toxins will start to kill the host before the fungus can properly colonize the host tissue, hence preventing the cadaver to develop mycosis.

The Cox regression model is more flexible in the analysis of biopesticide bioassay studies than previous models such as probit and logit analyses. Probit analysis and logit regression analysis have found wide use in modelling the probability of a response in relation to dose (Finney, 1971; Robertson & Preisler, 1992). Preisler and Robertson (1989) discussed problems related with the analysis of data from time-dose-response experiments. The classical way of conducting an independent data analysis on time or dose is to separate either the dose effect or the time effect from the time-dose-mortality data used in the bioassay tests (Dell *et al*., 1983; Shapiro *et al*., 1987 in Nowierski *et al*., 1996). Models that consider both time and dose effects appear to be more appropriate for evaluating the effectiveness of a pathogen or a pesticide on the target host (Robertson and Preisler, 1992). Another problem that typically arises with time-dose-mortality studies is that the observations made through time are not independent, and, hence, the mortality response through time may be correlated (Robertson and Preisler, 1992). These authors used complementary log-log models (CLL) that help to overcome this problem because they describe insect survival as a function of time and dose. Cox regression models worked well in modelling time-dose-survival relationships of fungal isolates attacking termites in the bioassay of these studies. Cox regression models are very similar with CLL models but easier to work with SPSS. In bioassay studies with biopesticides these kind of statistical approaches are more feasible since biopesticides do not have acute effects like insecticides, but kill relatively slowly. In the preliminary pathogenicity tests of this study survival analysis was used for data analysis, followed by Wilcoxon test. Both survival analysis and Cox regression models are based on the hazard function (Cox & Oakes, 1984). Therefore a comparison of results between experiments is possible.

However, it should be stressed that in this study the different bioassays were made in different time periods and also in different places (Ethiopia and Benin). Hence, there were

differences in environmental conditions during transporting termites from collection sites to the laboratory and also while conducting the experiments. There might have been differences in biological response of isolates during different time periods and differences in termites of different colonies and age groups among the bioassays conducted. Similar problems were discussed in relation to bioassays with entomopathogenic fungi on North American grasshoppers by Nowierski *et al*. (1996). These factors in part may explain the high variation in results between the different bioassays. According to Robertson *et al*. (1995), natural variation in chemical and microbial pesticide bioassays is one of the major factors that contribute to differences in LD_{50} values, even in termite individuals from the same colony. Robertson *et al*. (1995) observed substantial difference in LD_{50} values in repetitive tests using the same methodology and termite individuals of the same age and colony. Variability in termite bioassays has been also observed by Lai *et al*. (1982) and Wells *et al*. (1995). Moreover, Hänel (1981) recorded large differences in control mortalities (19-62%) in *N. exitiosus* 11 days after treatment despite using individuals of the same age group and from the same laboratory reared colony. Lower termites (families Mastotermitidae, Kalotermitidae, Hodotermitidae and Rhinotermitidae) can be reared in the laboratory comparatively easily and therefore most of the experimental work on termites has been carried out with them (Verma, 1982). Contrary to that higher termites (family Termitidae) are difficult to rear under laboratory conditions because of their complex social organization (Verma, 1982). Moreover, among the higher termites, fungus growing termites belonging to the subfamily Macrotermitinae are far more difficult to rear than species without fungus gardens (Gunther, 1970). Therefore emphasis should be given in developing more reliable bioassay techniques for testing fungus-growing termites, or the bioassays should be carried out as much as possible under field conditions.

3.4.3 Horizontal transmission (cross contamination)

In laboratory studies, Kramm *et al*. (1982), Grace and Zoberi (1992), Jones *et al*. (1996), Gitonga (1996) and Rosengaus and Traniello (1997) used either *M. anisopliae* or *B. bassiana* to demonstrate transmission of infection from treated to untreated termites through contact. In this study, the MSTs of initially healthy *M. subhyalinus* workers exposed to termites contaminated with sporulating cultures of *M. anisopliae* isolates, was shorter compared with healthy termites exposed to termites contaminated with spore suspension. Milner *et al.* (1998b) observed 100% mortality in 50 *Coptotermes* spp., four days after they were allowed to groom for 24h with a single worker termite that had been

rolled in pure conidia. They estimated the fungal dose applied to a single termite as 10^7 conidia. Gitonga (1996) observed 42.7% and 49.8 mortality in termites, five and ten days after first exposure to one termite contaminated with $1x10^7$ spores/ml for 24h. He observed 84.4% and 97.6% mortality among 50 healthy *M. michaelseni,* five and ten days after first exposure in termites exposed to one contaminated termite for the whole observation period. Rath and Tidbury (1996) concluded that liquid formulations are less repellent to termites than dust formulations, in part possibly due to the water-seeking nature of termites (Verkerk, 1990, in Rath, 2000). Thus the reason for shorter MSTs of termites contaminated with spore dust could be, that healthy termites might have recognized and groomed termites contaminated with spore dust faster than termites contaminated with spore suspension. The other possibility is that termites might have simply died faster with spore dust than spore suspension.

Moreover, in this study, when groups of 50 healthy termites were exposed to contaminated termites they died faster, compared to healthy termites that were exposed in groups of 30 individuals. Apart from Rosengaus *et al*. (1998b) there are not many published reports on the rate of transmission of fungal spores at different population densities of uncontaminated termites. Contrary to results of this study, Rosengaus *et al*. (1998b) observed that the survivorship of termites exposed to filter paper disks treated with different doses of spore suspension of *M. anisopliae* increased with increasing group sizes from one to 25 individuals. Other studies focused on the effect of different population sizes and exposure time of contaminated termites on transmission of disease to a fixed number of uncontaminated termites (Zoberi, 1995; Jones *et al*., 1996). The reason for shorter MSTs in termites in the larger group size in this study might have be increased stress due to a higher population density of termites in a Petri dish compared with termites in the smaller group size.

Kramm *et al*. (1982) observed grooming termites eating the fungal spores and speculated that mortality of such termites resulted from contamination of the groomer's mouthparts, because fungal spores are inactivated by the termites' digestive system. Whether transmission of infection happens as described by Kramm *et al*. (1982) or through body contact or other ways is of little importance. Rath (pers. obs., in Rath, 2000) observed that grooming is not effective in removing all the fungal conidia. He further states, that the ultimate death of treated termites, as well as microscopic inspection of the termite cuticles, indicated that effective quantities of *M. anisopliae* spores were still present after grooming.

The results of this study confirm that cross contamination can play an important role in the spread of fungal diseases in termite colonies. This is important in relation to the development of control strategies based on this technology. EcoScience Corporation (New Jersey, USA) claim that treating 5-10% of termites of a termite colony with their product Bioblast^{TM} can lead to 100% mortality in a colony as a result of horizontal transmission (Paragon Products, 1996). Therefore, emphasis should be given to developing application methods that can target enough termites that can transmit the disease to other termites in the colony. It would also be interesting in the future to devise a bioassay method that simulates the field condition better to investigate more carefully the efficiency of horizontal disease transmission in termite colonies. However, the effect of cross contamination is considerably reduced by termite defence strategies. By ingesting and deactivating the spores, grooming termites are removing spores out of the cross contamination circuit. Moreover, some termite species isolate infected conspecifics by walling them off (Strack, 2000).

Among the tested isolates, MM caused the shortest MSTs in termites in the cross contamination tests where different termite group sizes and spore formulations were used. This isolate has shown high virulence against *M. subhyalinus* workers both in the preliminary pathogenicity test and in the dose response bioassays. In this study time to death was determined by the speed of transmission of the spores and the virulence of the particular isolates. The range of MSTs obtained from these experiments (tables 14, 15 & 16) were comparable with those obtained in the direct contamination trial (table 7). Thus, time of transmission must have been very short. This is why the termites died faster in the cross contamination tests when isolate MM was used as source of contamination.

