ADDIS ABABA UNIVERSITY

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EVALUATION OF ETHIOPIAN ISOLATES OF ENTOMOGENOUS FUNGI AS POTENTIAL BIOLOGICAL CONTROL AGENT OF THE DESERT LOCUST, *SCHISTOCERCA GREGARIA*.

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

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This is to certify that this copy of the M.Sc thesis entitled: Evaluation of Ethiopian Isolates of Entomogenous Fungi as a Potential Biological Control Agent of the Desert Locust, Schistocerca Gregaria

by student Seneshaw Aysheshem is the final version that has accommodated the corrections and suggestions made by the examining committee.

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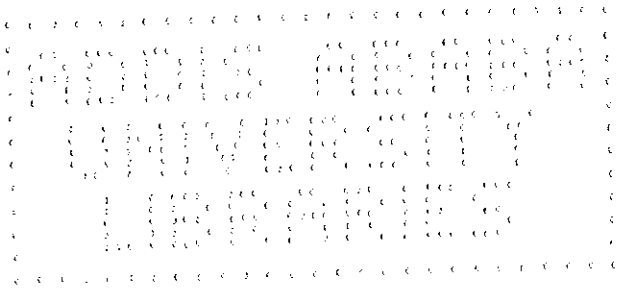

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ABSTRACT

Ten native fungal isolates were collected from the different regions of Ethiopia. Eight of the isolates were identified at International Institute of Tropical Agriculture, IITA, Benin. Five of the isolates were identified as *Beauveria*, two were *Paecilomyces* and one belonged to *Metarhizium* species. Eight of the tested fungal isolates showed pathogenecity to fifth instar *Schistocerca gregaria.* The *Beauveria* spp. isolate FF and *Metarhizium* spp. EE were more virulent compared to the rest. Thus two isolates were further assayed in peanut (*Arachis hypogaea)* and noug (*Guizotia abyssinica)* oil formulations, and compared with a known entomopathogen, *M. anisopliae* ICIPE 30. The results indicated that there were strong dose-response patterns. Peanut oil formulation enhanced infectivity of both FF and EE. Infectivity of EE was significantly ($P<0.05$) reduced in noug oil formulation. Comparison of virulence revealed that isolates FF and EE were more pathogenic than ICIPE 30. FF in peanut oil formulation had the lowest LC_{50} value of $2.02x10^5$ conidia/ml followed by EE, $2.93x10^5$ and ICIPE 30 with $4.98x10^5$ conidia/ml. The highest mortality was achieved by FF at $1x10^8$ conidia/ml, resulting in 50% and 100% mortality in 4.2 days and seven days, respectively. The median lethal time taken to fourth, fifth and adult FF treated insects were 3.47, 4.14 and 4.83 days; respectively that indicated fourth instars are more susceptible. Insects killed as a result of FF infection developed red pigmentation and produced white powdery mass of spores on the external surface. The *Metarhizium* isolates EE and ICIPE 30 produced green sporulation on the integument when the cadavers were kept at high humidity. FF and EE grew at a temperature range of 24^0C and 37^0C, with peaks at 24^0C and 28^0C for FF and 28^0C for EE. Malt extract agar favoured growth and sporulation of FF, whereas EE grew and sporulated faster on sabouraud dextrose agar. Germination of both isolates was significantly ($P<0.05$) high in peanut oil formulation than in noug oil.

1. INTRODUCTION

The desert locust, *Schistocerca gregaria,* is one of the most serious pests of agriculture in the Sahel (Geddes, 1990). During plague years, the desert locust has been noted to cause damage to the more humid zones of Africa and Asia (Brader, 1988). The devastation of crops and many types of vegetation by locusts have also been recorded since biblical times, and the cost for control has been very high (Brader, 1988).

The locust has a peculiar feature in that it can form swarms during favorable times. When repeated rains dampen the desert sands, thousands of eggs hatch. The hoppers constantly touch one another, resulting in a change of behavior and color and switching to a gregarious phase. Hopper bands and adult swarms then form large groups that can migrate very large distances by flying, following wind patterns (Conley, 1969; Lomer *et al.*, 1992). For example, in 1987, desert locust swarms have been noted to cross the Atlantic, confounding predictions of the experts that this is impossible (Greathead, 1992).

