ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

PATHOGENICITY OF ETHIOPIAN ISOLATES OF ENTOMOPATHOGENIC FUNGI AGAINST GLOSSINA MORSITANS MORSITANS .

A thesis submitted in partial fulfilment of the requirement for the degree of
Master of Science in Biology

MAMUYE HADIS
May, 1999

CONTENTS

PAGE

LIST OF TABLES AND FIGURES

ACKNOWLEDGEMENTS

I am deeply indebted to Dr Dawit Abate, my advisor, with out his help, encouragement, and guidance, in every aspect of the work, this work would have been impossible.

I thank my advisor Dr Getachew Tikubet for providing me with fungal isolates and for his advice.

I am also grateful to Ato Sinishaw Aysheshim for providing *Metarrhizium* and *Beauveria* isolates and for kindly sharing his experience with me in the laboratory work. I also thank him for the literature he provided me.

My thanks go to Dr Emiru Seyoum for helping me get relevant literature.

The help and encouragement rendered through out this study by my colleagues and friends at the Ethiopian Health and Nutrition Research Institute is greatly acknowledged.

I want to express my gratitude to the friends I made in the graduate school for their encouragement and making my stay at the Addis Ababa University so memorable.

Finally I wish to thank the Department of Biology in general and Dr. Kifle Dagne in particular for their help and advice.

Abstract

Fungal pathogens of insects have increasingly shown promise as effective control agents. In this work pathogenicity of five local isolates, *Metarrhizium anisopliae (EE)*, *Metarrhizium* sp. (MM), *Beauveria bassiana* (FF), *B. bassiana* (GG), *B. bassiana* (AK), and two exotic an isolates, *M. anisopliae* (ICIPE 30) and *B. bassiana* (temporarily coded as Bb) were determined against laboratory reared *Glossina morsitans morsitans*. Fifteen days old cultures of the respective isolates were harvested in sterile distilled water (0.1% Tween -20). After determining the concentration of spore suspensions using Neubauer improved hemocytometer, concentrations were adjusted to 1 X 10^8 (the screening concentration used). Adult flies, less than a week old, were exposed to spore suspensions. Flies were chilled at 1°c for seven minutes prior to exposure. Flies were then exposed to the spores by dipping them in the suspension for 15 seconds, after which they were recovered and put back to their cages and to the insectary (70% RH, 25° C). Mortality was recorded for a maximum duration of 20 days. Among the *Metarrhizium* species, *Metarrhizium* EE has demonstrated the highest performance (96.67%), followed by *Metarrhizium* ICIPE 30 (90.00%), and *Metarrhizium* MM (73.33%). The differences were not significant (P> 0.05). Among the *B.bassiana* isolates the Kenyan isolate has performed the best (81.67%). *Beauveria* FF, *Beauveria* GG, *Beauveria* AK, have shown per cent moralities 75.00%, 63.33%, 53.33%, respectively. The difference between *Beauveria* FF and *Beauveria* Bb was not significant (P<0.05) while the difference between *Beauveria* Bb and *Beauveria* GG was significant (P< 0.05). All, but *Beauveria* GG, were significantly better than *Beauveria* AK (P< 0.05). Spore production for *Metarrhizium* MM and *Metarrhizium* EE was determined on solid substrates, whole grains of rice, wheat, barley and sorghum. Both isolates have grown best on rice

giving a yield of 1.42 X 10^9 spores/gram of rice for *Metarrhizium* MM and 1.62 X 10^9 spore/ gram of rice for *Metarrhizium* EE. The next highest yield was on sorghum, 3.10 X 10^8 spores/gram for *Metarrhizium* MM and 2.25 X 10^8 spores/gram of wheat for EE. No correlation was observed between moisture content and the spore yield of grains. Mass production and utilization of selected fungal pathogens against tsetse as a bioinsecticide as a component of Integrated Pest Management is recommended.

1. INTRODUCTION

Trypanosomiasis, a disease caused by trypanosomes, is of great economic and medical importance in Africa. Human African trypanosomiasis, sleeping sickness, is exclusively transmitted by tsetse flies (*Glossina* species) while animal trypanosomiasis being transmitted mainly by tsetse flies could also be transmitted by other biting flies.

In addition to causing great human suffering, deaths, and loss of work efficiency, the disease could also force a whole population of people to move from an infected area to another area where the disease dose not occur. Absence of cattle and domestic animals in tsetse infected areas has a profound effect on agricultural development and productivity (Wilson *et al.*, 1963).

Control of trypanosomiasis involves measures against both the fly and the parasites, case detection and treatment and vector control through different techniques are employed. Some of the techniques in vector control include: spraying of insecticides in the natural habitat of the fly (Kuria & Bwogo, 1986), use of traps (Dransfield, 1991), insecticide impregnated screens (Grundler, 1991), pour-on on cattle (Meyer *et al.*, 1991), sterile insect technique (WHO, 1986), bush clearing or a combination of techniques (Wellde, *et al.*, 1989).

However, the techniques mentioned are known to have one or another drawbacks. Use of insecticide spray over a large area is known to be responsible for environmental pollution and other related problems. Use of traps and screens

requires regular maintenance (Leygues & Gouteux, 1989; ILRAD, 1989). Insecticide pour-on has to be applied at least once a month (Meyer *et al.*,1991) and cost of chemicals hamper their availability to farmers (Hendrickx & Napala, 1995).

Hence the search for better control methods is a continuing effort.

The need for Integrated Pest Management in tsetse control has been emphasized (Kaaya & Munyinyi, 1995), biological control could be one of the components in integrated tsetse control. Biological control has many attractions in pest management, one of which is the capability of providing long term suppression of the target pest (Greathead, 1987).

Recent studies on the use of entomopathogenic fungi against *G. morsitans morsitans* have shown the high potential of fungal agents in the control of tsetse flies. *Beauveria bassiana* , and *Metarrhizium anisopliae* were found to be pathogenic for adult *Glossina morsitans morsitans* causing mortality often reaching 100% by 15-18 days of post infection (Kaaya, 1989).

This work, therefore, aimed isolating local entomopathogenic fungi, evaluate their potential to control tsetse flies in the laboratory, and see the feasibility of mass production of entomopathogenic fungi using solid state fermentation.

2. LITERATURE REVIEW

2.1.Tsetse flies

2.1.1. Life cycle

Tsetse flies have a holometabolous life cycle, they pass through egg, larva, pupa, and adult. Unlike most flies which lay eggs tsetse flies give birth to a larva (Service, 1986).

Female tsetse fly reaches maturation usually 2-3 days after hatching. After the fly got inseminated and took a blood meal a single egg completes its maturation in about four days. Then the egg gets fertilized and hatches in the uterus. The larva passes through three instars in five days time before it gets deposited. The larva is normally deposited on loose soil (sand or humus) frequently in shaded areas, which could be under logs, bushes, and animal burrows (Service, 1986; Kettle, 1984).

Soon after deposition, the larva starts to bury itself in soil. In fifteen minutes time it changes into a pupa.

The pupal stage is normally long usually 4-5 weeks, after which the adult emerges and escapes to the surface and flies away. A newly hatched fly takes 16-20 day till it deposits the first larva. After the first larva the female is able to give birth every 9-12 days (Kettle, 1984; Service, 1986).

2.1.2. Adult behavior

Both sexes exclusively feed on blood. The hosts could be man, other mammals, reptiles and birds. However no tsetse fly species is known to depend on a single host, though most species show preferences for certain hosts. For example, in West Africa *Glossina palpalis* feeds predominantly on reptiles and man while *Glossina morsitans* feeds mainly on warthogs. They take blood meals every 2-3 days, depending on the weather this interval could be reduced or increased. They normally feed during the day time (Service, 1986).

Tsetse flies rest in dark and humid places. During day time they rest mainly on tree trunks, twigs, branches, and at night they rest on upper surfaces of leaves (Service, 1986).

No tsetse fly species is known to live in an open grass land, tsetse flies are associated with woody vegetation (Buxton, 1955). According to their habitat preferences, morphology, and behavior, tsetse flies are separated into three groups: *Fusca* group, *palpalis* group and *morsitans* group. The *fusca* group lives in low land rain forests or gallery forests while the *palpalis* group and *morsitans* group inhabit riverine vegetation and thickly wooded Savannah areas, respectively (WHO, 1986; Service, 1986).

Optimum temperature for tsetse flies is 25-26°C. However the optimum temperature range could vary from species to species according to their habitat difference. In

general , the upper limit for adults is 38°C, the lower limit is 17°C. For pupae 32°C and 16°C are the respective temperatures (FAO, 1986a)

2.1.3. Distribution, economic and medical importance

There are thirty species and subspecies of *Glossina* in sub-Saharan region of Africa in thirty seven countries, an area of over 10 million square kilometers or roughly a third of the continent (WHO, 1986; ILRAD, 1989). Their distribution is restricted between latitude 15° north and 20° south, however it extends to about 30° south along the eastern coastal area. Some species have relatively wider distribution , like *Glossina morsitans* which is found across west, central, and east Africa, and others are restricted to some geographical areas. *G. palpalis*, for example, is found only in west Africa (Service, 1986).

The distribution of tsetse flies is dictated by climate, vegetation, fauna, and human activities (Langridge *et al.*, 1963). Depending on these factors, the distribution of tsetse flies is subject to change (WHO, 1986).

Sleeping sickness is endemic in 36 countries of sub-Saharan Africa. About 15000-200000 new cases of sleeping sickness are reported annually, and it could be responsible for tens of thousands of deaths each year (TDR, 1993). And this figure is an under estimation of the actual figure , since only 3 to 4 millions of the estimated 50 million people at risk are currently under surveillance (Cottand, 1993). According to WHO (1979) in (UNDP/World Bank/WHO/, 1982) estimation, about

US 5 million is spent annually on sleeping sickness surveillance and vector control operations.

Africa's low livestock productivity is mainly ascribed to the wide spread occurrence of trypanosomiasis. In addition, the areas inhabited by tsetse flies, therefore avoided by man, are potentially the most agriculturally productive areas in Africa. Approximately a third of Africa's cattle population which totals 150 million, and equivalent numbers of small ruminants are considered to be at risk of infection. Losses in meat production alone are estimated at US 5 billion a year (ILRAD, 1989).

Lack of trained man power and funds, and the high cost of surveillance and tsetse control operations are the major obstacles for the continent in the fight against the disease (UNDP/World Bank/WHO, 1982).

2.1.4. Control of tsetse flies

2.1.4.1. Early methods

Bush clearing.

Attempt to control tsetse flies started in the early 1900's. One of the methods at this time was bush clearing where by the habitat of the fly is destroyed either completely or partially there by making the area unsuitable for the fly. However, bush clearing has become no longer ecologically or economically acceptable through time (Service, 1986; Cuisance, 1985). Total bush clearing, however, can still be used for some purposes such as preparing a barrier to prevent spread of tsetse from infested areas to tsetse free areas (FAO, 1986).

Game destruction

Killing game animals which serve tsetse flies as a source of food was practiced in the past to control tsetse flies (Hocking *et al.*, 1963). However, nowadays this method is no more used due to its damage to the wild life (Cuisance, 1985).

2.1.4.2. Traps

Since bush clearing and game elimination are ecologically damaging the search for environmentally friendly control methods was a necessity. Traps are ecologically safe methods.

Traps which attract flies and catch them are being widely used since 1970's (Lancein, 1985; FAO, 1992) to control tsetse flies. After several investigations (Vale, 1979; Hargrove and Vale, 1978) it was known that traps catch more flies when they are baited with host odor. Trials using traps baited with acetone and cow urine have been reported to reduce flies up to 90% (Dransfield *et al.*,1991). However, it was observed that not all flies attracted to the trap are caught (Vale, 1982); hence the need for devices which need not be entered to be effective were necessary.

2.1.4.3. Insecticide impregnated screens

Insecticide impregnated screens usually consisting of a black close, with a dimension of 1 meter square were later developed to kill tsetse flies (Vale *et al.*, 1988). Compared to traps, screens are cheaper, simpler and can remain effective with some damage (Laveissier *et al.*, 1990) . Screens coated with delthamethrin and

baited with odors, acetone or butanone, are reported to achieve a reduction of a population of *G. pallidipes* by 99.9% in Zimbabwe (Vale *et al.*, 1988).

However, regardless of the advantages of traps and screens, their wider usage was limited . Some of the reasons for their limited use are: deployment and maintenance of traps and screens over large area was difficult, and cost of odor attractants and insecticides, the need for skilled man power and infrastructure to design and follow up of the control program are beyond the capacities of African communities (Baylis & Stevenson, 1998).

2.1.4.4. Insecticide pour-on

More recently cattle treated with insecticides (synthetic pyrethroides) as pour-ons or dips are being used in tsetse control operations. Tsetse flies in an attempt to feed on insecticide treated cattle get a lethal dose of the insecticide. A reduction of 93% in tsetse population has been reported in two year time using cypermethrin pour-on (Leak *et al.*, 1995). The cost of the pour-ons and their use in permanent basis , however, is questionable (Meyer *et al.*,1991; Hendrickx & Napala, 1995).

2.1.4.5. Chemical control.

Applications of insecticides to the vegetation forming the habitats of the fly is still is the method of choice in large scale tsetse fly control campaigns (Seketeli, 1985). The most widely used insecticides in the control of tsetse flies are DDT and dieldrin; and relatively recently endosulfan (WHO, 1986).