3.4.4 Repellency test

In laboratory repellence tests using spore paste and dust of all the *M. anisopliae* and *B. bassiana* isolates, significantly more termites visited Petri dishes containing the spore paste (only up to 75 minutes after the start of the experiment) or dust (200 and 225 minutes after the start of the experiment) of isolate ICIPE30 than Petri dishes containing the spore paste or dust of isolate MM and the (control) Petri dishes without spore paste or dust. However, when spore pastes of isolates ICIPE30 and FF were compared, no significant differences in termites visiting treated and control Petri dishes were observed through out the whole observation period. On the contrary, in the choice tests where MM and FF spore

pastes were compared, termites avoided spore pastes of MM and FF and stayed at significantly larger numbers in the control Petri dish. Hence, termite response pattern to spores of different fungal isolates in different formulations, or the mound material used as substrate, or the fungal comb provided as attractant is not clear.

The reason for these highly variable results might be that the experimental set-up did not provide the termites with real differences in terms of olfactory cues, or might simply indicates that the termites did not recognize the spores at distance, i.e. indirectly. In future studies this methodological problem could be overcome by developing an olfactometer adapted to termite biology, and by providing differently directed olfactory cues. Several authors have studied direct repellency of *M. anisopliae* spores to termites. Staples (unpublished in Milner *et al*., 1998b) found quantitative differences in the behaviour of termites towards different isolates of *M. anisopliae* at lower dose but she also observed that all the tested isolates were similarly repellent at high doses. Kramm *et al*. (1982) and Zoberi and Grace (1990b) found that cadavers of *R. flavipes* killed by *M. anisopliae* and *healthy workers actively avoided B. bassiana*. However, Zoberi and Grace (1990b) also observed that 'mycelial mat' cultures (which included spores) were not avoided at all, resulting in high mortality in the workers. In a later laboratory study, Delate *et al*. (1995a) showed that agar culture of both *B. bassiana* and *M. anisopliae* were not repellent to *C. formosanus*. Rath (2000) believes that the nature of repellency has not been determined properly, and it is not clear whether the spores, the dead or mummified cadavers, or the high levels of foreign particulate material associated with contamination of termites during laboratory bioassay are repellent. Hence, there is a clear need for more experiments on potential repellency of termites to entomopathogenic fungi, focusing on different dosages and formulations of different isolates over different observation time, using different testing techniques. Milner (2000b) to some extent correlated repellency with virulence. However, he found *M. anisopliae* strains with little evident repellency also to be highly virulent against *C. lacteus*. Therefore, while dealing with selection of strains for termite control, the repellent effect of the strains needs to be investigated separately.

4 Sporulation potential of Ethiopian isolates of *M. anisopliae* and *B. bassiana* on different media

4.1 Introduction

Once a range of suitable isolates has been identified in bioassays, such isolates then need to be tested in large-scale field trials. A high level of inoculum is necessary for an efficient use of *B. bassiana* and *M. anisopliae* (Wraight & Robert, 1987). Efficient mass production techniques are a prerequisite for successful field applications of entomopathogenic fungi. An efficient and economic large-scale production, the formulation of fungal propagules into a product with an adequate shelf life, and a suitable application strategy are fundamental for the successful development of a mycoinsecticide.

The development of a good mycoinsecticide relies on the biological properties of the isolate. One of the properties that need to be considered in selecting an isolate as a potential microbial control agent after laboratory virulence and field performance tests is its productivity (Jenkins *et al*., 1998). According to Feng *et al*. (1994) and Gitonga (1996) rapid radial growth and high sporulation capacity *in vitro* and *in vivo* are important attributes of a good strain. The selection of strains with good growth and sporulation capacity on nutritionally poor media is important for mass production in an industrial set-up (Feng *et al*., 1994).

In this study, isolates that had already been tested in laboratory bioassays against *M. subhyalinus* were selected for sporulation potential studies. The *M. anisopliae* isolates MM, EE and TT, and the *B. bassiana* isolate FF were compared with the standard *M. anisopliae* isolate ICIPE30. The fungi were grown on two common mycological media, Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA), and rice. An attempt was also made to quantify their sporulation potential on termite cadavers.

4.2 Materials and methods

All experiments were carried out at IITA Benin.

4.2.1 Spore production on mycological media

4.2.1.1 Fungi

Five fungal isolates were used during this experiment. These were the Ethipoian *M. anisopliae* isolates EE and MM and the *B. bassiana* isolate FF, all obtained from AAU (Table 4). In addition, the *M. anisopliae* strain TT, isolated from termites in Ethiopia, and the ICIPE, Kenya, *M. anisopliae* isolate ICIPE30, serving as a standard isolate, were used in this study.

4.2.1.2 Spore inoculation

Conidial concentrations of $1x10^7$ conidia/ml spore suspensions of each isolate were prepared just before starting the experiments, following the protocol described in section 2.3.3. Thereafter, l ml of the spore suspensions of each isolate was transferred to separate SDA and PDA Petri dishes (9 cm in diameter) under a sterile airflow, using a sterile Pasteur pipette. The suspensions were evenly spread over the substrate using a sterile L-shaped glass rod. The plates were kept at 28ºC ± 2ºC in an incubation room under natural light for 10 days. There were three replicates per treatment.

4.2.1.3 Estimation of sporulation

The conidia were harvested by adding 50 ml sterile water containing 0.05% Tween 80 onto a Petri dish that contained 10 days old cultures of the fungal strains. The conidia suspensions of each isolate were transferred to 150 ml conical flasks and agitated on a shaker (Lab-Line, Melrose Park, UK.) at 1,500 rpm for three minutes to break up the spore clumps. Spore counts were carried out using a Neubauer Hemocytometer. Highly concentrated suspensions were further diluted down in one-magnitude steps. This was repeated if necessary.

4.2.2 Production of spores on solid substrate (rice)

4.2.2.1 Fungi

The spore production of the five selected fungal isolates was compared in this experiment.

4.2.2.2 Preparation of media

Liquid media were prepared by suspending yeast granules in hot tap water (20 g/l) over 15 minutes. The yeast solution was then homogenized in a commercial blender (Moulinex, Wareing, France) for 60 seconds. Locally purchased sugar (sucrose) (20 g/l) was then dissolved in the hot broth. Seventy-five ml of the broth was filled into 250 ml Erlenmeyer flasks. The flasks were plugged with cotton wool bungs and covered with aluminium foil. The solution was then autoclaved at 121ºC (15 psi) for 30 minutes.

4.2.2.3 Preparation of inoculum

After autoclaving and cooling the broth, 1 ml of spore suspension of a conidial concentration of 6 x 10^6 spores/ml of each fungal isolate prepared from 14 days old SDA culture (for a detailed description refer to section 2.3.3) was added to each 250 ml flask. The liquid cultures were then incubated on a rotary shaker set at 1,500 rpm for two days at 28ºC ± 2ºC room temperature and under florescent light.

4.2.2.4 Preparation of rice grain

On the day of inoculation, rice was washed and allowed to drain before addition of water at the rate of 300 ml/kg and groundnut oil at 20 ml/kg. The rice was then boiled for 20 minutes to accelerate the absorption of water before autoclaving. After being allowed to cool, batches of 1 kg of rice were placed in 38 x 52 cm locally made polypropylene carrier bags. The bags were finally autoclaved at 121ºC (15psi) for 60 minutes and then allowed to cool.