Until mid-1980's, locust control had depended largely on application of persistent chemical pesticides, such as dieldrin, which is not environmental friendly. Persistent pesticides are effective even when direct contact rate between spray and target insect is low because of the long activity of the spray residue (Brader 1988; Prior and Street, 1997). However, it is now apparent that there are widespread ecological consequences from excessive use of such chemicals such as soil and ground water contamination, impacts on the food chain and potential health care concerns. As a result, persistent chemical insecticides are now prohibited in most countries and replaced by less persistent pesticides such as fenitrothion and malathion (Brader, 1988; Lomer, *et al.*, 1992). These chemicals are much less effective and often require repeated applications within a season or extensive blanket spraying to achieve

satisfactory control, since they kill largely as a result of direct spray contact only (Brader, 1988).

In terms of cost also chemical pesticides are very expensive, particularly for developing countries. For example, during 1986-1989 the total cost of locust and grasshopper control programs (mainly in the Sahel and northwest Africa) amounted to over 400 million U.S. dollars (OTA, 1990). In 1986-7, about 4.6 million hectares (ha) of land in 10 countries received aerial or ground insecticide treatments against locusts and grasshoppers. In 1988, during the desert locust plague, 10 million hectares in 10 countries of northern and western Africa were sprayed with approximately 13 million liters of insecticide at a cost of approximately US$ 100 million. This resulted in increased cost and environmental damage of the control program (Brader, 1988; OTA, 1990; Kremer, 1993).

These factors coupled with increasing public awareness raised a growing interest of insect pest management in which biological control has an important role (Brader, 1988). Biological control of insect pests with microbial agents is an appealing prospect, since it would reduce inputs of chemical pesticides into the environment.

Entomopathogenic fungi, particularly *Metarhizium* and *Beauveria* in the Class Deuteromycetes, are attractive as biopesticides for use in integrated pest management (IPM) systems because they combine host specificity with proven safety (Bateman *et al.*, 1993)

Often, locusts are indigenous to the areas of infestation indicating that their natural enemies already surround them. Therefore, this research was carried out to find native isolates of fungi with virulence to *S. gregaria* with the following specific objectives:

- To survey, collect and identify native entomopathogenic fungi from the host insect and/or from the soil ecosystem.
- Screening of potent local isolates of fungi which are pathogenic to *Schistocerca gregaria*.
- To evaluate the efficacy of native entomopathogenic fungi against *S. gregaria*.
- To compare the efficacy of native isolates with a known entomopathogenic strain.

2. LITERATURE REVIEW

2.1. The desert locust

2.1.1. Economic importance and distribution

There are about 5000 species of grasshoppers, a few of these, which are capable of aggregating are called locusts (Barrass, 1980). Among the different locust species, perhaps the most destructive species is the desert locust, *Schistocerca gregaria* (Baron, 1972).

The desert locust has certain preferred types of habitat in the desert where vegetation has been able to spring to life following rain. However, since this occurs rarely, *S. gregaria* has adapted a highly nomadic habitat. It is capable of long distance flight, which no other insects can do. They are characterized by awesome expansion both in its numbers and territory when conditions are favorable (Baron, 1972). It can invade nearly 11 million square miles, an area that includes roughly 20% of the world's land surface (Conley, 1969). Its distribution is in the northern half of Africa as far east as northern India (Barrass, 1980).

A locust eats approximately its own weight in a day. Immature adults are the most voracious and mobile life stage and hence the most damaging. Accordingly, a swarm of locust with millions of number can destroy completely many tones of food a day (Barrass, 1980). Estimates of actual economic damage inflicted by desert locust are difficult to obtain because such damage often occurs in remote areas and on subsistence crops whose value is difficult to assess (Steedman, 1990).