Insecticides are applied to the habitat if tsetse fly in two ways, as ground application and aerial spraying.

1. Ground application of insecticides.

This technique is based on the application of residual insecticides in the resting places and larviposition sites of tsetse flies. The insecticide must stay effective at least for two months, the maximum duration for a pupal stage of tsetse fly. Insecticide applications are normally carried during the dry season to protect the insecticide from being washed away by rain (WHO, 1986; Seketeli, 1985).

2. Aerial spraying

The purpose of aerial spraying of insecticides in tsetse control is to destroy all or almost all flies at the time of treatment in the zone under attack (Hamon *et al.*, 1977). Hence the need for repeated (sequential) applications of the insecticides usually 5 times at about 10 day intervals (to kill newly emerging flies), which makes aerial application expensive (Hamon *et al.*,1977; WHO, 1986). For this reason, aerial applications are reserved for emergency situations when it is necessary to stop transmission rapidly (UNDP/World Bank/WHO, 1982).

In general tsetse fly control has been based on large scale use of insecticides on the ground or from the air (Cuisance, 1985). However, large scale use of insecticides is, nowadays, heavily criticized for various reasons such as soil and water contamination, impacts on the food chain, proliferation of resistance, affecting beneficial fauna, cost, etc. (Pfeiffer & Grigliati, 1996; Hall & Papierok, 1982).

2.1.4.6. Sterile Insect Technique (SIT)

By this control method large numbers of mass produced males are sterilized by X-rays or gamma radiation and released into the natural habitats of the target pest. The concept behind sterile insect technique is that the released sterile males will mate with the wild virgin females which store sterile sperm for subsequent fertilization rendering them infertile. As a result, the density of the natural population will decrease and subsequent releases of sterile males further decrease the natural population with a possible elimination of the target pest from the treated area . Prior to releasing sterile males reduction of the natural population is first carried by conventional methods to reduce the number of sterile males to be released (IAEA, 1992).

SIT has been successfully used to eradicate the new world screwworm in the USA and Mexico (Krafsur *et al.*, 1987; IAEA, 1992), and Libya (Lindquist *et al.*,1992).

Attempts are now being made to use SIT to control tsetse flies. In Nigeria and Burkina Faso *G. palpalis gambiensis* has been eliminated form considerable areas using SIT (IAEA, 1992). Recently, *G. austeni* has been eradicated from Zanzibar in a four year (1994-1997) program (Tsetse and Trypanosomiasis Information Quarterly, 1998a).

Ethiopia is going to launch a program to eradicate *G. pallidipes* from the Southern Rift Valley, an area estimated to be 25 thousand square kilometer using SIT in collaboration with joint FAO/IAEA and Seibersdorf Laboratory (Tsetse and Trypanosomiasis Information Quarterly, 1998b).

Though SIT is environmentally friendly and target specific, it is considered very sophisticated and expensive (Krafsur,1987; IAEA, 1992).

2.1.4.7. Biological control

Interest in biological control of economically important pests is growing from time to time as environmentally friendly approach. More over, the low reproductive potential of *Glossina* makes it an attractive candidate for biological control (Jordan, 1985). However, little has been done regarding tsetse flies, though a number of natural enemies of tsetse flies have been recorded (Simmonds *et al.*,1977).

Predators

Numerous natural enemies, predators and parasites of different stages of tsetse fly have been reported in nature; but the mortality that can be ascribed to them is not known (Jordan, 1985).

Birds, Insects, spiders are known to prey on adult tsetse flies while ant, beetles, birds and small mammals are known to prey on pupae of tsetse flies (Gruvel, 1977; Cuisance, 1985; FOA, 1986). Ants feeding on tsetse pupae, and robber flies , wasps and spiders feeding on the adults are considered the most important predators (FAO, 1986).

Parasitoids and parasites

Different species have been recorded to prasitize pupae of tsetse flies: *Syntomosphyrum* sp., *Mutilla* sp., *Thyridanthrax* sp. etc. (Simmonds *et al.*, 1977). *Syntomosphyrum* had been released to the field in an attempt to control tsetse flies in Malawi, Nigeria, and Tanzania (Lloyd et al., 1927; Lamborn, 1925; Nash, 1933, in

Simmonds *et al.*, 1977). All these attempts were not successful, the maximum parasitism being 9.9% in Tanzania. The potential of these parasitoids in tsetse control is considered to be minimal due to their seasonality and lack of specificity to tsetse flies (Cuisance, 1985).

Pathogens

Very little work has been done with pathogens of tsetse flies, and no field trial has ever been reported (Kaaya, 1989). Different bacterial species have been associated with mortality of tsetse flies due to contamination in the laboratory feeding systems (Poinar *et al.*, 1977), but the possibilities of contamination in nature is very rare (Cuisance, 1985), hence, their use in biological control of tsetse flies is slim or impossible .

Recently a relatively detailed experiment on fungal infection of tsetse have been carried. In laboratory experiments using entomopathogenic fungi, *B. bassiana* and *M. anisopliae,* it was able to cause heavy mortality (up to 100%) against *G .m. morsitans* (Kaaya, 1989). Horizontal transmission of both pathogens from infected flies to non infected ones was also demonstrated (Kaaya & Okech, 1990). Larvae which were made to pupate in a sand and spore mixture of the pathogens have led to the deaths of emerging flies (Kaaya & Munuyinyi, 1995). These findings have raised the hopes of using these entomopathogens in tsetse control operations.

2.2. Fungal control

Fungal diseases in insects are known to be common and are reported to cause epizootics in nature. This phenomenon is an indication that some fungi have potential as microbial control agents (Goettel, 1992). More than 700 species of entomopathogenic fungi belonging to 90 genera are known to occur world-wide (Goettel, 1992; Wraight & Roberts, 1987) and they are known to vary in different important features like temperature and moisture optima, host range, epizootic potential, ease of mass production etc, providing wide spectrum of target insects (Wraight & Roberts, 1987; Ferron, 1978). Commercialized fungal products include *Verticillium lecanii,* used for aphid and whitefly control in green houses, *Hirsutella thompsonii,* used against citrus rust mite, *Metarrhizium anisopliae* against spittlebugs, and *Beauveria bassiana* against corn borer and Colorado potato beetle (Gardner & McCoy, 1992 ; Wraight & Roberts, 1987). More than a dozen of fungal species are also currently under development (Gardner & McCoy, 1992).

2.2.1. Taxonomy

The classification of fungi is difficult and differs from authority to authority (Ingold & Hudson, 1993; Alexopoulous & Mims, 1979). According to Dent (1991), the entomopathogenic fungi are distributed among the five classes of fungi, Deuteromycetes, Zygomycetes, Oomycetes, Chytridiomycetes and the Trichomycetes. The majority of entomopathogenic fungi belong to the class Zygomycetes, while fungi with higher potential for pest control, *Beauveria*, *Metarrhizium*, *Nomurae*, *Verticillium*, *Hirsutella*, are in the class Deuteromycetes (Dent, 1991).

2.2.2. Hosts

Major insect hosts of entomopathogenic fungi are: Culicidae (mosquitoes), Aphidae (aphids), Delphacidae (planthoppers), Cicadellidae (leafhoppers), Cercopidae (spittlebugs), Aleyrodidae (whiteflies), Coccoidae (scales), Thysanoptera (thrips), Coleoptera (beetles), and Lepidoptera (caterpillar) (Wraight & Roberts, 1987). Fungi are also known to attack arachnids (Dent, 1991). This wide host range of entomopathogenic fungi endows them with a very high potential for use against most insect pests (Goettel, 1992). However, individual fungus can display variation in host range. *M. anisopliae*, for example, is known to have diverse insect hosts, in the orders Coleoptera, Lepidoptera, Orthoptera, Hemiptera and Diptera (Hall & Papierok, 1982), while *Lagenidium giganteum* is known to infect only mosquitoes (Rawlins, 1984). Variation in specificity and virulence is also known to occur for different isolates of the same species (Goettel, 1992; Clarkson, 1996) showing genetic variation in host range with in a species. In addition, many factors like the nature of cuticle and host defense in the haemolymph are expected to determine host specificity of the fungi (Hall & Papierok, 982)

2.2.3. Pathogenesis

Mode of action

Entomopathogenic fungi infect their hosts by direct penetration of the body wall unlike other microbial pathogens which need to be ingested (Clarkson & Charnley, 1996). This feature of fungi has enabled them to infect insects with sucking mouth

parts, which otherwise can not be infected with other microbial pathogens (Carruthers & Soper, 1987). However, infection through the spiracles (Ferron, 1978) or alimentary tract (Broom *et al.*,1976; Veen, 1966) have been also reported.

The cuticle (exoskeleton) of insects is composed of proteins and chitin associated with lipids and phenolitic compounds (Ferron, 1978). Penetration involves both physical force and enzymatic reactions (Clarkson & Charnley, 1996). It also involves specific recognition of the host by chemical communication with the fungus (Gow, 1993). The first step in fungal infection of insects is fungal contact with the host cuticle by conidia, blastospore, or zoospores (Domnas & Warner, 1991). Different factors are involved in the attachment of spores into the insect's cuticle. For example, conidia of entomophthorales are covered with amorphous mucus, and spores of certain Dueteromycetes are slimy, therefore, helps them to attach to insect cuticle (Tanada & Kaya, 1993). On the other hand the hydrophobocity of the conidial wall and the insect epicuticle also seem to help adhesion (Boucias *et al.*, 1988).

In many pathogenic fungi fungal contact is followed by the germination of conidia on the host surface and differentiation into an infection structure known as appressorium. From the appressorium an infection hypha penetrates through the host cuticle and gradually emerges into the insects homocoel (Clarkson, 1996). Appressorium is a swelling produced at the end of short germ tube which attaches to the cuticle and send infection pegs into the host (Roberts & Yendol, 1971). Successful penetration is presumed to depend on the ability of spores to adhere to the

cuticle, germinate and penetrate enzymatically (Hall & Papierok, 1982). The fungus then starts to multiply in the hemolymph as yeast like blastospores, hyphal bodies, or protoplasts (Clarkson & Charnley, 1996.)

Different studies have shown that involvement of different enzymes during penetration. Gabriel (1968) has shown that the fungi *Entomophthora apiculata, E. thaxteriana, E. virulenta* and four strains of *E. coronata* are capable of producing lipolytic, chitinolytic and proteolytic enzymes. And production of chitinase was shown to be suppressed when chitin was not included in the medium, suggesting that the enzyme chintinase could be adaptive. Smith and Grula (1983) have also shown that chitinase produced by *B.bassiana* is an inducible enzyme by D-glucosamine and N-acetyl glucosamine . On the other hand Leopold and Samsinnakova (1970) have reported the presence of chitinase, cellulase, protease and lipases in culture of *B.bassiana*; chitinase being constitutive. These conflicting reports might tell that chitinase could be constitutive or adaptive depending on the species or strain of the fungus. Study on *M. anisopliae*, *B. bassiana* and *V. lecanii* (St. leger *et al.*, 1986) has shown that cultures of these species produce a variety of enzymes, endoproteases, aminopeptidases, carboxypeptidase, lipase, esterase, chitinase and N-acetylglucosaminidase. Enzyme production levels has shown considerable variation between species and within species (in *M. anisopliae*), except for endoproteases which were produced at high level in all the isolates. Enzymes were released sequentially, the proteolytic enzymes, aminopeptidase and carboxypeptidase, appearing first, followed by N-acetylglucosaminidase and chitinase. The sequential

release of enzymes has also been shown in an earlier study on *B. bassiana* by Smith *et al.* (1981).

The exact role that individual enzymes play in cuticle penetration and pathogenesis is not yet well understood (Clarkson, 1996). Paris and Segretain (1975) in Domnas & Warner (1991) believed that a direct relationship exist between the presence or absence of lipase and virulence, while Paris and Ferron (1979) working on *B. brongniarti*, a species similar to *B.bassiana,* found that lipase production didn't correlate with virulence.

The usual pattern of development of fungal infection in insects can be summarized as:

- the infective unit conidium or zoospore attaches to the insect cuticle
- the infective unit germinates on the cuticle
- the cuticle is penetrated either directly by germ tubes or indirectly by infection pegs from appressoria
- the fungus multiplies in yeast phase (hyphal bodies) in the homocoel
- toxic metabolites are produced
- the host dies

- fungus grows in the mycelial phase with invasion of virtually all host organs
- hyphae penetrate from the interior through the cuticle to the exterior of the insects
- Finally infective units are produced on the exterior of the insect (Robert, 1981, in Dent, 1991)

Toxins

Death of insects while infection is confined to the haemolymph and hypodermis had led to the conclusion that toxins are important in entomopathogenic fungi (Roberts & Yendol, 1971). Toxins are produced as microbial secondary metabolites, their toxicity being a secondary effect of their occurrence in a susceptible host (Lysenko & ,Kucera, 1971). Microbial toxins are generally defined as non enzymatic, low molecular-weight products of micro-organisms or micro-organism-host interactions that are harmful to the host at low concentrations (Roberts, 1981 in Gardner & McCoy, 1992).

Fungal metabolites can be screened for their biological activity either by injecting into the hemocoel of the insect, or by cuticular contact or ingestion. Since injection into the homocoel is not similar with the natural route of infection, ingestion and cuticular contact, it doesn't give an accurate evaluation of the potential of the toxin as an insecticide (Gardner & McCoy, 1992). Injection, however, can be useful in elucidating the modes of action of the toxin (Roberts & Yendol, 1971).