4.2.2.5 Inoculation of rice

The two days old broth of each isolate prepared as described under section 4.2.2.3, was diluted with 250 ml cold sterile water and shaken gently by hand. Under a laminar air flow cabinet, 150 ml of the ready diluted liquid inoculum was added to each kilogram bag of sterile rice and the bags were thereafter kneaded to distribute the inoculums through the

rice. Each bag was then placed in disinfected plastic basins (38 cm in diameter and 21 cm deep) and loosely folded to minimize the entrance of contaminants. The basins were covered with plastic lids (38 cm diameter). The basins were adapted to allow passive aeration during growth and conidiation through four aeration holes, plugged with sterile polyurethane bungs. Plastic basins that contained the same isolates were stuck on top of each other. The plastic basins were placed in a clean incubation room under natural light conditions at 28ºC ± 2ºC. Separate rice cultures of each isolate were prepared to estimate sporulation at different time intervals after 1, 3, 7 and 11 days, respectively. Three replicates were prepared for each observation.

4.2.2.6 Estimation of sporulation

The spores incubated for 1, 3, 7 and 11 days, respectively, were then extracted from the bags by adding 600-2,000 ml of kerosene into each bag and kneading the bags. Then the resultant aliquots of each isolate were filtered through a 75 micron sieve and filled into plastic buckets. The volume of the aliquots of each isolate was measured using a 2 l graduated measuring cylinder. One ml of aliquot of each isolate was added to 2.5 x 8 cm vials containing 9 ml of kerosene, using a sterile Pasteur pipette before counting. Spore counts were carried out using a Neubauer Hemocytometer. Highly concentrated suspensions were further diluted down in one-magnitude steps. This was repeated if necessary.

4.2.3 Sporulation on cadavers

Estimation of sporulation on cadavers was made for the five before mentioned isolates that had been earlier tested for sporulation on artificial media. For each isolate, ten termite cadavers that had been previously treated with 10^7 spores/ml of the respective fungal strains were carefully removed from the bioassay arenas (for details refer to section 3.2.4.2). Each cadaver was washed with 10 ml of water, containing 0.05% Tween 80, in a 2.5 x 8 cm vial. The spore concentration in the resulting suspension was estimated using a Neubauer Hemocytometer.

4.2.4 Statistical analysis

The total numbers of spores were first subjected to log transformation and the data were then analysed by means of one-way- and two-way-ANOVAs (SPSS, 1999). Comparison of means was carried out using the Tukey test. The significance level was set at $p<0.05$.

4.3 Results

4.3.1 Spore production on mycological media

There were no significant interactions in percentage spore production between the different isolates and the mycological media. When comparing the different isolates on the bases of each medium, no significant differences in terms of spore production were recorded among the five tested strains grown on PDA media (table 18). However, on SDA the *M. anisopliae* isolate MM produced significantly more spores than the *B. bassiana* isolate FF but it did not differ significantly from the *M. anisopliae* strains ICIPE30, EE and TT. No significant difference was recorded among ICIPE30, EE, TT and FF. Moreover, no significant difference in spore production was observed when the two mycological media were compared across all the isolates (for PDA 1.54 x 10^9 ± 2.3, and for SDA 2.52 x 10^9 ± 1.18).

Table 18 Mean number of spores per Petri dish harvested from *Metarhizium anisopliae* isolates ICIPE30, MM, EE and TT and from *Beauveria bassiana* isolate FF grown on SDA and PDA media at 28ºC ± 2ºC under natural light for 10 days.

Isolates	Mean number of spores/Petri dish ± SE x 10^9					
	Media					
	PDA			SDA		
ICIPE30*	1.14	±	0.3a	2.15	±	0.4ab
MM	2.78	±	0.5a	5.75	±	1.3a
EE	1.91	±	0.7a	2.10	±	0.5ab
TT	1.52	±	0.3a	1.72	±	0.4ab
FF	0.94	±	0.0a	0.88	±	0.2b

* Standard isolate. Means followed by the same letter, in the same column are not significantly different at $p<0.05$, using the Tukey test. Data were first subjected to arc sin transformation before analysis of variance.

4.3.2 Spore production on rice

The average number of spores produced per gram of rice by four *M. anisopliae* and one *B. bassiana* isolates measured at intervals of 1, 4, 7, and 11 days after inoculation, respectively, is shown in table 19. The mean spore yield of the *M. anisopliae* isolate ICIPE30 reached a maximum already after seven days. However, ICIPE30 and MM did not differ significantly across the whole observation time, with EE yielding the same amount of spores after 11 days. These three *M. anisopliae* isolates produced significantly higher yields than the *B. bassiana* isolate FF after one and four days of incubation. On day seven, the *B. bassiana* isolate yielded as much as MM and EE, but not any longer after 11 days. Isolate TT yielded significantly less compared to the other *M. anisopliae* isolates across the whole observation time.

Table 19 Mean number of spores per g substrate harvested from *Metarhizium anisopliae* and *Beauveria bassiana* isolates grown on rice at 28ºC ± 2ºC and natural light over a period of 11 days.

Isolate	Age of culture (days)			
	1	4	7	11
	Mean number of spores/g ± SE x 10^5	Mean number of spores/g ± SE x 10^8	Mean number of spores/g ± SE x 10^9	Mean number of spores/g ± SE x 10^9
ICIPE30[1]	21.8 ± 10a	13.5 ± 0.7a	2.9 ± 0.3a	3.0 ± 0.1a
MM[1]	6.5 ± 0.9ab	14.8 ± 1.08a	1.9 ± 0.3ab	2.8 ± 0.3a
EE[1]	2.3 ± 0.6b	0.4 ± 0.03b	1.2 ± 0.2ab	2.1 ± 0.1a
TT[1]	0.0 ± 0.00c	0.1 ± 0.02c	0.3 ± 0.04c	0.7 ± 0.01c
FF[2]	0.0 ± 0.00c	0.2 ± 0.04c	0.9 ± 0.2b	1.0 ± 0.03b

*Standard *M. anisopliae* isolate; 1 = *M. anisopliae* isolates; 2 = *B. bassiana* isolate; means followed by the same letter, in the same column are not significantly different at $p<0.05$, using the Tukey test. Data were subjected to log transformation before analysis of variance.

4.3.3 Sporulation on cadaver

Contrary to the results on the artificial media, the *B. bassiana* isolate FF produced significantly more spores on termite cadavers than the *M. anisopliae* isolates ICIPE30, MM and TT (table 20). There was no significant difference in average spore production on cadavers between FF and the *M. anisopliae* isolate EE. No significant difference was observed between isolates ICIPE30, MM and TT. Isolates MM and EE did not differ significantly in spore production on termite cadavers either.

Table 20 Mean number of spores per cadaver of *Macrotermes subhyalinus* workers treated with 10^7 spore/ml spore suspensions of *Metarhizium anisopliae* and *Beauveria bassiana* isolates.

Isolates	Mean number of spores/cadaver ± SE x 10^7		
ICIPE30*	0.88	±	0.11a
MM[1]	0.95	±	0.12ab
EE[1]	1.30	±	0.17bc
TT[1]	0.63	±	0.2a
FF[2]	3.40	±	0.65c

* Standard *M. anisopliae* isolate; 1 = *M. anisopliae* isolates; 2 = *B. bassiana* isolate; means followed by the same letter, in the same column are not significantly different at $p<0.05$, using the Tukey test. Data were subjected to arc sin transformation before analysis of variance.

Based on its overall performance in the pathogenicity, dose response bioassays, cross contamination and sporulation potential studies, the *M. anisopliae* isolate MM was selected for further field studies.