In Ethiopia, several major plagues have occurred. In 1958, for instance, Ethiopia has lost about 167,000 tons of grain, enough to feed more than a million of people for a year (Conley,

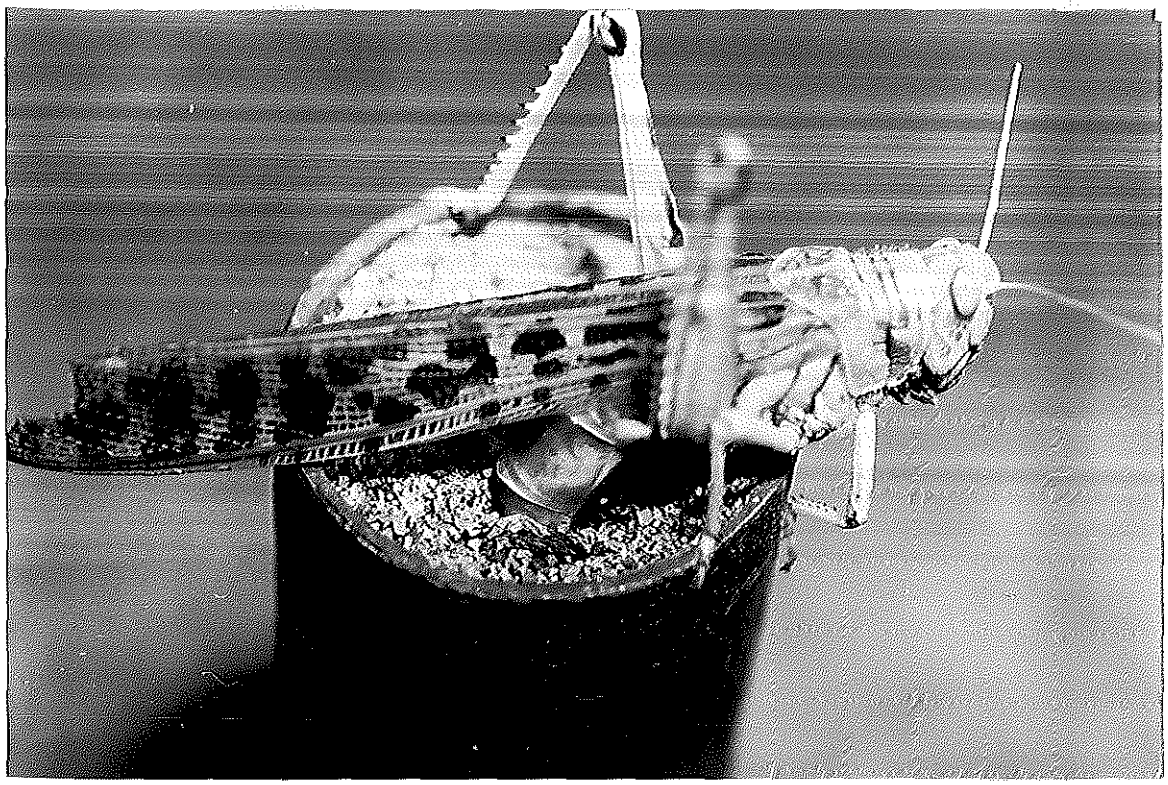
1969). In March and April 1998, the country has also been repeatedly attacked by outbreaks of desert locust. According to media broadcast the swarm was estimated to be about 3.5 to 5 billion in number and could feed up to 15,000 tons of grains a day.

2.1.2. The life cycle

The life cycle of *Schistocerca gregaria* comprises three stages, namely egg, nymph (hopper) and adult. The time spent in each stage varies considerably depending on environmental conditions (Steedman, 1990).

The female locust, using a pair of short black curved hooks protruding from the end of the tail, digs a hole about 10 cm deep in the sand (Fig. 1a). Eggs are laid in the lower two thirds of this hole bound together by a frothy secretion that hardens to form a tubular egg-pod (Barrass, 1980). The number of eggs an egg pod contains may vary from 20-100. The whole process of probing, digging and laying takes about two hours (Steedman, 1990). Incubation period of the eggs takes about 12 days at 30-33°C under laboratory condition (Pers. observation).

The nymphs emerging from the eggs are also called vermiform larvae because they wriggle up through the frothy tube to the surface of the sand (Barrass, 1980).