Entompathogenic fungi are known to produce a number of insecticidal toxins *in vitro*. Several toxic compounds have been isolated from different entomopathogenic fungi. Toxins known as destruxins have been isolated from *M. anisopliae* (Roberts 1969; Paris & Ferron, 1981). From *B. bassiana* beauvericin has been isolated (Hamil *et al.*, 1969). Other toxic substances from *E. coronata, E. virulenta* have also been reported (Yendol *et al.*, 1968). However, no commercial pesticide based on toxins is available due to their toxicity to non-target organisms, including mammals (Roberts, 1981 in Paterson *et al.*, 1987).

Effect of toxins on target hosts has been given some attention in a general review by Ferron (1978). The effects include: inciting progressive degeneration of the host tissues & dehydration of tissues cells through fluid loss, disturbance of the nervous system, perturbation of molting, disturbance in fecundity, diapause, and resistance to cold.

2.2.4. Host defense responses

The major route of infection of fungal pathogen is through the integument (Charnley, 1992), though there are reports that infection could occur through the alimentary canal (Broom *et al.*, 1976; Veen, 1966) and the spiracles (Ferron, 1978).

One of the function of the integument is protection against infectious micro-organisms and metazoan parasites (Gunnarsson, 1988). Failure of infection through the alimentary canal is ascribed to anaerobiosis, digestive enzymes, adverse pH, and

protection by the peritrophic membrane (Charnley, 1992) and anti-fungal toxins from gut bacterial flora (Dillon & Charnley, 1986).

The active defense mechanisms of insects is known to involve two components, cellular and humoral (Hall & Papierok, 1982).

The first response to fungal infection is the appearance of host produced brown or black pigment in insect cuticle (melanization) around the site of fungal penetration (Charnley, 1991). Melanization (deposition of melanin) is dependent on the localized production or adsorption of phenoloxidase (Lackie, 1988). Melanization has been shown to retard production of chymoelastase (Pr1) (a basic protease) by *M. anisopliae in vitro*. However, this protection offered by melanin is incomplete as pure Pr1 releases melanin from cuticle by hydrolysing the associated protein (St. Leger, 1988).

In a study on infection of *Schistocerca gregaria* by *M. anisopliae* it was shown that haemocytes aggregated beneath the site of infection; and this happened before the invading hyphae reached the haemocoel. The aggregates then became melanized (Gunnarson, 1988). How the aggregation of the haemocytes is induced is not known. Osmotic factors have been suggested as one of the reasons (Nyhlen & Unestam, 1980). However, haemocyte aggregation & melanization didn't stop fungal hyphae from growing through melanized cell aggregate into the haemocoel. In Chironomid larvae, humoral encapsulation has been also seen in the cuticle, upon fungal infection (Gotz & Vey, 1974).

After crossing the integument, cellular (Charnley, 1992) or humoral (Gotz & Vey, 1974) encapsulation of the fungus is followed in the haemolymph. A nodule is then formed following encapsulation. The infection is either blocked or the fungus may overcome the encapsulation and invade the rest of the organism (Ferron, 1978).

However, there are evidences of host defense evading mechanisms by fungi. One is the dimorphic nature of most entomopathogenic fungi, existing as yeast like hyphal bodies in the haemolymph during the pathogenic phase, and changing to mycelial form prior to sporulation. Growth of fungus as blastospores in the haemolyph could aid the fungus in dispersion and colonization of the haemocoel, and optimize nutrient acquisition by increasing surface area and dissipate the efforts of the host cellular immune system (Clarkson & Charnley, 1996). Altered cell wall composition due to this change in morphology could be responsible for the failure of the cellular defense (Charnley, 1991). Destruxins produced by *M. anisopliae* have also been reported to interfere with the immune system of the desert locust (Huxham *et al.*, 1989 in Charnley, 1991).

2.2.5. Mass production, , formulation, storage and application.

2.2.5.1. Mass production

Fungi are produced as conidia, mycelia or blastospores. The form in which (conidia, mycelia, blastospore) a fungus is produced depends upon consideration of different factors such as stability of the infecting form, ease of production and application related problems (Wraight & Roberts, 1987). They are produced on solid substrate or liquid fermentation or a mixture of both techniques depending on the desired end product (Hall & Papierok, 1982; Genkins & Goettel, 1997).

Solid substrate fermentation

Many fungi grow on moist solid media and produce conidia (Goettel & Roberts, 1992). The solid substrates are normally grains such as wheat, rice, bran, sorghum, straw etc., (Auld, 1992; Jenkins & Goettel, 1997). Plastic bags and trays are among the commonly used equipment (Auld, 1992). The use of solid substrates provides larger surface area and by doing so increases yield (Jenkins & Goettel, 1997). For successful production, however, pH, moisture, particle size, temperature, aeration, nutrient status of the substrates need to be controlled (Auld, 1992; Jenkins & Goettel, 1997). Solid substrate fermentation relatively takes longer time (around 15 days) and could be costly in terms of labor, time and material according to the socio-economic condition of a country (Hall & Papierok, 1982; Auld, 1992).

Spore yields up to 10^{10}spores per gram of substrate can be achieved using solid substrate fermentation (Hall and Papierok, 1982).

Compared to blastospores which are produced in submerged fermentation, conidia are considered to be robust (Hall & Papierok, 1982).

Submerged culture

The technology for mass culture of fungi is already developed to harvest fungal metabolites and yeast biomass production in submerged culture. Therefore the method can also be used for mass production of entomopathogens. However, only few entomopathogenic fungi produce infectious propagules in liquid fermentation (Goettel & Roberts, 1992)

Most hyphomycetous fungi produce blastospores in submerged culture (Jenkins & Goettel, 1997). However blastospores are less resistant to environmental stresses and difficult to preserve (Van Winkhelhof & McCoy, 1984; Ferron, 1978). Hence large scale production of blastospores is used only for *Hirsutella thompsoni*, which grows slowly on solid media (McCoy *et al.*, 1972).

In submerged culture fungi are grown in liquid medium either in shake flasks or in deep tank fermenters which could have 10000 liter capacities. Conditions of submerged cultures can easily be controlled compared to solid substrates (Jenkins and Goettel, 1997).

Some dueteromycetes are known to form conidia in liquid culture (Hall & Papierok, 1982). For example, some isolates of *B. bassiana* and *M. anisopliae* do produce conidia in submerged culture (Campbell *et al.*, 1983; Thomas *et al.*, 1987). Comparison of virulence and viability among aerial (solid substrate) conidia,

submerged conidia and blastospores has shown that blastospores were slightly more virulent than both conidia types, while both conidia types showed greater viability in storage than blastospores (Hegedes *et al.*, 1992 in Jenkins & Goettel, 1997).

Diphasic liquid -solid fermentation

Diphasic liquid -solid fermentation is a combination of liquid and solid fermentations to get the advantages of both techniques; the production of high biomass of the fungus in liquid fermentation and production of stable aerial conidia on a solid substrate (Jenkins & Goettell, 1997). Blastospores produced in liquid medium are poured into a solid substrate to promote fast growth of mycelial layer which will produce conidia. This technique has been used to mass produce *B. bassiana* in the USSR (Ferron, 1978) and *M. anisopliae* in Brazil (Aquino *et al.*,1975 in Auld, 1992).

The type of media used can affect yield (Vilas Boas, 1996), virulence (Goral, 1978 in Daoust & Roberts, 1983), and toxin production (West & Brigs, 1968 in Roberts & Yendol, 1971). Reduction of virulence has also been reported upon repeated culturing on artificial media (Samsinnakova & Kalalova, 1983).

However virulence can be restored to its original level upon passage through insect hosts (Daoust & Roberts, 1982). To avoid loss of virulence on artificial media, fresh spores obtained from infected insects must be preserved as seed for further spore production (Roberts & Yendol, 1971).

On the other hand virulence can be boosted using different techniques. Al-aidros and Seifert (1980), have selected a hyper- virulent mutant of *M. anisopliae* from a genetically uniform wild type strain by treating with a chemical mutagen.

Currently genetic engineering, whereby genes responsible for pathogenicity and other important features like sporulation rate are increased in number & expression and moved from one entomopathogenic fungi to another is being considered (Clarkson, 1991). However, utilization of genetically manipulated micro-organisms still remains as a controversial issue.

2.2.5.2. Formulation

Formulation is required to improve shelf life, persistence and efficacy in the field (Hall & Papierok, 1982; Goettel & Roberts, 1992). To achieve these ends, a formulated pathogen may contain additives such as wetters, stickers, humectants, UV protectants, and thixotropic agents (Moore & Caudwell, 1997; Hall & Papierok, 1982). However, it must be Known before hand that each additive must not have adverse effect on the viability of the product (Hall & Papierok, 1982).

Formulations could be in the form of dust, granules, wettable powder and liquid (Goettel & Roberts, 1992; Gardner & McCoy, 1992). The choice of formulation depends on target host habitat and application method (Goettel & Roberts, 1992). In addition the safety of a formulation to non target insects must be well considered (Moore & Caudwell, 1997).

Oil formulations have shown to perform better than acqueos solutions. Prior *et al.* (1988), by comparing oil and water formulations of *B. bassiana* conidia against the cocoa weevil pest, *Pantorhytes plutus* have found that oil formulation was more than 34 times more effective than the water formulation. The explanation given to the difference was, adhesion of the spores to the cuticle due to cutinophilic nature of the oil. On the other hand, conidial suspensions in oil might be more effective for field applications due to the non drying property of oil which would allow it to be applied at small droplet size than water based formulations (Prior *et al.*, 1988). Conidia survival in the oil formulation was also longer (12 days) than the water formulation (3 days) at room temperature.

On another study oil formulations of conidia have shown greater performance against the desert locust. More than 100 times more effective than water based formulation at low humidity (RH 35%) in the laboratory , showing the potential of the fungus to control pests even at low humidity environments (Bateman *et al.*,1993).

Blastospores which are not known to be stable (Van Winkelhof & McCoy,1984) have been spray dried and formulated in water based formulation (20% molasses, 80% water) and produced 100% mortality against *Schistcerca gregaria* in semi field trials, in arid situations (10-20% RH and 25-30°C) (Stephan *et al.*, 1997). The success of this formulation has been presumed to be due to the protection of blastospores from desiccation and ultraviolet radiation by molasses and due to the adhesive capacity of molasses. In a similar study Stephan and Zimmerman (1998)

have found a similar result against *Locust migratoria* by adding a skimmed milk powder at concentrations of 10 or 20%.

For pathogens which do not easily produce condia such as the entomphthoralean pathogens, production of mycelium and using it to control pests is another alternative (Wraight & Roberts, 1987). Alginate pellet formulations are being recommended to use mycelial biomass. Alginate pellets can be stored dry and then reactivated up on rehydration. These formulations are considered to protect the fungus from UV radiation and other environmental factors (Knudssen *et al.*,1990).

2.2.5.3. Storage

Storage is considered the most overlooked aspect of study (Hall & Papierok, 1982). Longevity (viability) of spores is related to temperature, humidity, gaseous atmosphere, and light during storage. Spores must be used within few weeks or months of their production unless proper storage is used (Roberts & Yendol, 1971).

Different techniques are being used to store fungal preparations of conidia, blastospores and mycelia.

Conidia, can be stored either in the dry form (pure conidia or with carriers like clay) or in oil formulations (Moore & Caudwell, 1997). Dry conidia are usually stored with carriers like clay. Fragues *et al.* (1983) have shown that naked blastospores of *B. bassiana* were inactivated after three weeks of incubation in soil due to biodegradation by soil micro-organisms ; on the other hand, clay coated blastospores were still active after two months. Carriers during storage help to protect the

biopreparation from the effects of its metabolism and resultant by products (Moore & Caudwell, 1997).

When oil formulations are intended to use conidia are normally stored in oil which could be vegetable, mineral, or a mixture (Moore & Caudwell, 1997). Oil formulation of *B. bassiana* conidia have showed a prolonged conidia survival than water based formulation. Formulation in water (0.01% Tween-80) lost infectivity in three days, while the oil formulation has maintained infectivity for twelve days at room temperature (Prior *et al.*, 1988). Storage in oil must avoid contact of oil with oxygen which could produce antioxidants which have anti microbial activity and damage conidia (Moore and Caudwell, 1997). Stephan *et al.* (1997) has reported that blastospores can be stored for 13 months with resultant viability of 73.1% by spray-drying them and keeping them at 5°C.

Mycelial preparations have been stored at room temperature for several months with 100% viability when formulated as alginate pellets (Knudsen *et al.*, 1990).

Lower relative humidity was shown to have a significant effect on lowering the viability of conidia of *Entomophthora* spp. (Yendol, 1968). Spore survival and virulence of *M. anisopliae* against mosquitoes was seen to be critically affected by temperature and relative humidity (RH). Longer survival time was recorded for moderate temperature (19°C or 26°C) and higher relative humidity (97%) or lower temperature (4°C)and lower humidity (0%). No loss of virulence occurred at 19°C - 97% RH or 4°C - 0% , while conidia stored at 26°C - 97% RH have shown 50% reduction in virulence against *Culex pipiens* after 18 months of storage (Daoust &

Roberts, 1983). Viability of conidia of *Entomophaga grylli* was reported to be inversely related to the duration and intensity of exposure to light (Carruthers *et al.*, 1988).