4.4 Discussion

4.4.1. Spore production on mycological media, rice and cadavers

Surface culture techniques are used for the routine maintenance of isolates and for the production of conidia. One of the most commonly used media by insect pathologists is Sabouraud's Dextrose Agar, supplemented with yeast extract (SDAY); however, many other media such as cornmeal, Czapeck-Dox, malt extract, PDA and Sabouraud's maltose agar are also often used (Goettel & Inglis, 1997). In this study, the *M. anisopliae* isolate MM had the highest spore yield (8.9 x 10^7 spores/cm^2) on SDA plates. However, MM did not yield significantly higher spore numbers than ICIPE30 (3.3 x 10^7 spores/cm^2) and EE (3.2 x 10^7 spores/cm^2). The *B. bassiana* isolate FF yielded 1.3 x 10^6 spores/cm^2. In comparison, spore yield of *M. anisopliae* and *B. bassiana* obtained from SDA plates by Gitonga (1996) ranged from 1 x 10^{10} - 8 x 10^{10} spores/cm^2 and 3 x 10^{10} - 9.5 x 10^{10} spores/cm^2, respectively. Senshaw (1998) obtained a spore yield of 1.5 x 10^8 spores/cm^2 and 4.7 x 10^7 spores/cm^2 of the *M. anisopliae* isolate EE and the *B. bassiana* isolate FF on SDA plates, respectively.

In industrial terms, liquid fermentation of microbial control agents is more advanced than solid-state fermentation and has the potential for considerably lower production costs (Jenkins & Goettel, 1997). However, the conidia of *M. anisopliae* and *B. bassiana* are hydrophobic and only produced at an interphase between substrate and air (Lomer *et al.*,

2001). There is a wide range of solid substrates available for use in the production of fungi for biological control. Rice is an ideal substrate for the cultivation of entomopathogenic deuteromycete fungi (Mendonca, 1992; Ibrahim & Low, 1993; Milner *et al*., 1993). This is probably due to a combination of factors, including nutritional balance, costs, worldwide availability, a physical characteristic such as grain size and shape, hydration properties and structural integrity even after colonization by fungi (Jenkins *et al.*, 1998). In this study, on cooked rice, the *M. anisopliae* isolates MM, ICIPE30 and EE produced similar spore yields after 11 days of incubation. However, ICIPE30 grew faster, reaching the highest spore yield (3×10^9 spores/g of rice) already after seven days. In comparison, Gitonga (1996), Mamuye (1999) and Mendonca (1992) reported yields of $1{,}3 \times 10^9$, 1.62×10^9 and an exceptional 10^{10} spores/g of rice, respectively. All three isolates were comparably good spore producers (J. Langewald, IITA Benin, pers. comm.)

The *B. bassiana* isolate FF was the worst spore producer on both artificial media, but together with *M. anisopliae* isolate EE it produced the highest number of conidia on termite cadavers. The conidial production of isolates MM, TT and ICIPE30 on cadavers was similar but lower compared with EE and FF. In this study, conidial yield of *M. anisopliae* isolates ranged from 0.63 to 0.95×10^7 spores per cadaver, whereas FF, the only *B. bassiana* strain tested, produced 3.4×10^7 spores per cadaver. Almeida *et al*. (1997) reported a range of 3.5×10^6 to 2.11×10^7 spore yield of *B. bassiana* per cadaver. The spore size of *B. bassiana* (1.5-2.0 µm) is smaller than that of *M. anisopliae* (7-11 µm) (Goettel & Inglis, 1997). Therefore, on an individual cadaver of the same surface area *B. bassiana* can produce more spores than *M. anisopliae*, leading to higher spore yield per cadaver.

The conidial production on SDA correlated well with production on rice for all the isolates. Spore production on the host cadaver correlated well with the production on rice for isolates EE, MM and ICIPE30, but not for TT and FF. Almeida *et al*. (1997) reported similar results with the spore yield on different mycological media correlated with the yield on rice for some but not all isolates.

The extent of sporulation on cadavers or artificial substrates is important for two reasons: First, good sporulation on cadavers is an important criteria in the selection of entomopathogenic fungus strains for termite control, as this characteristic may provide better conditions for the initiation of epizootics in termite nests (Hänel, 1982b; Wells *et al*.,

1995, Almeida *et al*., 1997). Second, good growth and sporulation on artificial substrates determines the economics of industrial mass production (Feng *et al*., 1994). Additionally, different isolates within the same species vary in their requirements for nutrition, pH, water activity of the substrate, temperature optima for mycelial growth and conidiation, light and incubation period (Johnpulle, 1938; Bartlett & Jaronski, 1988; Latge & Moletta, 1988; Kleespiess & Zimmermann, 1992). Thus, a set of particular parameters needs to be identified for the optimisation of production for each isolate (Jenkins *et al*., 1998). The results from this study show that the Ethiopian *M. anisopliae* isolate MM produced rather large amounts of conidia both on host cadavers and on artificial media. However, it did not differ from the standard isolate ICIPE30 and the *M. anisopliae* isolate EE. High competitiveness of these isolates on SDA media is a good quality indicator, since many other steps in the process, like quality control, are usually based on SDA cultures (Jenkins *et al*., 1998).

Although white rice is by far the most commonly used substrate for mass production of fungal conidia, it is an expensive imported food commodity in Ethiopia. Other indigenous cereals were tested as substrate for growing *M. anisopliae* but yielded 10 times less than rice (Mamuye, 1999). Because rice and other cereals also serve as food, mass production on waist by-products should be developed and their use emphasized. Yet in terms of mass production economics, the proportional costs of the substrate are rather low (Cheery *et al*., 1999).

Based on its overall performance in pathogenicity, virulence tests and spore production tests on host cadavers and artificial media, the *M. anisopliae* isolate MM was selected for further field studies in Ethiopia. Unfortunately, the standard strain ICIPE30 could not be imported into Ethiopia, because it is considered as an exotic strain by the local authorities.

5 Field test on the use of *M. anisopliae* for the control of *M. subhyalinus* colonies in Ethiopia

5.1 Introduction

The association of subterranean termites with soil is an important advantage for using a fungus pathogen for their control (constant temperature, absence of light and high humidity) (McCoy, 1974). Additionally, some authors consider the social behaviour of termites, such as grooming, trophallaxis and cannibalism as advantageous for the spread of the pathogens, once they have been introduced into the nests (Kramm *et al*., 1982, Hänel, 1982a)

However, recently behavioural defence reactions of termites to entomopathogenic fungi have been observed. Some of the behavioural defence reactions are, alarm signals in colonies of certain termite species like *Zootermopsis angusticollis* Hegen. (Isoptera: Hodotermitidae) (Rosengaus *et al*., 1998a), triggered by the presence of entomopathogenic fungi, grooming behaviour since it is also a way of removing fungal spores, deactivation of ingested spores by the termites' digestive system and the burying away of cadavers of contaminated termites from the termite colony (Milner, 2000b).

Despite these limitations, a number of strategies have been developed for use of *Metarhizium* spp. for termite control (Milner, 2000a). Blowing of conidia dust into termite mounds (Fernandes & Alves, 1991; Alves *et al*., 1992; Milner & Staples, 1995; Milner *et al*., 1997) and use of *M. anisopliae* as a repellent to protect building structures (Gunner *et al*., 1994; Milner & Staples, 1996; Rath & Tidbury, 1996; Milner *et al*., 1997) are examples. International patents involve the use of *M. anisopliae* for termite control (Milner *et al*., 2000a), and Bioblast^{TM}, a biological termicide, is based on an *M. anisopliae* isolate (EcoScience, 1997 in Rath, 2000). Gitonga (1996) was the only one studying the use of *M. anisopliae* and *B. bassiana* under field conditions in Africa (i.e. Kenya). His results indicate the possibility to create epizootics in mounds of *M. michaelseni* using three different formulations of indigenous *M. anisopliae* strains. There are no published reports on field control of termites using entomopathogenic fungi in Ethiopia.