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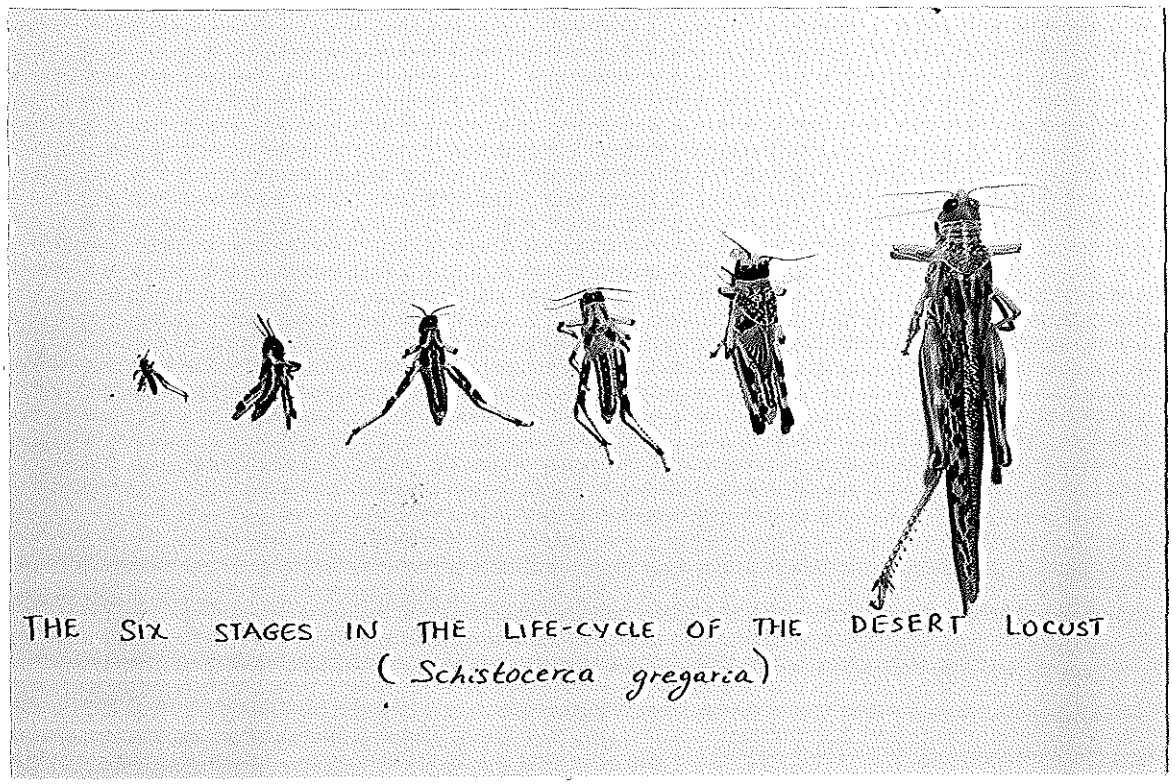


b)

Fig. 1. a) A female *S. gregaria* in the process of egg laying. b). The six life stages of the desert locust.

The life cycle of *S. gregaria* is geared to survival in an environment of extreme hostility, which involves a fight against desiccation caused by excessive heat and low humidity. To overcome this situation, the locust has a hard exoskeleton incorporating a waxy outer layer, which checks transpiration (Baron, 1972).

The exoskeleton, in addition to giving protection, provides a surface to which muscles are attached. When the cuticle is hardened, it becomes inflexible as a result of which further growth is retarded (Barrass, 1980). Therefore, the locust periodically casts or molts the cuticle five times in the course of their nymphal development until the adult winged stage (Fig. 1b).

The rate of development of each instar is mainly dependent on temperature and may vary from 22 days under hot conditions (37°C) to over 70 days under cool conditions (22°C). The last molt is from the fifth instar to the adult stage that comprises well-developed wings. The adult locust gradually becomes hard and able to fly (Steedman, 1990).

The adult male is a brilliant yellow and usually matures before the female. The female maturity is hastened by a skin odor given off by the male. The odor also affects other males so that the process of maturation of the whole swarm is speeded up (Baron, 1972). The maturation period of the adults is very variable ranging from three weeks under suitable condition up to eight months in cool and/or dry condition (Steedman, 1990).

The life span of the desert locust also varies considerably. Under laboratory condition, they have been noted to live more than nine months (Pers. observation). However, in the field situations their life span could vary between two and five months (Steedman, 1990). Their life span has been found to depend on the time taken to attain sexual maturity. The quicker

they mature, the shorter their life span (Steedman, 1990).