2.2.5.4. Application

Application of entomopathogenic fungi (mycoinsecticides) could be in the form of colonization where relatively small inocula of the material in the form of diseased insects or cultured material are applied into the environment, or in the form of microbial insecticides where large amount of material is needed which can be applied with equipment used for chemical insecticides (Roberts & Yendol, 1971; Gardner & McCoy, 1992). Mycoinsecticides can be applied either as dusts, granules or sprays and could be applied in the form of conidia, blastospores, or mycelial fragments (Gardner & McCoy, 1992).

Ultra low volume (ULV) applications are recommended to minimize cost and to cover large area at a smaller volumes (Bateman, 1997). Oil suspensions of conidia are considered effective for ULV applications since conidia suspends easily in oil due to their lipophilic nature and the oil also protects evaporation there by protecting the ULV applications from drying (Bateman *et al.*, 1993).

Once the pathogen comes into contact with the target host the possibility of infection is high even under low relative humidity (Bateman *et al.*, 1993).

Since viability of entomopathogenic fungi are affected by light, temperature and humidity (Carruthers *et al.*, 1988; Daoust & Roberts, 1983) and the surface, soil,

foliage, water etc on which the fungus is applied (Gardner & McCoy, 1982) their application must consider all these factors to maximize their use. Many soil types, for example, are known to have fungistatic properties due to their natural microbial flora and fauna which reduce viability of entomopathogenic fungi. *Penicillium urticae* was reported to produce inhibitor of *B. bassiana* in soil (Lingg & Donaldson, 1981). Application must also coincide with the presence of optimum environmental factors like humidity, temperature and susceptible stages of the host (Roberts & Yendol, 1971; Gardner & McCoy, 1992).

3. MATERIALS AND METHODS

3.1. Insects

In this study *G. m .morsitans* was used as a target insect to evaluate the efficacy of entomopathoginic fungi for tsetse fly control.

G. m. morsitans were obtained from the insectary in the Vector Biology Laboratory of the Ethiopian Health and Nutrition Research Institute. The flies are kept at temperature of 25° C and 70% relative humidity in cages made of white mesh and plastic containers.

3.2. Fungi

Three *B. bassiana* isolates, coded as FF, GG, And AK and One *M. anisopliae* (EE) were obtained from, the culture collections of the Mycology Laboratory, Biology Department, Addis Ababa University, collected by Aysheshim (1998). One *M. anisopliae* (ICIPE 30) and one *B. bassiana* (Bb), isolates were obtained from the International Center for Insect Physiology and Ecology (ICIPE). One *Metarrhizium* sp. (MM) was isolated from soil from Arbaminch, southern Ethiopia, the vicinity of which is known to harbor tsetse flies, as part of this study

3.2.1. Isolation of fungus

The common sources of entomopathogenic fungi are dead insects and the soil particularly from insect breeding sites.

3.2.1.1. Isolation from insects.

Attempts to isolate fungal pathogens from field collected cadavers were made by scraping parts of insets which seem to show fungal growth with sterile loop and transferring it to a fungal medium, Sabourauds' dextrose agar plates with antibiotic, to prevent bacterial growth, which subsequently were incubated at 28°C for one week.

3.2.1.2. Isolation from soil

Soil samples were collected in plastic bags from Arba Minch area where tsetse flies are known to occur . One gram of soil was suspended in 9 ml of sterile water (0.2% tween 20) and shaked to homogenize the soil sample. The suspension was further diluted to 10^{-2} and 10^{-3} in sterile water. An aliquot of O.1 ml of each dilution was transferred to SDA plates. After growth was eminent, fungi supposed to belong to the entomopathogenic genera, *Beauveria* and *Metarrhizium* spp. were sub-cultured to get pure cultures of the suspected entomopathogens.

3.3. Characterization

Both microscopy and colony morphology were used to identify fungus using 15 day old SDA cultures. Conidial size was determined under light microscope fitted with a micrometer. Identification was based on Domsch *et al.* (1980).

3.4. Storage of fungi

Pure cultures of entomopathogenic fungi isolates were cultured on agar slants and kept at 4°C. Insect cadavers were also kept at 4°C in glass vials. Soil samples in plastic bags were kept at room temperature.

3.5. Media preparation

Sabouraud dextrose agar (SDA)with the following formula was used :

Ingredient	gms/ liter.
Mycological peptone	10.0
Glucose	40.0
Agar	15.0

Desired amount of SDA was suspended in a distilled water in the proportion of 65 gms/liter. The suspension was boiled and sterilized at 121°C for 15 minutes. After it is cooled until hand hot an antibiotic, chloramphinicol was added (0.4 gm/liter). Then the media was transferred to sterilized petridishes .

3.6. Culture preparation and harvesting fungi

Fungi were cultured under aseptic condition on SDA plates for 15 days at 28°C. Spores were harvested by adding sterile distilled water (0.1% tween-20, Sigma,

WA) to 15 days old agar cultures and gently scrapping the culture with a spatula. A suspension of spores and hyphal fragments were obtained. The suspension was then filtered through cotton to remove hyphal fragments. About 1 ml of the spore suspension was then agitated on a vibrant test tube shaker (Paramix 3, Julabo Labortechnik, GMBH, WG) to break up spore clumps for 7 minutes.

Spore concentration was determined using Improved Neubauer Haemocytometer (Superior, west Germany). Spore concentration was then adjusted to 1 X 10^8 for efficacy evaluation against *G. m. morsitans*

3.7. Assessment of pathogenicity

Each fungal isolate was screened against 3 groups of 20 male flies. A slightly modified method of Kaaya (1989) was used. Spore suspensions (1 X 10^8 spore/ml) were prepared in 50 ml flat bottom flasks into which chilled flies (1°C for seven minutes) were dipped and shaked for 15 seconds. Then the suspension were poured out through a gauze leaving chilled flies on the gauze, after which the flies are transferred to respective cages. Control flies were dipped in sterile distilled water for the same period. The flies were then kept in an insectary (25°C, 70 RH) for 21 days including the day of infection. Mortality was recorded daily.

3.8. Dose-mortality relation ship

A group of 25 male flies were exposed to spores of *Metarrhizium* EE and *Metarrhizium* MM to concentrations of 10^8, 10^7, 10^6, and 10^5 spores/ml, while

control flies were treated with sterile distilled water. Mortality was recorded for 20 days after the day of infection.

3.9. Re-isolation of entomopathogens

Flies from the experimental groups were surface sterilized by dipping them in absolute ethanol for few seconds and in 0.5 % sodium hypochlorite for 2 minutes. They were then rinsed in distilled sterile water for few seconds before they were placed on SDA plates. The plates were incubated at 28°C until growth was visible. The re-isolated fungus was compared with the original to verify identity of the pathogen and prove that it is the pathogen responsible for the disease and death of the insect.

3.10. Growth optimization and Spore yield

Besides cultivation of the fungi on agar media , solid substrates were chosen and compared for spore production of selected entomopathogens .

Spore yield on solid substrates of rice, wheat, barley and sorghum was compared in two ways: a) Seventy five gms of each grain in two duplicates were soaked in 100 ml of water in 250 ml Erlenmeyer flasks overnight. After discarding the excess water the grain types were autoclaved at 120°C for 30 minutes. The substrates were inoculated with 1.25 ml of 1 X 10^7spores/ml of either *Metarrhizium* MM or *Metarrhizium* EE. Then the flasks were kept at 25 °C and 70% RH for 20 days.

Spore yield of the substrates was determined by taking a 2 gm sample from each flask and transferring them to a 50 ml flask with 10 ml of distilled water (2% Tween-20). The flasks were shaken till the grains are clean of spores as confirmed visually. One ml of the spore suspension was transferred to a vial and agitated by a test tube shaker for 6 minutes and the concentration was determined using the haemocytometer. b) Another investigation was carried out to compare the effect of boiling and soaking before sterilization. The experiment was carried in the same way except that in this group boiling of grains in 22.5 ml of water (6.5% vegetable oil) till all the water is absorbed (LU.BI.LO.SA, 1996) instead of soaking was carried.

Moisture content of soaked grains was determined by soaking 15 gms of each grain in 20 ml of water in 50 ml flask overnight. After pouring of the water 5 gm of each grain was taken from each flask and was dried in an oven (200°C) for 2 hours. Samples were re-weighed to see the percentage moisture content from the difference in weight before and after drying.

Moisture content of boiled grains was determined by boiling 15 gms of each grain in 4.5 ml of water and 0.3 ml of oil till all the water is absorbed. Moisture content was determined in the same manner as it was for the soaked grains.

3.11. Growth rate and temperature

To determine the growth rate and optimum temperature of the entomopathogenic fungi, circular agar discs , 9 mm in diameter, from a 10 day old cultures of MM and

EE were transferred to SDA plates. The cultures were incubated at 4°C, 28°C, 37°C, and 55°C for 10 days. Growth was determined by measuring the diameter of the culture after 10 days.

3.12. Viability

Spore germination was determined microscopically.

Spores of EE and MM were inoculated to a broth media (Glucose-1gm, NH_4SO4-0.5gm, Yeast extract-0.5gm, and KH_2PO4-0.25gm in 1 liter) in such away that the concentration of the spores in the broth is 10^6 spore/ml. After 24 hours of incubation germination rate was determined under the microscope by counting germinating and non-germinating spores. Budding spores were considered as germinating.

3.13. Statistical Analysis

Analysis of the data was performed using students t-test at 95% level of confidence. SPSS software was employed for the analysis. All the graphs were ploted using Harvard Graphics (HG) software.

4. RESULTS

4.1. Isolation of fungal pathogen

In the attempt to isolate entomopathogenic fungi effective against the tsetse fly one isolate identified as *Metarrhizium* sp. coded as MM was isolated from soil. Attempts to isolate entomopathogens from insect cadavers have failed.

4.2. Characterization and identification

Fifteen days old cultures of *Metarrhizium* MM on SDA were greenish, and spores have cylindrical shape. The spores have a size of 3.60-7.20 X 2.40 -2.88 μm. The isolate was identified as *Metarrhizium* sp.

4.5. Assessment of pathogenicity

Among the *Metarrhizium* species, the highest performance, 96.67% mortality was achieved by *Metarrhizium* EE. *Metarrhizium* ICIPE 30 and *Metarrhizium* MM have showed 90% and 73.3% mortality, respectively (Table 1). The differences however, were not significant.

Among the *B. bassiana* isolates, the exotic isolate *Beauveria* Bb has performed the best, 81.67%. *Beauveria* FF, *Beauveria* GG, *Beauveria* AK have shown per cent

mortality, 75.00%, 63.33%, 53.33% respectively (Table 2). The difference between *Beauveria* FF and *Beauveria* Bb was not significant (P> 0.05) but *Beauveria* Bb was significantly better than the other isolates, *Beauveria* GG and *Beauveria* Ak (P< 0.05). *Beauveria* FF and *Beauveria* GG had not significant difference (P> 0.05). *Beauveria* FF was significantly better than *Beauveria* AK (P< 0.05), while the difference between *Beauveria* GG and *Beauveria* AK was not significant (P > 0.05).

Increased mortality has been seen with increased concentration of spores (Fig 1).

4.4. Growth optimization

4.4.1. Spore yield on solid substrates

Both isolates, *Metarrhizim* MM and *Metarrhizium* EE, have grown on all four grains (Fig.). However, rice was found to be the best substrate, about 10 times better than the rest of the substrates.

The spore yield of *Metarrhizium* MM on soaked grains was in the order of rice, sorghum, wheat and barley, while on boiled grains it was in the order of rice, sorghum, barley and wheat (Fig. 2) . For *Metarrhizium* EE, the order of yield was rice followed by wheat, sorghum, and barley for both soaked and boiled grains (Fig. 3). The difference between rice and the rest of the grains was significant, P < 0.05 for *Metarrhizium* MM and P < 0.01 for *Metarrhizium* EE. The yield between *Metarrhizium* MM and *Metarrhizium* EE either soaked or boiled, on rice was not significant (P > 0.05).

No significant difference in yield was observed whether rice was soaked or boiled for both fungus ($P>0.05$).

No significant difference was also observed among wheat, barley, and sorghum when soaked for *Metarrhizium* EE ($P > 0.05$). The same was true for *Metarrhizium* MM, except the yield of barley was significantly lesser than sorghum's ($P < 0.05$).

The highest moisture content was observed for rice followed by barley, wheat, and sorghum, whether the grains were soaked or boiled . The moisture content of all the grains, however, was higher when the grains were soaked rather than boiled (Fig. 6). No relationship was observed between spore yield and moisture content of substrates (Fig. 4).

4.4.2. Radial growth and temperature

No growth has been seen at 4°C and 55°C for both *Metarrhizium* MM and *Metarrhizium* EE isolates after ten days of incubation. Both grew better at 28°C than at 37°C, 211.1% of the original size of the innoculum (9 mm in diameter) for *Metarrhizim* MM and 244.4% for *Metarrhizium* EE. At 37°C *Metarrhizium* MM grew better(150%) than *Metarrhizium* EE (116%) (Fig.5).

4.5. Germination rates

Both *Metarrhizium* MM and *Metarrhizium* EE have shown similar germination rates, 41.72% for *Metarrhizium* EE and 42.36% for *Metarrhizium* EE.

5. DISCUSSION

All the seven isolates tested have incurred mortality ranging from 53.33% (by *Beauveria* AK) to 96.67% (by *Metarrhizium* EE) against *G. m. morsitans* in 20 days post exposure at $1X10^8$ spores per ml (Table1&2). Poinar *et al.* (1977) have reported 30% mortality by the same fungal species against the same fly. Kaaya (1989) and Kaaya and Munyinyi (1995), on the other hand have reported different mortality rates ranging from 59.70% to 95.45%, at $2.0X10^7$spores per ml in 18 days post exposure.