The main objective of this study was to determine the efficacy of the spore dust of a selected indigenous *M. anisopliae* isolate against *M. subhyalinus* under field conditions,

using different spore doses and application methods. An attempt was also made to determine the possibility of protecting wooden structure from *M. subhyalinus* by dressing the structure with spore suspensions of the *M anisopliae* isolate.

5.2 Materials and methods

5.2.1 Trial sites

All field experiments were carried out at Sasiga and Lekemt Zuri, Eastern Wellega zone in the Oromia regional state. The sites are 370 and 335 km west of Addis Ababa at an altitude of 1,520 and 1,600 m above sea level, respectively.

Like the Western Wellega zone, these areas, also have a single rainy season between April and October. The mean annual rainfall in Sasiga is about 1,375 mm (Abdurahman, 1990) and at Lekemt Zuria it varies from 1,012 to 1,130 mm (NMSA, pers. comm..). At Sasiga, the mean minimum monthly temperature ranges from 13.5 to 15.3°C (Abdurahman, 1990) and at, Lekemt Zuria from 12.67 to 14.75°C. Similarly mean maximum temperature ranges from 30.7 to 31.8°C at Sasiga (Abdurahman, 1990) and 27.07 to 28.14°C at Lekemt Zuria (NMSA, pers. comm..).

The soil types and crops grown in the Sasiga and Lekemt Zuria area are similar to the ones in Mendi and Gimbi (for details refer to section 2.2.1.2).

5.2.2 Isolate

The isolate used in the field test was the *M. anisopliae* isolate MM, the most promising Ethiopian isolate. Before conducting this experiment a SDA slant culture of MM was sent to IITA Benin for mass production. Sachets of dry spores of MM were obtained from IITA's mass production unit 15 days before the experiment. Spore viability was tested as described in section 3.2.1.2 three days before every field trail and only spores with a higher than 80% germination rate were used.

5.2.3 Mound selection

The field experiments were carried out on *M. subhyalinus* mounds. Two to three years old active colonies of *M. subhyalinus* were selected 15 days before the experiment by asking farmers about the age of each mound. The activity of each mound was determined at the

time of selection using a "hole repair method". Here fore, rectangular holes at the side of the active mounds of *M. subhyalinus* were dug down to the level of the termite comb. The volume of the mound material removed was measured using a 50 cm ruler. The size of the holes was again measured after 24 hours. Mounds with holes sealed within 24 hours time were considered as active mounds. All mounds were found in maize, teff and Niger seed fields. Distances between mounds varied between 100 – 200 m.

5.2.4 Picket dressing

This trial was conducted at Sasiga between June and August 2000, using 2-3 years old active termite mounds.

Fifteen cm deep holes were applied at the top and the side of each mound, using dry eucalyptus pickets. Oven-dry eucalyptuses pickets of 30 x 1.5-2 x 1.5-2 cm were used in this trail. The oven-dry weight of each picket was recorded before starting the experiment. The spore doses used were, 1, 0.4 and 0.1 g spores per picket (or control pickets dressed with vegetable oil and without oil). The spore mass of the different doses was weighed separately on aluminum foil on a beam balance (Gallenkamp, Leicestershire, UK) and placed on separate plastic sheets. Then, 20 cm of the length of the pickets was dipped into vegetable oil, and by holding up the pickets for 1 minute the excess oil could drip off the pickets. Each picket was then rolled in the different amounts of spore mass on the aluminum sheet. Spore mass, which did not stick on the pickets was carefully scrapped off the aluminum sheet with a scraper and painted on the pickets afterwards. Then 15 cm of the length of the treated pickets was placed in the holes on each mound. Each of three mounds received one picket of each different treatment. There were three replications per treatment. The oven dry weight of the pickets was again taken after 2.5 months.

5.2.5 Mound treatment

5.2.5.1 Application of spores of *M. anisopliae* isolate MM to *M. subhyalinus* mounds through ventilation holes, using a bicycle pump

This trial was conducted both at Sasiga and Lekemt Zuria between August 2000 and March 2001 on 2-3 years old active mounds. The activity of each mound was determined one day before (for details see section 5.2.3).

At Lekemt Zuria 20, 9.3 and 4.6 g spores per mound were used. At Sasiga, the spore doses were reduced to 4.6 and 9.3 g spores per mound because of a lack of sufficient spore material. The spore mass of the different doses was weighed separately as described in section 5.2.3. At the top and the centre of each mound, holes were applied in order to reach the ventilation hole of the termite mound. Then, all mounds were treated before noon by blowing the dry spores of each dose from a small container connected to a bicycle pump through a rubber hose into the ventilation holes. During the operation a cardboard was used to cover the hole to prevent the spore dust from puffing out of the artificial hole. Three technicians treated three replicate mounds of each dose at a time. After the application was completed, the holes were covered with plastic sheets to prevent rainwater entering the holes. There were three replications per treatment in each site.

5.2.5.2 Monitoring termite activity

The activity of termites was determined using the same protocol as described in section 5.2.3. Termite activity was estimated over 15 days intervals, over nine weeks in Lekemt Zuria and eight weeks at Sasiga.

Additionally the activity of the termite colonies was tested, comparing the foraging activity of the colonies on Eucalyptus pickets (refer to section 5.2.4 for details on the size and placement of the pickets). Four pickets were placed on each mound. The pickets were removed in 15 days intervals over a period of nine weeks, the oven dry weight taken and returned back into their respective holes.

Mounds, which did not show termite activity (no hole repair or further decrease of dry weight of the pickets) after nine weeks were broken up and the state of the colony was assessed by looking for live termites.

5.2.5.3 Application of spore dust into ventilation holes by hand

This trail was conducted at Lekemt Zuria. All aspects of the trial were as previously described in section 5.2.5.1 except that spore dust was applied into mounds by introducing it carefully into two to four ventilation holes of each mound by hand. The hole repairing method was used to evaluate termite activity of *M. subhyalinus* as described in section 5.2.3.

5.2.6 Statistical analysis

Percentage weight of the pickets and percentage replaced soil volume were first subjected to arc sin transformation and analysed across all observation intervals using repeated measure ANOVAs (SPSS, 1999), with time and dosages or control being the independent factors. Additionally one-way ANOVAs were carried out for each separate observation interval. A comparison of means was carried out using LSD ranking test. The significance level was set at $p<0.05$.

5.3 Results

5.3.1 Picket dressing

Sixty days after treatment, percentage weight loss, between pickets treated at three concentrations of *M. anisopliae* and control, or among the pickets treated with the different dosages was not significantly different (table 21).

Table 21 Percentage weight loss in eucalyptus pickets treated with suspensions of *Metarhizium anisopliae* at three spore concentrations and placed on *Macrotermes subhyalinus* mounds, 60 days after placement at Sasiga.

Treatment	Percentage weight loss		
Control	30.20	±	22.25a
Oil	27.73	±	20.23a
1g spore/picket	7.92	±	6.12a
0.4g spore/picket	12.38	±	9.02a
0.1g spore/picket	17.84	±	10.18a

Means followed by the same letter in the same column are not significantly different $p<0.05$, using LSD ranking test. Data were first subjected to arc sin transformation before analysis of variance.

5.3.2 Mound treatment

5.3.2.1 Application of spore dust of *M. anisopliae* isolate MM through ventilation holes using a bicycle pump.

The percentage termite mound soil volume replaced over 24 hours by *M. subhyalinus* workers after application of spore dust at different doses of *M. anisopliae* isolate MM through artificial holes into mounds is presented in table 22a (three doses) and 22b (two doses) for the two different sites. The percentage termite mound soil volume replaced by *M. subhyalinus* workers in mounds treated with different doses of isolate MM was

significantly different from untreated mounds when comparing data over the whole observation period (repeated measure ANOVA and subsequent LSD test) in Lekemt Zuria (table 22a). Moreover, in this site the time effect was significant, too (df=5; F=27.7; $p<0.001$). Additionally a significant interaction between time and treatment was observed (df=15; F=3.9; $p<0.05$). Results in Sasiga were similar, (Time effect: df=3; F=21.5; $p<0.001$; Time - treatment - interaction: df=6; F=5.7; $p<0.01$). When comparing the data on a daily basis using one-way ANOVAs, one day before treatment, workers replaced 100% of the removed soil in all termite mounds (table 22a). In both sites 15 and 30 days after treatment, termite activity was significantly lower in mounds treated with spores across all dosages compared to the non-treated control mounds, respectively, with no difference in dose response. The observation remained the same until day 75 after treatment. Three mounds, treated with 4.6 and 20 g spore/mound and 9.3 g spores/mound at Lekemt Zuria and Sasiga, respectively, were found inactive 15 and 30 days after treatment, and continued to remain inactive throughout the experimental period. After 75 days, when the mounds were distracted, *Xylaria* mushrooms growing on the mound were observed, indicating that the termite fungus gardens were not longer maintained by the colonies.

There was no significant difference between percentage weight losses in pickets placed on mounds treated with different doses of isolate MM or non treated when comparing data over the whole observation period (repeated measure ANOVA with subsequent LSD rank test (table 23). Changes over time, however, were significant (df=4; F=20.1; $p<0.001$). The time-treatment interaction was significant (df=12; F=2.7; $p<0.01$). When comparing the data on a daily basis using one-way ANOVAs, up to the third observation after treatment no significant differences in percentage weight losses in pickets were recorded among treated and untreated mounds (table 23). However, from day 60 after treatment onwards, the percentage weight loss in pickets on mounds showed some significant response to dose (table 23).

5.3.2.2 Application of spore dust into ventilation holes by hand

The results of observations on the percentage termite mound soil volume replaced by *M. subhyalinus* on mounds treated by placing three different doses of *M. anisopliae* in ventilation holes over 75 days in Lekemt Zuria are presented in table 24. When compared

using repeated measure ANOVA with subsequent LSD rank test, the percentage termite mound soil volume replaced by *M. subhyalinus* workers in mounds treated with different doses of isolate MM was significantly different from untreated mounds, with the high dose being significantly different from the low and medium dose, when comparing data over the whole observation period. The time effect was significant, too ($df=4$; $F=6.4$; $p<0.01$). There was no significant time-treatment interaction ($df=12$; $F=1$; $p>0.05$). When comparing the data on a daily basis using one-way ANOVA, a significant response to dose was observed at day 45 and 75 after treatment, the medium (9.3 g spores/mound) and the highest doses (20 g spores/mound) differed significantly from the lowest dose (4.6 g spores/mound) and untreated control; at day 30 and 60 only the highest dose differed significantly from the control (table 24).

Table 22a Percentage termite replaced soil volume by *Macrotermes subhyalinus* workers within 24 hours in mounds treated with three dose rates of spore dust of *Metarhizium anisopliae* using bicycle pump, at different intervals over a period of 75 days at Lekemt Zuria, Ethiopia.

a.

Treatment (across observation time effects)	Percentage soil volume replaced														
	Days after treatment														
	1 DBT			15			30			45			60		
Control a	100.0	±	0.0a	100.0	±	4.0a	100.0	±	0.0a	100.0	±	0.0a	100.0	±	0.0a	100.0	±	0.0a
4.6g spore/mound b	100.0	±	0.0a	54.0	±	27.1ab	45.0	±	22.8b	38.9	±	21.6b	38.9	±	5.2b	23.7	±	7.5b
9.3g spore/mound b	100.0	±	0.0a	35.0	±	18.2b	37.3	±	12.6b	36.0	±	8.2b	36.0	±	3.1b	33.7	±	12.0b
20g spore/mound b	100.0	±	0.0a	35.8	±	17.9b	34.7	±	17.4b	26.0	±	14.9b	26.1	±	13.0b	20.4	±	10.3b

DBT = Day before treatment; means followed by the same letter in the same column are not significantly different at $p<0.05$, using LSD ranging test. Data were first subjected to arc sin transformation before analysis of variance.

Table 22b. Percentage soil volume replaced by *Macrotermes subhyalinus* workers within 24 hours in mounds treated with two dose rates of spore dust of *Metarhizium anisopliae* using a bicycle pump, at different intervals over a period of 60 days at Sasiga, Ethiopia.

b.

Treatment (across observation time effects)	Percentage soil volume replaced											
	Days after treatment											
	1 DBT			30			45			60		
Control a	100.0	±	0.0a	100.0	±	0.0a	100.0	±	0.0a	100.0	±	0.0a
4.6g spore/mound b	100.0	±	0.0a	57.8	±	18.1b	49.9	±	23.0b	42.6	±	7.0b
9.3g spore/mound b	100.0	±	0.0a	35.0	±	17.7b	33.5	±	22.0b	32.1	±	19.3b

DBT = Day before treatment; means followed by the same letter in the same column are not significantly different at $p<0.05$, using LSD ranging test. Data were first subjected to arc sin transformation before analysis of variance.

Table 23 Percentage weight loss in eucalyptus pickets over a 75-day period placed on *Macrotermes subhyalinus* mounds treated with three doses of spore dust of *Metarhizium anisopliae* by bicycle pump into ventilation holes at Lekemt Zuria, Ethiopia.

Treatment (across observation time effects)	Percentage soil weight loss				
	Days after treatment				
	15	30	45	60	75
Control a	12.2 ± 6.3a	15.0 ± 6.9a	23.6 ± 6.9a	25.5 ± 6.9a	34.5 ± 8.0a
4.6g spore/mound a	3.7 ± 2.2a	12.0 ± 5.1a	22.0 ± 5.8a	20.2 ± 5.8ab	20.9 ± 5.8ab
9.3g spore/mound a	4.7 ± 2.9a	7.1 ± 3.6a	8.2 ± 3.6a	8.2 ± 3.6bc	8.6 ± 3.5bc
20g/spores/mound a	3.5 ± 2.5a	4.6 ± 2.9a	7.6 ± 4.4a	4.3 ± 4.3c	4.3 ± 4.3c

DBT--Day before treatment; means followed by the same letter in the same column are not significantly different at $p<0.05$, using LSD ranging test. Data were first subjected to arcsine transformation before analysis of variance.

Table 24 Percentage termite replaced soil volume by *Macrotermes subhyalinus* workers within 24 hours on mounds treated with three dose rates of spore dust of *Metarhizium anisopliae* by hand, at different intervals over a period of 75 days at Lekemt Zuria, Ethiopia.

Treatment (across observation time effects)	Percentage soil volume replaced				
	Days after treatment				
	1 DBT	30	45	60	75
Control a	100.0 ± 0.0a	100.0 ± 0.0a	100.0 ± 0.0a	100.0 ± 0.0a	100.0 ± 0.0a
4.6g spore/mound b	100.0 ± 0.0a	83.7 ± 16.3ab	77.3 ± 12.1b	72.0 ± 22.2ab	70.4 ± 9.4b
9.3g spore/mound b	100.0 ± 0.0a	78.4 ± 13ab	77.3 ± 15.9bc	72.2 ± 14.7ab	64.7 ± 9.2bc
20g/spores/mound c	100.0 ± 0.0a	55.9 ± 7.0b	49.4 ± 5.3c	42.0 ± 28.1b	46.7 ± 4.1c

DBT = Day before treatment; means followed by the same letter in the same column are not significantly different at $p<0.05$, using LSD ranging test. Data were first subjected to arc sin transformation before analysis of variance.