Compared with works of Kaaya (1989) and Kaaya and Munyinyi (1995), the performance of the isolates of the present study seems to be low owing to the fact that the concentration of spores they have used is five times lower than the present study. Variation in pathogenicity or other features of the same species of different isolates or strains is common phenomenon (Goettel and Roberts, 1992; Clarkson, 1996).

Some investigators have tried to relate pathogenicity of an isolate to the original host it is isolated from; that is, strains of a species isolated from a host is more virulent for that host rather than strains isolated from taxonomically unrelated host (Tanada and Kaya, 1993). However, no explanation is given why this could be. On the other hand, Feng and Johnson (1990) have reported that *B. bassiana* strains isolated from taxonomically not related hosts were more virulent than the strain which was isolated

from a taxonomically related host.

All the local isolates were not isolated from tsetse flies and the strains evaluated by Kaaya (1989) came from Europe and their original host is not indicated.

Though the performance of the isolates in this study seems to be relatively lower , there is a possibility that the pathogenicty level could be just enough or more to achieve control. On the other hand , the fact that the isolates are local have the advantage of avoiding importing foreign isolates which would be in a hard currency, if there is a chance to use these fungal species to control any local pest. Another advantage of local isolates is that they are considered better adapted to the natural environment they are isolated from than an imported one , hence considered better for control operations in that area (Prior and Street, 1997).

Interest in biological control of pests is rising due to various problems associated with conventional control methods (St. Leger & Roberts, 1997) and entomopathogenic fungi are considered to have a great potential in pest control (Clarkson & Charnley, 1996).

From the present and previous successive studies by Kaaya (1989), Kaaya and Okech (1990), and Kaaya and Munyinyi (1995), tsetse flies can be considered as candidate target hosts for fungal control. Horizontal transmission of entomopathogens between infected and non infected flies when kept together even for only 30 minutes and causing mortality reaching up to 75%, reduced longevity of adults emerging from exposed pupae (Kaaya & Okech, 1990; Kaaya & Munyinyi, 1995) raises the

potential of entomopathogens to control tsetse flies, since both pupae and adults can be targeted.

However, application of the entomopathogens into the breeding habitats of the fly when targeting the pupae seems to be a difficult task. Pupae under normal conditions are buried under soil; and treating a whole area of tsetse breeding area could be very difficult.

Ecology of tsetse on the other hand seems to be favorable for fungal control due to their preference for a higher humidity (> 70%) which is more or less the relative humidity required for fungal sporulation (Ramoska, 1984) and development of fungal epizootics (Ferron, 1978). For riverine tsetse flies which inhabit water side vegetation whose relative humidity could reach 100%, the chance of epizootics is very high (Kaaya, 1989). It is now known that fungal pathogens could infect and kill at very low humidity though epizootics is unlikely (Bateman *et al.*, 1993; Ramosk, 1984).

Of the four temperatures compared for growth comparison, 0°C, 28°C, 37°C and 55°C, no growth was seen at 0°C and 55°C. Walstad *et al.* (1970) have reported that the range of temperature for *B. bassiana M.. anisopliae* is 15-35°C. At 28°C *Metarrhizium* EE grew better 244.4% of the original innoculum size than *Metarrhizium* MM which showed 211.1% growth. Gitonga *et al.* (1996)have suggested that there exists a strong correlation between radial growth and virulence. In this study too, *Metarrhizium* EE has performed better than *Metarhizium* MM, though statistically not significant ($p>0.05$), where the isolates were cultured at 28°C

and exposed flies were kept at 25°C. However, at 37°C *Metarrhizium* MM grew better than *Metarrhizium* EE, though compared to the growth rate at 28°C both had shown a slower growth rate.

Since, the range of temperatures at which the growth rates compared was too wide it is difficult to speak of the optimum ranges for the isolates.

The development of mycopesticide needs mass production of the fungal agents. Conida production of both isolates was highest on rice (Fig.2,3) Wheat, barley, and sorghum had similar production rates (Fig.2,3). Rice is of course known for its high production of *M. anisopliae* and *B. bassiana* compared to other substrates (Vilas Boas,1996; Ibrahim & low, 1993) and it is widely used for mass production of *B. bassiana* and *M. anisopliae* (Mendonca, 1992; Goettel & Roberts, 1992).

Attempts were made to see if there was any difference in conidia yield when the different grain types were simply soaked in water or boiled. However, the results were inconsistent (Fig. 2,3). For example, with *Metarrhizium* MM , yield was better on the three substrates wheat, barley and sorghum when they were soaked while the reverse was true for rice (Fig.2). With EE only barley produced higher yield when boiled while the rest performed well when they were soaked. Since boiling does not seem to increase yield, soaking can be used for mass production of these fungi; soaking could be very economical in areas where energy is scarce.

According to Jenkins and Goettel (1997), parameters such as pH, temperature, nutrient status, aeration are difficult to control in solid state fermentation. In this

experiment differences in aeration and moisture content from flask to flask, and some other subtle differences in different batches of the same grain might have played role for the inconsistent results.

Moisture content of rice was the highest in both treatments , that is ,soaked or boiled (Fig. 6)however moisture content did not correlate with spore yield. For example, barley which gave the least yield had the second highest moisture content (Fig. 4). Gitonga (1996) had also reported that rice with different moisture contents did not differ in conidia yield of *M. anisopliae* and B. *bassiana.*

The highest spore yield in this work is 1.62 X 10^9 spore/gm of rice. Gitonga (1996) has reported 1.25 X 10^9 spores/ gm of rice. Mendonca (1992) had also reported a yield of 1 X 10^{10} spores of *M. anisopliae* per gm of rice . Comparing the different reports of yield is, of course, difficult for they could vary in the methodology used for their production, the size of the innoculum, and the age of the culture during harvesting.

According to Auld (1992), solid fermentation is less costly to developing countries due to cheap labor, provided the substrates are available.

Solid substrate fermentation on rice is used in Brazil to produce *M. anisopliae* to control sugar cane froghopper, *Mahanarva posticata* (Mendonca, 1992). And in the USSR to produce *B. bassiana* to control Colorado potato beetle (Wraight and Roberts, 1987).

Once the potential of entomopathogens to control tsetse flies is established mass production of mycopesticides on solid substrates can easily be practiced in tsetse infested areas.

Field trials against tsetse flies with the existing isolates and looking for strains with better pathogenicity and better spore yield are recommended for the future.

Table 1. Mean cumulative per cent mortality in adult male *G. m. morsitans* by different isolates of *Metarrhizium* species at different days of post exposure.

Isolate	Day 5	Day 10	Day 15	Day 20
M.anisopliae (EE)	21.67 ± 0.94	78.33 ± 3.09	96.67 ± 0.47	96.67[a] ± 0.47
M.anisopliae (ICIPE 30)	26.67 ± 0.94	73.33 ± 2.88	88.33 ± 2.62	90.00[a] ± 2.61
Metarrhizium sp. (MM)	10.00 ± 1.60	38.33 ± 3.09	65.00 ± 1.41	73.33[a] ± 2.49
Control	0.00	0.00	3.33 ± 0.47	8.33 ± 1.70

[a]same letters indicate no significant difference at 5 % level of confidence.

Table 2. Mean cumulative per cent mortality in adult male *G. m. morsitans* by different isolates of *Beauveria bassiana.*

Isolate	Day 5	Day 10	Day 15	Day 20
B. bassiana (FF)	6.67 ± 0.94	51.67 ± 1.25	61.67 ± 1.25	75.00^{ab} ± 0.00
B. bassiana (Bb)	18.33 ± 1.88	51.67 ± 4.64	68.33 ± 2.49	81.67^{a} ± 1.25
B. bassiana (GG)	6.67 ± 1.25	30.00 ± 2.16	60.00 ± 0.81	63.33^{bc*} ± 1.25
B. bassiana (AK)	1.67 ± 0.47	23.33 ± 1.25	43.33 ± 1.70	53.33^{c} ± 1.25
Control	0.00	0.00	0.00	0.00

*same letters indicate no significant difference at 5 % level confidence.

Fig 1. Dose-Mortality Relationship In Adult Male *G.m.morsitans* in 20 days post exposure.

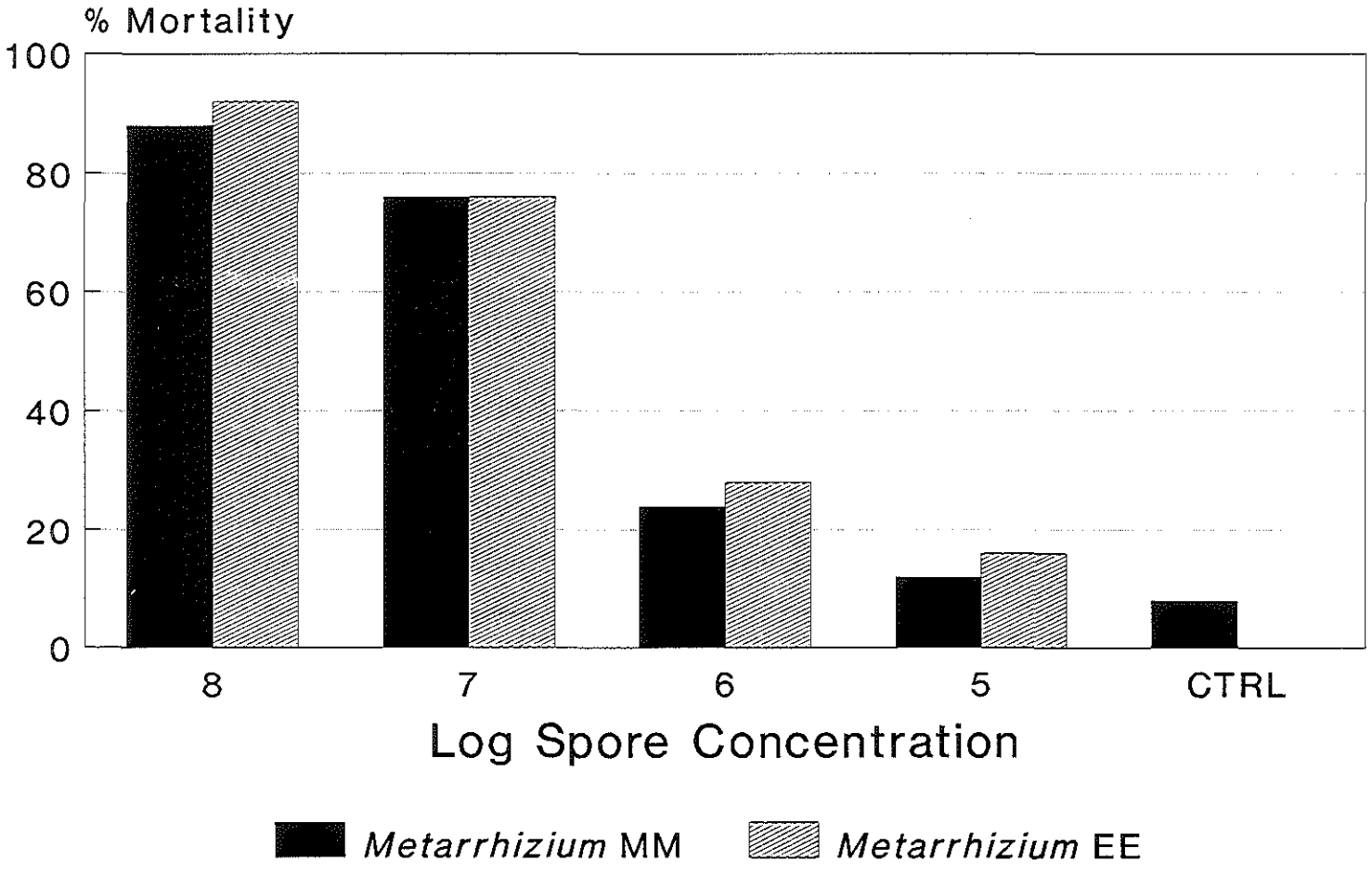


Fig 2. Mean Spore Yield (spores/gm of subsrate) of *Metarrhizium* MM on different grain types (Boiled Vs Soaked)