5.4 Discussion

5.4.1 Picket dressing

In the field tests *M. subhyalinus* workers did not avoid *M. anisopliae* treated pickets. There was no significant difference in weight loss between treated and non-treated pickets, indicating that termites attacked treated pickets as much as non-treated pickets. This supports results from the laboratory repellency study, where in certain cases termites did not distinguish between pathogen-treated zones compared to control zones. However, experimental set-ups in the field and laboratory studies largely differed. In the laboratory the termites could simply react to an odour, while when used as paint, the direct contact repellency might be more important. Milner and Staples (1996) believed that treating soil with *M. anisopliae* repels termites rather than killing them after observing three years protection of timber surrounded by treated soil under cool, dry condition. Under tropical conditions protection was reduced to six months. In this study the actual barrier represented by the repellent spores was rather thin, compared with a barrier of contaminated soil surrounding the pickets as in Milner and Stapless' experiment. The weather conditions at the trial site in Ethiopia during the experiment were cool and rainy. The rain might have had a detrimental effect on the fungal spores too, thus reducing the repellent effect. Similar experiments carried out in Benin, resulted in some degree of protection. Depending on the applied spore dose and formulation, the damage on pickets by termites was reduced (J. Langewald, IITA Benin, pers. comm.) However, compared with chemical treatments, the effect was rather weak. In the USA, in soil treatment tests that simulated conditions under buildings, using wooden blocks placed in the soil, chemical insecticides like chlorpyrifos, permethrine, diazinon and isofenphos gave protection to the wooden blocks against termite attack between five and 14 years, depending on the type of insecticide and concentration used (Edwards & Mill, 1986). Metal salts and oxides can be effective for 3-5 years (Pearce, 1997). However, the picket bioassay set-up proved to be a good tool to monitor termite colony activity (see below).

5.4.2 Application methods and spore doses

Despite the apparent lack of success in destroying significant numbers of termite mounds, it was possible to kill three termite colonies at each spore dose and to cause a reduction in

termite activity, indicating ongoing epizootics in the remaining treated colonies. The termites in the treated mounds remained affected throughout the 75-days study period, as shown by the significantly reduced hole sealing and foraging (on the pickets) activity from 15-30 and 75 days after application, respectively. Results of this study as a whole differ from tests conducted by Hänel and Watson (1983) on *N. exitiosus* as in their case the highest mortality after application of *M. anisopliae* was observed during the first three weeks, with a decline in mortality up to the 6th week. In this study, such effects were recorded considerably later, corroborating earlier findings of Milner *et al*. (1998b) who observed, that *C. acinaciformis* colonies died gradually at six months after application. Gitonga (1996) also observed a gradual increase in mortality and infection percentage in *M. michaelseni* in mounds treated with contaminated rice, sawdust bait and conidial dust of *M. anisopliae*.

In the present study, treated mounds did to some degree respond differently to spore doses. There was no difference between the application methods, except that the colonies that received manual treatments reacted more slowly. According to Fernandes and Alves (1991), 5 g/mound of either *M. anisopliae* or *B. bassiana* was effective enough to cause 100% mortality in *C. cumulanus* colonies within 10 days after application. On the other hand, Milner and Staples (1995, 1996) and Milner *et al*. (1998b) reported dose-response to treated mounds and recommended 10 g/mound or higher conidial dose for a complete colony kill. This study could, however, demonstrate that *Macrotermes* colonies can be killed within 15 days, corroborating results of Fernandes and Alves (1991) for a termite species that form comparatively smaller colonies. Possibly in this study some of the *M. subhyalinus* mounds treated with different doses might eventually have died after the end of the observation period, as also observed by Milner *et al*. (1998b) for Australian termites. Differences in results between different studies may be not only linked to the difference in size of the colony and the difference in species, but also to social structure, size and behaviour of the termites, and other mound characteristics (Gitonga, 1996).

Suppression of the pathogenic effects due to interactions among fungal species already present in the mound, poor growth due to lack of light, grooming and walling-off treated areas are factors that may hinder proliferation of a disease (Hänel & Watson, 1983; Zoberi & Grace, 1990a; Logan *et al*., 1990). Milner and Staples (1996) believe that treatment of mound nests by damaging small sections and applying *Metarhizium* based dust is

ineffective as the termites wall-off such treated areas. Beattie *et al*. (1994) found that the outer layers of the mounds are very low in organic matter and have a very low matrix potential, both of which could limit microbial growth. However, they believe that the nursery area was suited for microbial growth, though antimicrobial substances may be produced to defend this part of the nest. *Z. angusticollis* line the nest chambers and galleries with pellets of the faeces which can inhibit germination of *M. anisopliae* spores (Rosengaus *et al*., 1998a). Hänel and Watson (1983) speculated that termites might produce fungistatics that prevent germination of fungal spores in the mound. Rath (2000) indicated that grooming could both increase the disease incidence in termite populations and conversely increase survivorship. Traniello *et al*. (2002) demonstrated that after exposure to *M. anisopliae*, *Z. angusticollis* individuals develop immunity as group member and resulted on higher survivorship. Furthermore, termites significantly improved their ability to resist infection when they were placed in contact with previously immunized nest mates. The most limiting factor, however, might be the application methods which most often reach only a small portion of a termite colony, and do not necessarily have an direct impact on the queen, which in the case of *Macrotermes* spp. is well hidden in a royal chamber and produces on average 40,000 eggs per day (Harris, 1971). Despite these limitations several strategies have been developed for use of *Metarhizium* spp. in termite control (Milner & Staples, 1996), and the possibility of developing more commercial products is currently explored (Milner, 2000a). The control of termites with entomopathogenic fungi does not need to rely on fungal growth within the mound, as control can also be achieved if the pathogens simply survive on the interior surface of the nest (Rath, 2000). Results of this study have shown to some extent that *M. anisopliae* has the potential for control of *M. subhyalinus* under field conditions, and can survive extended periods in termite galleries.

6 General discussion

The initial stages of the development of a pest control strategy using entomopathogenic fungi is either the collection of fungal isolates and subsequent screening for virulence to the target pest, or the introduction and testing of non-indigenous fungal strains, available in international collections such as the American Type Cultural Collection or the collection of the International Mycological Institute (IMI) (Milner, 1992). In this study, several *M. anisopliae* isolates and one *B. bassiana* isolate were isolated from infected termites and termite mound material from termite prone areas in the Ethiopia.

However, since only limited numbers of isolates were obtained, surveys for entomophathogenic fungi should be extended. A broad range of fungal isolates increases the chances of selecting a highly efficient and virulent strain for pest management programs (Brownbridge *et al*., 1993). Milner *et al*. (1998ab) suggested that it is best to search for suitable strains in the environment of the target pest, and at the same time testing isolates from other hosts and soil types, since specific termite-associated isolates do not exist and most isolates are virulent for termites. Results of this confirm this hypothesis. The most virulent *M. anisopliae* isolate against *M. subhyalinus* was not isolated from termites but from ordinary soil. Thus for future for collection exercises, a broad survey approach, focusing not only on termite affected areas but also a wide range of different ecological zones, seems to be most promising.

Ethiopia's diverse natural and environmental conditions that contributed to its wealth of flora and fauna have also created a rich diversity of microbial genetic resources (Fassil, 1999). Hence, there could be also good opportunities for encountering a large variety of entomopathogenic fungi with possible high potentials for pest control in general and termite control in particular. Prior and Street (1997, in Milner *et al.,* 1998b) advocated the use of indigenous entomopathogenic fungi for microbial pest control, as they are often better adapted to survive in their natural environment and are thus more likely to be efficient. Moreover, the development of a mycopesticide based on an indigenous fungal strain renders the registration process easier. However, over the past decades the concept of using non-indigenous biological control agents for the control of both indigenous and alien pests became rather successful (Waage, 1996). For the development of microbial control products this concept is interesting because of the considerable investments involved,

particularly for the development of the extensive registration dossier (including expensive mammalian toxicity tests) required in certain countries.

However, the question whether to develop indigenous or exotic isolates is discussed very controversially, and should be treated with care. Importing non-indigenous biocontrol agents which have been commercialized in one part of the world may be a commercially a more preferable option than to invest in new research for the development of a local species for the same purpose. Moreover, global diversity of natural enemies constitutes a pool from which the best natural enemy product for a particular pest and environment may be drawn. In this context, whether the pest or the natural enemy product is indigenous or alien has become a secondary issue. The discovery of strains of *Bacillus thuringiensis* Berliner with high levels of virulence to beetles and flies through similar exploration research is a good example for the benefit of non-indigenous species and strains in microbial control(Waage, 1996). Economics and the benefits of selecting from the global natural enemy pool, has made commercial introductions of non-indigenous natural enemies commonplace today (Waage, 1996). However, this does not mean that indigenous strains should totally be ignored. Like in the case of Green Muscle^{TM} for grasshopper control (Waage, 1996), local strains that are highly virulent against certain indigenous pests can be encountered. Those countries that insist on the sole use of indigenous strains of pathogens should accept a coronal definition of indigenous in order to exploit the full benefits of biological control.

In the present study, the laboratory bioassays and the field tests in general showed that *M. anisopliae* and *B. bassiana* strains found in nature are pathogenic against *M. subhyalinus* and can initiate epizootics in termite colonies. Although certain results from the bioassays were inconsistent, across all experiments there was a general trend showing that some isolates were more virulent compared with others. Generally, *M. anisopliae* isolates were more virulent than *B. bassiana* isolates. The experiments also demonstrated the importance of social behaviour for microbial control of termites. The common grooming behaviour can enhance the control potential of entomopathogenic fungi through cross contamination. On the other hand, this behaviour can also limit the efficacy of the pathogens. By ingesting and deactivating the spores, grooming termites remove spores from the system. Additionally some termite species isolate infected conspecifics by walling them off (Strack, 2000). All these activities take spores out of the cross contamination circuit. In order to exploit the

control potential of an entomopathogenic fungus one should consider social defence mechanisms in termite colonies. For termite control, a fungal strain with a medium speed of kill and higher sporulation potential is preferable (Carruthes & Soper, 1987). Strains which cause a medium speed of kill would give a contaminated termite enough time to cross contaminate untreated termites before death. However, a strain should not be too slow in killing the target insect since that would give termites more time to apply the before mentioned defence mechanisms that hinder transmission of spores and might stop an epizootic. In this study the *M. anisopliae* isolate MM collected in Ethiopia also remained effective at decreasing doses. A higher sporulation potential is also important for initiating epizootics. In this study, MM showed several properties for an ideal isolate for termite control. It caused a medium speed of kill and a lower LD_{50}, hence being more virulent both in the first few days (1-3 days) and at the end of the bioassay testing period (9 days). Additionally, MM has a high sporulation potential on cadavers.

The inconsistency of the laboratory bioassays was related to major difficulties in keeping workers of *M. subhyalinus* alive outside the termite colony for a sufficient period of time. Termites in general are very fragile insects and particularly *Macrotemes* spp. cannot be maintained in the laboratory easily. In general, lower termites (families Mastotermitidae, Kalotermitidae, Hodotermitidae and Rhinotermitidae) can be reared in the laboratory comparatively easily and therefore most of the experimental work on termites has been carried out with them (Verma, 1982). Yet, higher termites (family Termitidae) are difficult to rear under laboratory conditions because of their complex social organization (Verma, 1982). Moreover, among the higher termites, fungus-growing termites, belonging to the subfamily Macrotermitinae, are far more difficult to rear than species without fungus gardens (Gunther, 1970). Finally the susceptibility of termite colonies as a whole might be very different from the susceptibility of individual termites and castes under laboratory conditions (Rath, 2000). In general, emphasis should be given to develop more reliable bioassay techniques for testing fungus-growing termites, or the bioassays should be carried out as much as possible under field conditions. Moreover, when judging laboratory bioassay results, it is important to consider the behaviour of termites. Apart from direct control of termites through fungal infections, the repellent effect of entomopathogenic fungi might protect wooden structures from termite attack. Therefore, while dealing with selection of strains for termite control, the fungus itself, the formulation or the amount of spores that are repellent to termites should also be investigated separately. In this study, an

attempt to assess difference in the repellent effect of different *M. anisopliae* and *B. bassiana* strains against *M. subhyalinus* was not entirely successful. Most likely the experimental set-up did not separate olfactory cues good enough to provide the termites with a real choice.

From this study can be concluded that microbial pesticides will not serve as a 'stand alone' treatment for *M. subhyalinus*, but could become a component of an integrated termite control strategy. More work on the development of improved formulation and application methods is required. In future studies design and methodology of field experiments need to be further optimised. Moreover, such trials should be repeated over a longer period of time, using different *M. anisopliae* and *B. bassiana* isolates and different application methods that might trigger epizootics at reasonable costs. The possibility of repeated applications, as well as the survival of fungi in galleries should be assessed over a longer period of time. It would also be useful to determine the impact of *B. bassiana* and *M. anisopliae* on termites that build diffuse subterranean nests, including the genera *Microtermes* and *Odontotermes* that attack crops in the field and which are difficult to control by chemical and mechanical means. Langewald and Cherry (2000) indicated that one of the greatest challenges in the inundative use of microbial control agents is to find economically competitive means of mass production. They further stated, that for robust fungal strains or species, that are less sensitive to contaminants during mass production, cottage style production could be a cheap and feasible approach. Moreover, experiences in countries such as Brazil, Costa Rica and the Philippines have shown that *Metarhizium* spp. can be mass-produced in small local facilities to serve local needs; though product quality often remains a problem (J. Langewald, IITA Benin, pers. comm..). These production systems provide *Metarhizium* spp. mainly for the control of cash crop pests (e.g. sugarcane). Therefore, the challenge is to develop a strain that is both virulent and robust. In this study, attempts have been made to the compare the sporuation potential of *M. anisopliae* and *B. bassiana* isolates on different substrates. The *M. anisopliae* isolate MM had a higher sporulation potential both on mycological media and rice, which is the most common substrate for mass production of *Metarhizium* spp. (Aquino *et al.,* 1977; Mendonca, 1992).

In Ethiopia, particularly in the places where this study was conducted, the government organizes termite control campaigns through different projects. These control campaigns have been conducted every 5-8 years after termite population started building up. Emphasis

has not been given to integrated control strategies that could maintain termite population below a yet to be defined economic threshold. Termite control campaigns that were conducted in the past have shown that the use of synthetic pesticides alone could not provide a sustainable solution to the termite problem.

Whether farmers in Ethiopia are ready to purchase a fungus-based product for termite control remains to be studied. However, large government operations could switch to *M. anisopliae* if large-scale field applications have been demonstrated to be successful and economically competitive.

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