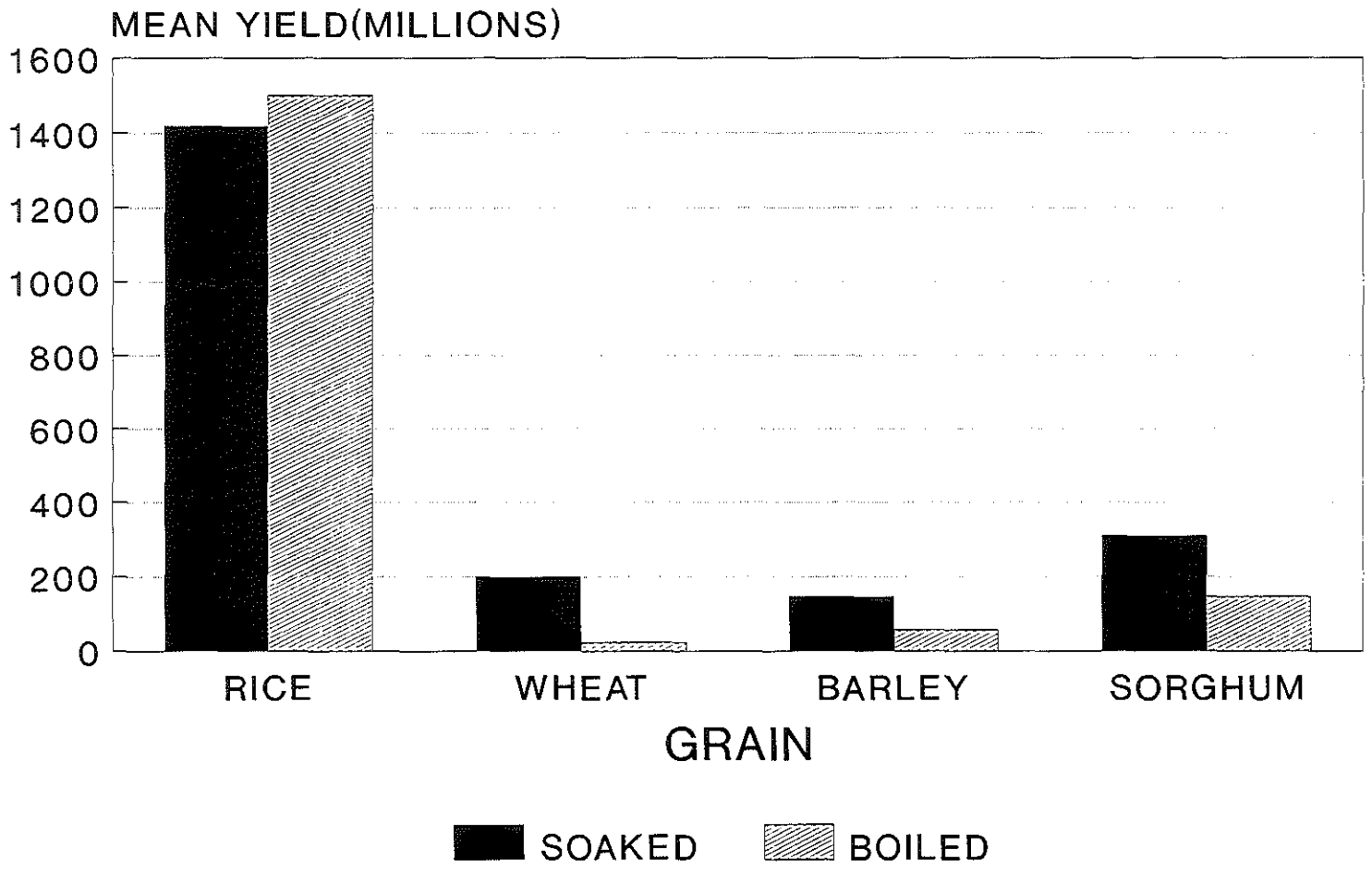


Fig 3. Mean Spore Yield (Spores/gm of substrate) of *Metarrhizium* EE on different grain types (Boiled Vs Soaked)

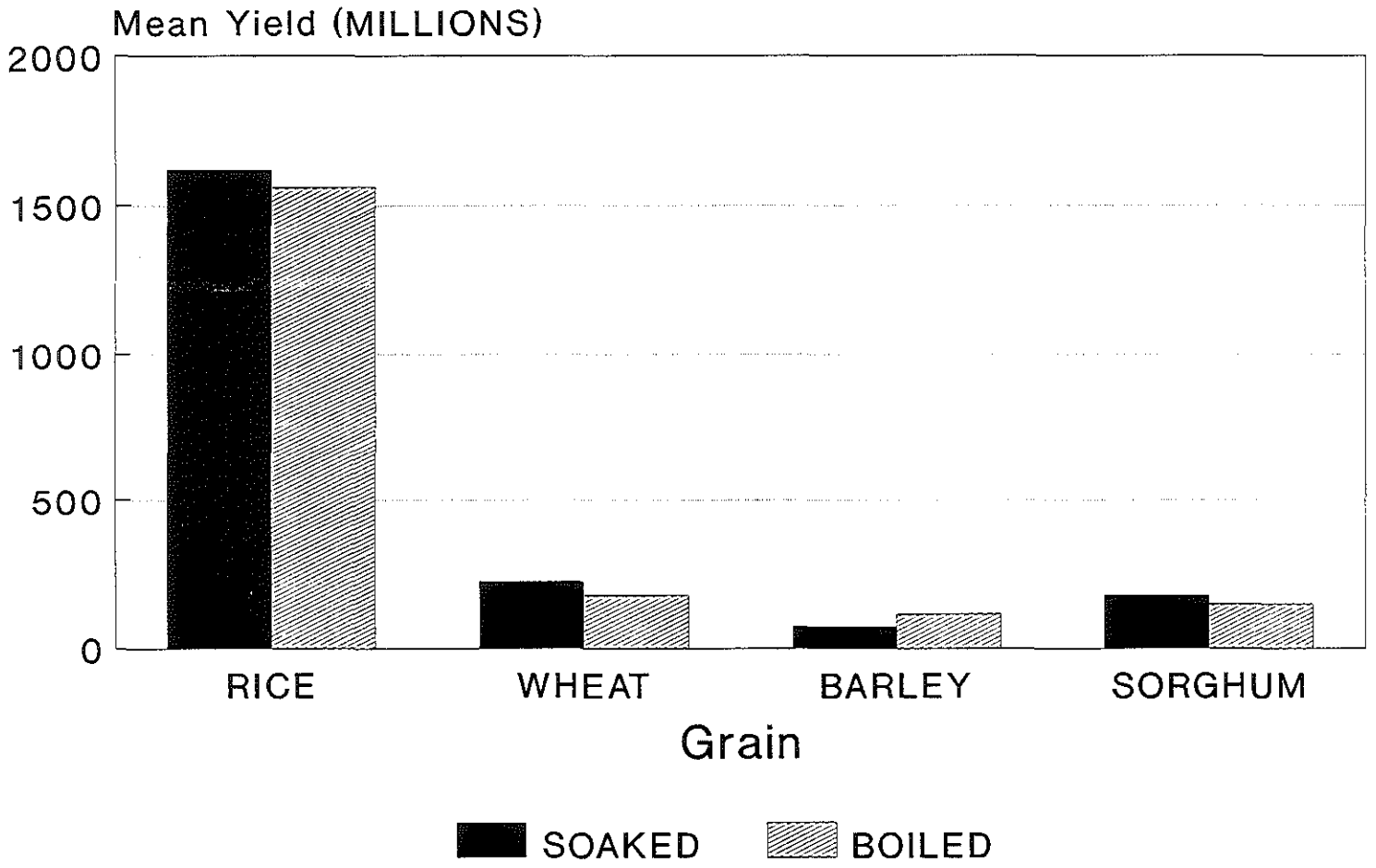


Fig.4. Mean Spore Yield (Spore/gm of substrate) of *Metarrhizium* MM & Per cent Moisture Content of Soaked Substrates

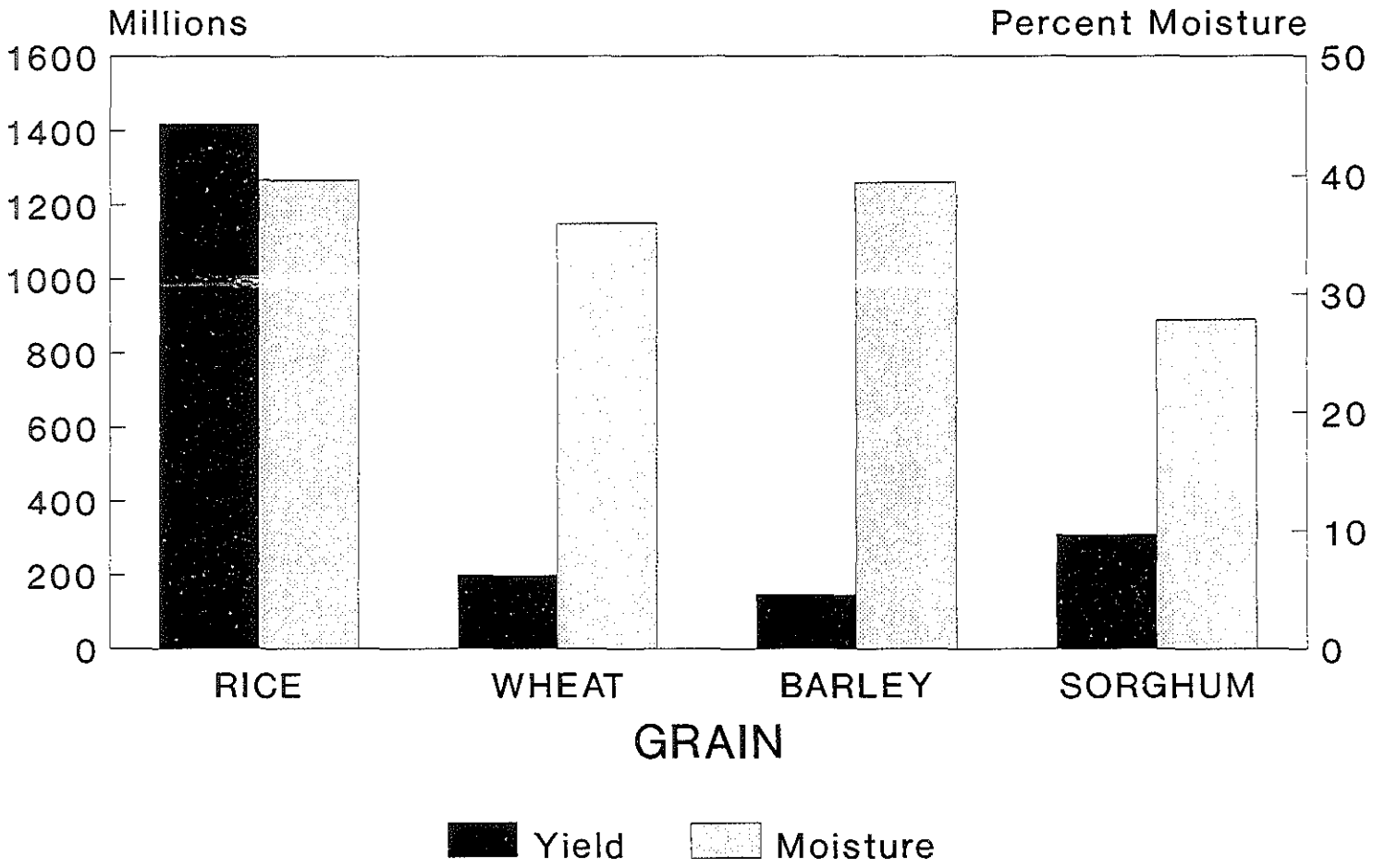


Fig. 5. Radial growth and temprature

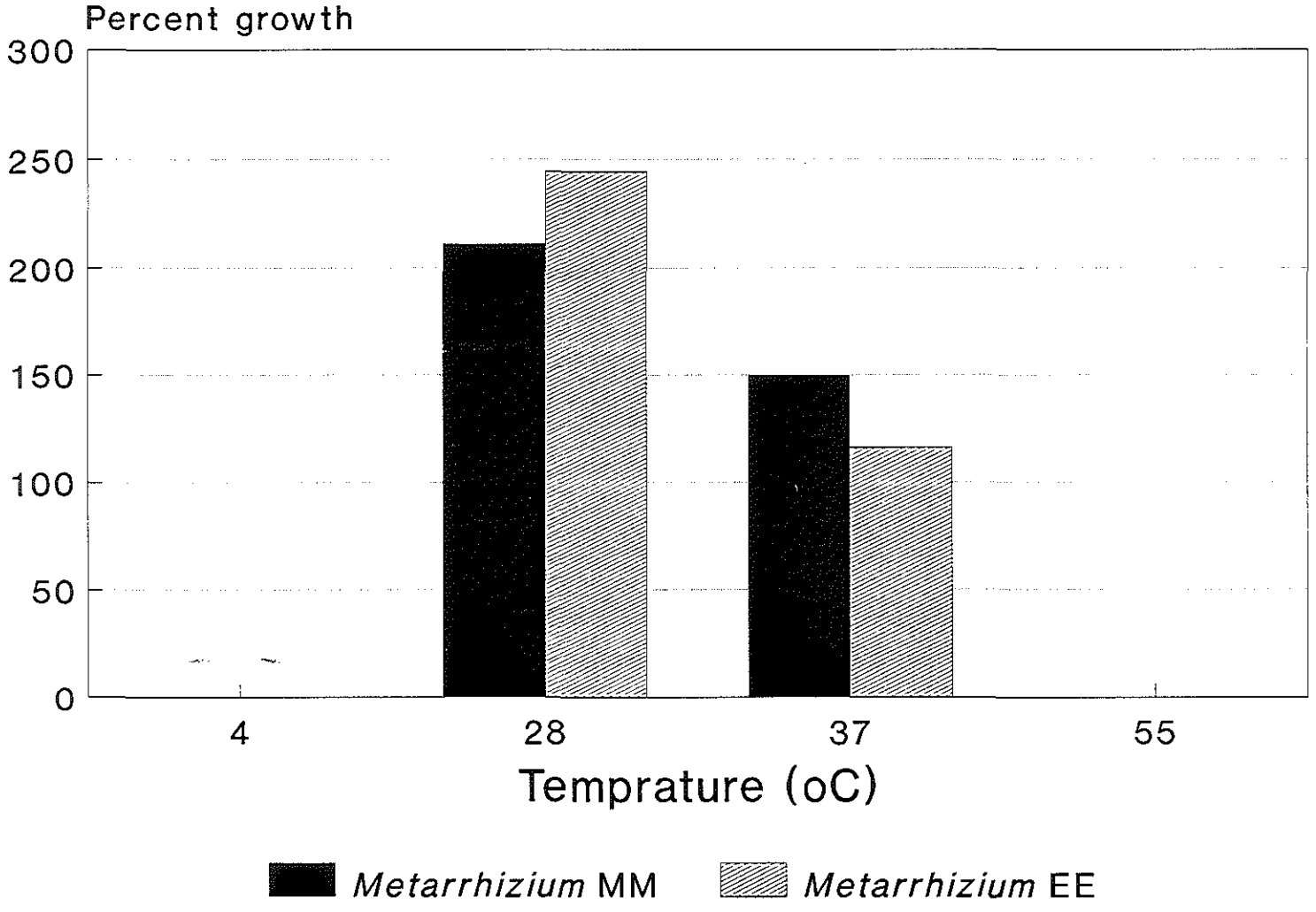
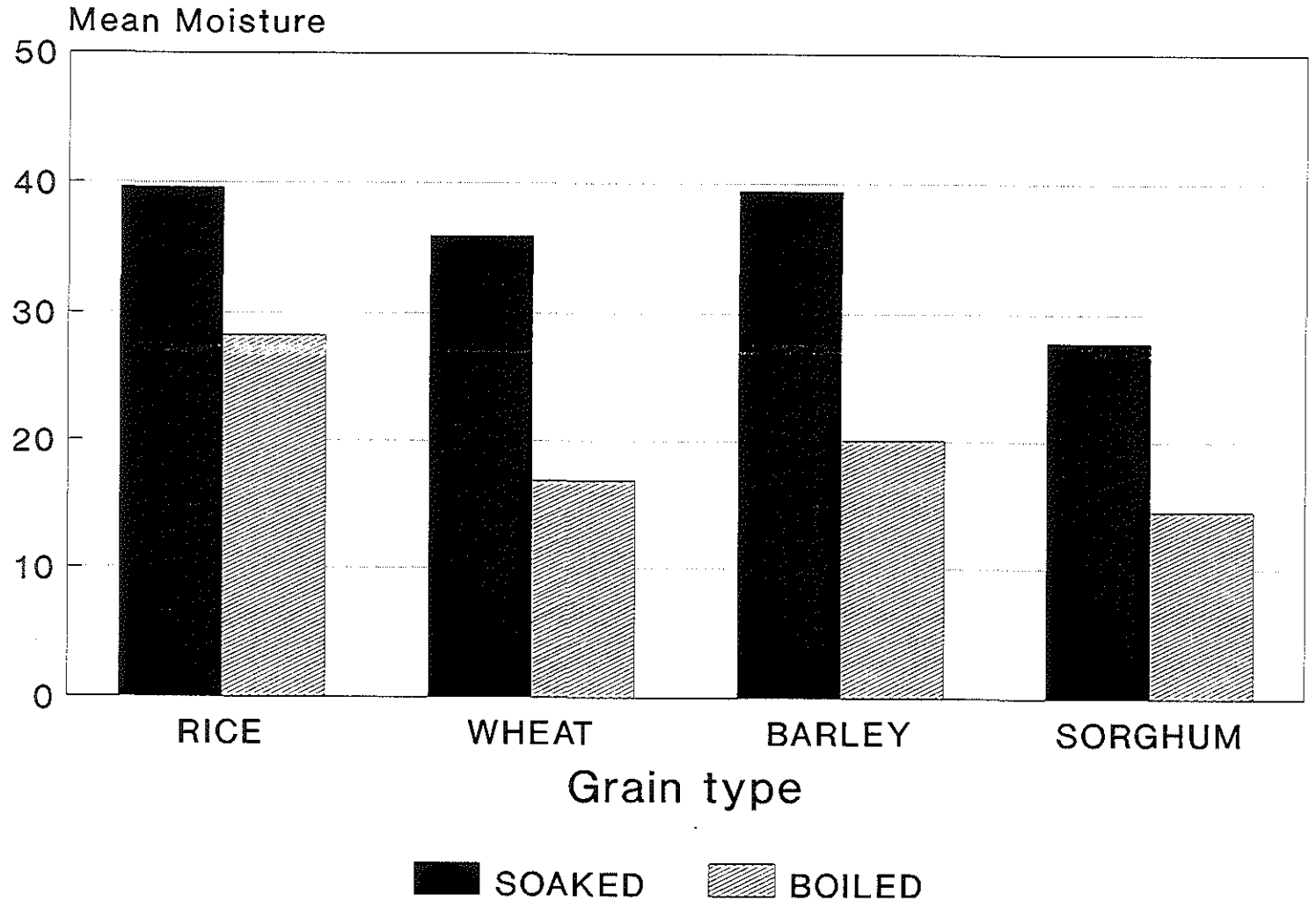


Fig. 6 Mean Moisture Content Of Grains



6. REFERENCES

A UNDP/ WORLD BANK/ WHO CONSULTATION (1982). Control of sleeping sickness due to *Trypanosoma gambiense. Bulletin of the World Health Organization,* **60**, 821-825.

Al-Aidroos, K. & Seifert, A.M. (1980). Polysaccharide and protein degradation, germination and virulence against mosquitoes in the entomopathgenic fungus *Metarrhizium anisopliae. Journal of Invertebrate Pathology,* **36**, 29-34.

Alexopoulos, C. J. & Mims, C.W. (1979). *Introductory Mycology.* 3rd edition. New York: John Wiley & Sons.

Aysheshim, S., (1998). *Evaluation of Ethiopian Isolates of Enotomogenous Fungi as Potential Biological Control of the Desert Locust* , Schistocerca gregaria. M. Sc. Thesis. Addis Ababa University.

Auld, B. A. (1992). Mass production, formulation and application of fungi as biocontrol agents. In: *Biological Control of Locusts and Grasshoppers.* (Editors, Lomer, C. J. & Prior, C.). *UK:CAB* International, pp . *219-229.*

Bateman, R. (1997). Methods of application of microbial pesticide formulations for the control of grasshopper & locusts. In: Goettel, M. S. & Johnson, D.C. (Editors). *Memoirs of Entomological Society of Canada, 171,69-81.*

Bateman, R. P. Carey, M., Moore, D. & Prior, C. (1993). The enhanced activity of *M. flavoviride* in oil formulations to desert locusts at low humidities. *Annals of Applied Biology,* **122**,145-152.

Baylis, M. & Stevenson, P. (1998). Trypanosomiasis and tsetse control with insecticidal pour-ons fact and fiction. *Parasitology Today*, **14**, 77-82.

Boucias, D.G., Pendland, J.C. & Latge, J.P. (1988). Nonspecific factors involved in attachment of enthomopathogenic Duetromycetes to host insect cuticle. *Applied and environmental Microbiology,* **54**, 1795-1805.

. Broome, J.R., Sikowski, P.P., & Norment, B. R. (1976). A mechanism of pathogenicity of *Beauveria bassiana* on larvae of the imported fire ant, *Solenopsis richteri*. *Journal of Invertebrate Pathology*, **28**, 87-1.

Buxton, P. A. (1955). *The Natural History of Tsetse Flies*. London: H. K. Lewis.

Campbell, R.K., Barnes, G. L., Cartwright, B. O. & Erkenbary, R. D. (1983). Growth and sporulation of *Beauveria bassiana* in basal medium containing various carbohydrate sources. *Journal of Invertebrate Pathology*, **41**, 117-121.

Carruthers, R. I. & Soper, R. S. (1987). Fungal Diseases. In: *Epizootiology of Insect Diseases*. (Editors, Fuxa, J. R. & Tanada, Y). New York: Wiley-Interscience, pp.357-416.

Carruthers, R. I., Ziding Feng, Ramos, M. E. & Soper, R. S. (1988). The effect of solar radiation on the survival of *Entomophaga gryli* (Entomophthorales: Entomophtharaceae) conidia. *Journal of Invertebrate Pathology*, **52**, 154-162.

Charnley, A. K. (1991). Mechanisms of fungal pathogenesis in insects with particular reference to locusts. In: *The Biological Control of Locusts & Grasshoppers.* (Editors, Lomer, C. J. & Prior, C.). UK: C.A.B. International, pp. 181-190.

Clarkson, J.M. (1996). Molecular biology of fungi for the control of insects. In: *Molecular Biology of the Biological Control of Pests and Diseases of Plants.* (Editors, Gunasekaran, M. & Weber, D. J.). Boca Raton, Florida: CRC press, pp. 123-135

Clarkson, J.M. & Charnley, A. K. (1996). New insights into the mechanisms of fungal pathogens in insects. *Trends in Microbiology,* **4**, 197-203.

Cottand, P. (1993). *Human African Trypanosomiasis. Meeting of interested parties on management and financing of the control of tropical diseases other than malaria.* Geneva: 15 September, 1993. Division of Control of Tropical Diseases, World Health Organization. CTD/MIP/WP.93.9

Cuisance, D. (1995). *Other approaches to Tsetse Control (excluding traps-screens). Expert Committee on the epidemiology and control of African Trypanosomiasis.* Geneva: World Health Organization, October 16-23. TRY/EC/WP/85.28.

Daoust, R.A. & Roberts, D.W. (1982). Virulence of natural and insect passaged strains of *Metarrhizium anisopliae* to mosquito larvae. *Journal of Invertebrate Pathology,* **40**, 107-117.

Daoust, R.A. & Roberts, D.W. (1983). Studies on the prolonged storage of *Metarrhizium anisopliae* conidia: effects of growth substrate on conidial survival and virulence against mosquitoes. *Journal of Invertebrate pathology,* **41**, 143-150.

Dent, D. (1991). *Insect Pest Management.* UK: C.A.B International.

Dillon, R. J. & Chanley, A. K. (1986). Inhibition of *Metarhizium anisopliae* by the gut bacterial flora of the desert locust, *Schistocerca gregaria* : Evidence for antifungal toxin. *Journal of Invertebrate Pathology,* **47**,350-360.

Domnas, A.J. & Warner, S.A. (1991). Biochemical activities of entomophagous fungi. *Critical Reviews of Microbiology*, **18**, 1-13.

Domsch, K. H., Gams, W., Anderson, T.H. (1980). *Compendium of Soil Fungi.* Volume 1. Toronto: Academic Press.

Dransfield ,R.O., Williams,B.G., & Brightwell, R. (1991). Control of tsetse flies and trypanosomiasis: might or reality? *Parasitology Today,* 7:287-291.

FAO (1986a). *Training Manual for Tsetse Control Personnel. Ecology and Behavior of Tsetse.* (Editor, Pallock, J. N.). Rome.

FAO (1986b). *Training Manual for Tsetse Control Personnel. V3: Control Methods and Side Effects* (Editor, Palloc, J.N.). Rome.

FAO (1992). Training Manual for Tsetse Control Personnel. V4:Use of Attractive Devices for Tsetse Survey and Control. Rome. .

Feng, M. G. & Johnson, J.B. (1990). Relative virulence of six isolates of *Beauveria bassiana* on *Duraphis noxia* (Homoptera: Aphidae). *Environmenal entomology*, **19**, 785-790.

Ferron, P. (1978). Biological control of insect pests by entomogenous fungi. *Annual Review of Entomology*, **23**, 409-442.

Fragues, J. O., Resinger, O., Robert, P.H. & Aubart, C. (1983). Biodegradation of entomopathogenic hyphomycetes: influence of clay coating on *Beauveria bassoon* blastospore survival in soil. *Journal of Invertebrate Pathology*, **41**, 131-142.

Gabriel, B. P. (1968). Enzymatic activities of some entomophthorous fungi. *Journal of Invertebrate Pathology,* **11**, 70-81.

Gardner, W. A. & McCoy, C.W. (1992). Insecticides and herbicides. In: *Biotechnology of Filamentous Fungi. Technology and Products.*(Editors, Finkelstein, D.B. & Ball, C.) Boston: Butterworth-Heinemann , pp. 335-359.

Gitonga, W. (1996). Metarrhizium anisopliae (Metschnikoff) Sorokin *and* Beauveria bassiana (Balsamo) Vuillemin *as Potential Biological Control Agents of* Macroterms michaelseni (Sjostedt). (Isospora: Termitdae*) in Kenya*. Ph.D. Thesis. Department of ecology and Molecular Biology, Royal Veterinary and agricultural University Copenhagen, Denmark.

Goettel, M. S. (1992). Fungal agents for biocontrol. In: *The Biological Control of Locusts and Grasshoppers.* (Editors, Lomer, C. & Prior, C.). UK: C.A.B. International, pp. 122-132

Goettel, M. S. & Roberts, D.W. (1992). Mass production formulation and field application of entomopathogenic fungi. In: *Biological Control of Locusts and Grasshoppers*. (Editors, Lomer, C. J. & Prior, C.). UK: CAB International, pp . 230-238.

. Gotz, P. & Vey, A. (1974). Humoral encapsulation in Diptera (Insecta): defense reactions of *Chironomus* larvae against fungi. *Parasitology*, **68**, 193-205.

Gow, N.A.R. (1993). Nonchemical signals used for host location and invasion by fungal pathogens. *Trends in Microbiology,* **1**, 45-49.

Greathead, D. G. (1987). Prospects for biological control in the tropics. *Insect Science and Its Application*, **8**, 787-789.

Grundler, G. (1991). Strategies for tsetse control in Cot d' Ivoir. *Tropical Medicine and Parasitology*, **42,** 451-452.

Gruvell, J. (1977). Predators. In: *Tsetse: The Future for Biological Methods in Integrated Control.* (Editor, Laird, M. L.). Ottawa: IDRC-O77E, pp. 45-55.

Gunnarsson, S.G.S. (1988). Infection of *Schistocerca gregaria* by the fungus *Metarrhizium anisopliae*: Cellular reactions in the integument studied by scanning electron and light microscopy. *Journal of Invertebrate Pathology*, **52**, 9-17.

Hall, R.W. & Papierok, B. (1982). Fungi as biological control agents of arthropods of agricultural and medical importance. *Parasitology*, **84**, 205-240.

Hamill, R.L., Higgens, C. E., Boaz, H. E., & Gorman, M. (1969). The structure of beauvericin a new depsipeptide antibiotic toxic to *Artemia salina. Tetrahedron Letters*, **49**, 4255-4258.

Hamon, J., Baldry, D. A. T., Parker, J. D., Callier, A. & Stiles, A. R. (1977). Status and Future of Control. In: *Tsetse: The future for biological methods in integrated control.* (Editor, Laird, M.) Ottawa: IDRC- 077E, pp. 35-44.

Hargrove, J.W. & Vale, G. A. (1978). The effect of host odor concentration on catches of tsetse flies (Glossinidae) and other diptera in the field. *Bulletin of Entomological Research,* **68,**607-612.

Hendrickx, G. & Napala, A. (1995). Preliminary report on the effect of different control methods of African animal typanosomiasis : Comments on their cost effectiveness and feasibility in Togo. In: *Twenty-second meeting of the International Scientific Council for Trypanosomiasis Research and Control* (Editor, Sories, K. R.), Kampala, Uganda, (25-29 October, 1993). Nairobi: OAU/STRC. PP.310-311.

Hocking, K S., Lamerton, J.F. & Lewis, E.A. (1963). Tsetse fly control and eradication. *Bulletin of the World Health Organization,* **28**, 811-823.

IAEA. (1992). *Laboratory Training Manual on the Use of Nuclear Techniques in Insect Research and Control.* A joint undertaking by FAO and IAEA (3rd edition). Vienna: IAEA. Technical Report Series, No. 336.

ILRAD. (1989). *Annual Report of the International Laboratory for Research on Animal Diseases.* Nairobi: Kenya.

Ingold, C. T. & Hudson, H.J. (1993). *The Biology of Fungi.* London: Chapman & Hall.

Jenkins, N.E. & Goettel, M. S. (1997). Methods for mass productions of microbial agents of grasshoppers & locusts. In: Goettel, M. S. & Johnson, D.C.(Editors). *Memoirs of the Entomological Society of Canada,* **171**,37-48.

Jordan, A.M. (1985). The vectors of African trypanosomiasis research towards non-insecticidal methods of control. *British Medical Bulletin,* **14**,181-186.

Kaaya, G. P. (1989). *Glossina moristans morsitans.* Mortalities caused in adults by experimental infection with enthomopathogenic fungi. *Acta Tropica,* **46**,107-114.

Kaaya, G. P. & Dargi, N. (1989). Mortalities in adult tsetse, *Glossina moristans moristans*, caused by enthomopathogenic bacteria. *Journal of Invertebrate Pathology*; **54,** 32-38.

Kaaya, G. P. Okech, M. A. (1990). Horizontal transmission of mycotic infection in adult tsetse, *Glossina moristans moristans* . *Enthomophaga*, **35**,589-600.

Laveissiere,C., Vale, G. A., Goutex, J.P. (1990). Bait methods for tsetse control. In: *Appropriate Technology in Vector Control* (Editor, Curtis, C.F.). : Boca Raton, Florida: CRC Press, pp. 48-74.

Leak, S.G.A., Mulatu, W., Rowland, G. A. & d'Ieteren, G.D.M. (1995). The control of *Glossina pallidipes*, *Glossina fuscipes* & *Glossina moristans submoristans* in south-west Ethiopia using cyperpermethrin 'pour-on ' insecticide. In: *OAU/STRC*, 1995 (No. 9123). pp. 257-263.

Leopold, J. & Samsinnakova, A. (1970). Quantitative estimation of chitinase and several other enzymes in the fungus *Beauveria bassiana*. *Journal of Invertebrate Pathology*, **15**, 34-42.

Leygues, M & Gouteux, J.P. (1989). Community participation in the control of an endemic tropical disease: supernatural beliefs and tsetse traps in the Congo. *Social Science and Medicine*, **28**, 1255-1267.

Lindquist, D. A., Abusowa, M., & Hall, M.J.R. (1992). The New World screwworm fly in Libya: a review of its introduction and eradication. *Medical and Veterinary Entomology*, **6**,2-8.

Lingg, A.J. & Donaldson, M.D. (1981). Biotic and abiotic factors affecting stability of *Beauveria bassiana* conidia in soil. *Journal of Invertebrate Pathology*, **38**, 1917-200.

LO. BI. LO. SA. (1997). *Insect Pathology Manual.* (Editors, Lomer C. H. & Lomer C. J.)

Lysenko, O. & Kucera, M. (1971). Micro-organisms as sources of new insecticidal chemicals: toxins. In: *Microbial Control of Insects and Mites.* (Editors, Burges, H. D. & Hussey, N. W.). New York: Academic Press, pp. 205-227.

McCoy, C. W., Hill, A., Kanavel, R.F. (1972). A liquid medium for the large scale production of *Hirsutella thompsoni* in submerged culture. *Journal of Invertebrate Pathology,* **19**, 370-374.

Mendonca, A. F. (1992). Mass production, application and formulation of *Metarhizium anisopliae* for the control of sugarcane froghopper, Mahanarva postica, in Brazil. In: Biological Control of Locusts and Grasshoppers (Editors, Lomer, C. J. & Prior C.). UK: C.A.B. International, pp.239-244.

Meyer, F., Bauer, B., Kabore, I. & Liebisch, A. (1991). Simultaneous control of tsetse flies and ticks by the application flumethrin pour-on on cattle in Satiri, Burkina Faso. *Tropical Medicine and Parasitology,* **42**, 451.

Moore, D. & Caudwell, R.W. (1997). Formulation of entomopathogenes for the control of grasshoppers and locusts. In: Goettel, M. S. & Johnson, D.C. (Editors). *Memoirs of Entomological Society of Canada,* **171**,49-67.

Nyhlen, L. & Unestam, T. (1980). Wound reactions and *Aphanomyces astaci* growth in crayfish cuticle. *Journal of Invertebrate Pathology*, **36**, 187-197.

Paris, M. & Ferron, P. (1981). Depsipeptides from *Metarrhizium anisopliae. Phytochemistry*, **20**, 715-723.

Paris, S. & Ferron, P. (1979). Study of the virulence of some mutants of *Beauveria brongniartii* (= *Beauveria tenella*). *Journal of Invertebrate Pathology,* **34**, 71-77.

Paterson, R.R.M., Simmonds, M. S. J. & Blaney, W. M. (1987). Mycopesticidal effects of characterized extracts of penicillium isolates and purified secondary metabolites (including myco-toxins) on *Drosophila melanogaster* and *Spodoptora littoralis. Journal of Invertebrate Pathology*, **50**, 124-133.

Pfeiffer, T. A. & Grigliati, T. A. (1996). Future perspectives on insect pest management: Engineering the pest. *Journal of Invertebrate Pathology,* **67**,109-119.

Poinar, Jr. J. O., Van der Geest, L., Helle, W. & Wassink, H. (1977). Pathology and nematode parasitism. In: *Tsetse: The Future for Biological Methods in Integrated Control.* (Editor, Laird, M. L.). Ottawa: IDRC-077E, PP. 75-92.

Prior, C. , Jollands, P.& Patourel, G. L. (1988). Infectivity of oil and water formulations of *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) to the cocoa weevil pest *Pantorhytes plutus* (Coleoptera: Curculionidae). *Journal of Invertebrate Pathology*, **52**, 66-72.

Prior, C. & Streett, D. A. (1997). Strategies for the use of entomopathogens in the control of the desert locust and other acridoid pests. In: Biological control of Grasshoppers and locusts (Editors, Lomer C. J. & Johnson, D. L.). *Memoirs of Entomological Society of Canada,* pp.5-25.

Ramosk, W. A. (1984). The influence of relative humidity on *Beauveria bassiana* infectivity and replication in the chinch bug, *Blissus leucopterus. Journal of Invertebrate Pathology,* **43**, 89-394.

Rawlins, S.C. (1989). Biological control of insect pests affecting man and animals in the tropics. *Critical Reviews in Microbiology,* **16**, 235-252.

Roberts, D.W. & Yendol, W.G. (1971). Use of fungi for microbial control of insects. In: *Microbial Control of Insects and Mites.* (Editors, Burges, H. D. & Hussey, N. W.). New York: Academic Press, pp. 125-149.

Roberts, D.W. (1969). Toxins from the entomegenous fungus *Metarrhizium anisopliae*: isolation of destruxins from submerged cultures. *Journal of Invertebrate Pathology*, **14**, 82-88.

Samsinnakova, A. & Kalalova, S. (1983). The influence of single spore isolate and repeated subculturing on the pathogenicity of conidia of the entomogenous fungus, *Beauveria bassiana. Journal of Invertebrate Pathology*, **42**, 156-161.

Seketeli, A. (1985). *Chemical Control of Tsetse Flies: Ground Application of Residual Insecticides. Expert Committee on the Epidemiology and Control of African Trypanosomiasis.* Geneva: October, 16-23. TRY/EC/WP/85.27.

Service, M. W. (1986). *Lecture Notes on Medical Entomology.* London: Butler & Tanner Ltd.

Simmonds, F.J., Jordan, A.M. and Toure, S.M. (1977). Parasitoids. In: *Tsetse: The future for Biological Methods in Integrated Control.* (Editor, Laid, M. L.).. Ottawa: IDRC- 077E, PP. 57-74.

Smith, R. J. & Grula, E.A. (1983). Chitinase is an inducible enzyme in *Beauveria bassiana. Journal of Invertebrate Pathology,* **42**, 319-326.

Smith, R. J., Sue Perkul, & Grula, E.A. (1981). Requirement of sequential enzymatic activities for penetration of the integument of the corn earworm (*Heliothis zea*). *Journal of Invertebrate Pathology,* **38**, 335-344.

Tsetse and Trypanosomiasis Information Quarterly. (1998a). **21,** 4-5.

Tsetse and Trypanosomiasis Information Quarterly. (1998b). **21**, 49-50.

Vale, G. A. (1979). Field studies of the responses of tsetse flies (Diptera: Glossinidae) to colors of men, lactic acid and carbondioxide. *Bulletin of Entomological Research,* **69**,459-467.

Vale, G. A. (1982). The improvement of traps for tsetse flies (Diptera: Glossinidae) *Bulletin of Entomological Research,* **72**, 95-106.

Vale. G. A., Lovemore, D.F., Flint, S. & Cockbill, G. F. (1988). Odour-baited targets to control tsetse flies, *Glossina* spp. (Diptera: Glossinidae), in Zimbabwe, *Bulletin of Entomological Research,* **78**, 31-49.

Van Winkelhof, A.J. & McCoy, C.W. (1984). Conidiation of *Hirustella thompsonii* var synnmatosa in submerged culture. *Journal of Invertebrate Pathology,* **43**, 59-68.

Veen, K.H. (1966). Oral infection of second instar nymphs of *Schistocerca gregaria* by *Metarrhizium anisopliae. Journal of Invertebrate Pathology,* **8**, 254-256.

Vilas Boas, A.M., Andrade, R. M., & Oliveira, J.V. (1996). Diversification of culture media for production of entomopathogenic fungi. *Arquivos de Biologia e Technologia*, **39**, 123-128.

Walstad, J. D., Anderson, R.F. & Stambough, W.J. (1970). Effects of environmental conditions on two species of muscardine fungi (B*eauveria bassiana & Metarrhizium anisopliae). Journal of Invertebrate Pathology,* **16**, 221- 226.

Wellde, B.T. Waema, D. Chumo, D. A., Reardon M.J., Oloof, F., Njigu, A. R., Opio, E.A., &, Mugutu, S.(1989). Review of tsetse control measures taken in the

Lambwe Valley in 1980-1984. *Annals of Tropical Medicine and Parasitology*, **83**, (Supplement 1), 119-125.

WHO. (1986). *Epidemiology and Control of African Trypanosomiasis, Report of a WHO Expert Committee*. Geneva: World Health Organization, Technical Report Serious, No 739.

Wilson, S.G., Morris, K.R.S., Lewis, I.J., & Krog, E. (1963). The effects of typanosomiasis on rural economy: with special reference to the Sudan, Bechuanaland and West Africa. *Bulletin of the World Health Organization,* **28**,595-613.

Wraight, S. P. & Roberts, D.W. (1987). Insect control efforts with fungi. *Developments in Industrial Microbiology, Supplement no.2,* **28**, 77-87.

Yendol, W.G. (1968). Factors affecting germination of *Entomophthora* conidia. *Journal of Invertebrate Pathology*, **10**, 116-121.

Yendol, W.G., Miller, E.M., & Behnke, C.N. (1968). Toxic substances from enthomophthoraceous fungi. *Journal of Invertebrate Pathology*, **10**, 313-319.