2.2. Control approaches

2.2.1. Chemical control

Chemical pesticides have long been the principal means of controlling *Schistocerca gregaria*. The insecticide most favored in outbreaks prior to 1980s was dieldrin, a very persistence and broad-spectrum organochlorine compound (Brader, 1988). However, because of its long persistence in the environment and toxicity to mammals, it was banned by most donor and recipient countries (Lomer *et al.*, 1992).

The insecticide used most widely in recent years has been fenitrothion, an organophosphorous compound of moderate mammalian toxicity (Brader, 1988). It is less persistence, with a half-life of less than 3 days, or even less than 24 hours under harsh environments, but has high toxicity to non-target aquatic and terrestrial arthropods (Sekizawa *et al.*, 1992). The current strategy of chemical application is blanket spraying of very large areas. However, the scale and cost of such operations have led to questions about their efficacy and undesirable side effects to non-target organisms (Prior and Street, 1997).

In an attempt to reduce the dependence on chemical treatments, particular emphasis has been given to potential biological control agents for incorporation into integrated pest management system (IPM). Biological control involves the use of a variety of manipulative methods to enhance the effects of a pest's natural enemies so that they reduce pest numbers to an acceptable level. Potential control methods with which biological control might be integrated include non-persistent, quick-acting chemicals, insect growth regulators, botanical pesticides and pheromones (Nasseh *et al.*, 1992; Prior and Streett, 1997).

2.2.2. Natural enemies

There are several parasites and predators of all stages of the desert locust. Numerous species of wasps, ants, ant lions, birds, reptiles and mammals are known to be natural enemies of the desert locust (Baron, 1972). However, the various natural enemies are neither numerous enough on the ground nor mobile enough in the air to challenge swarms significantly (Steedman, 1990).

2.2.3. Microbial control

Several groups of pathogens have been and are being considered for possible microbial control of the desert locust. Few protozoa species belonging to Order Microsporadia have been considered as a potential candidate of locusts and grasshoppers (Maddox, 1987; Johnson, 1997). Several species of nematodes have also been recorded in numerous grasshoppers and locust species (Baker and Caprinera, 1997). Mermithids are commonly found in grasshopper population that live in humid environments and are considered as one of the factors that regulate their population dynamics (Kaya, 1987; Kaiser, 1991). Epizootics due to bacterial and virus diseases have been observed among locusts and grasshopper species (David, 1975; Zelazny *et al.*, 1997; Street *et al.*, 1997).

2.3. Fungi as potential agents for *S. gregaria* control

Fungal diseases of insects have long been known since 1834 when the Italian, Agostino Bassi, showed the fungal nature of white muscardine disease of silkworms (Hall and Papierok, 1992). At present about 700 species of fungi in 90 genera are recognized to cause infection

among insects (Goettel, 1992). Nevertheless, few species have received much attention with a view to their use as biological control agents.

Entomopathogenic fungi are widely distributed throughout the fungal kingdom, the majority being in the class Deuteromycetes and Zygomycetes. The class Deuteromycetes alone contains about 150 species of insect pathogens (Samson, 1981).

Fungi are unique among all the groups of insect pathogens in that they can penetrate directly through the cuticular barrier and do not have to be ingested (Charnley, 1992). Given favorable environmental conditions, fungi exert spectacular natural control of insect populations, particularly when pest population is very high. The aim of using fungi, however, is to prevent population build up of the pest to damaging levels, by artificial application of fungal propagules (Hall and Papierok, 1992).

Metarhizium and *Beauveria* are the best-known genera in the class Deuteromycetes because of their wide geographical distribution and host range (Hall and Papierok, 1992). The genus *Metarhizium* contains two species, *M. anisopliae* and *M. flavoviride*. *M. brunneum* and *M. album*, are considered to be synonyms of *M. anisopliae* (Tulloch, 1976). *M. anisopliae* is by far the most important species, known to affect more than 200 insects (Veen, 1966) and consists of two forms, characterized by the size of their conidia (Tulloch, 1976; Samson, 1981).

Collections of *Beauveria* infected insects, from tropical ecosystems, have revealed considerable variation between isolates that has made species identification difficult (Mugnai *et al.*, 1989). According to Mugnai *et al.* (1989) the following species of *Beauveria* are recognized: *B. alba*, *B. amorpha*, *B. bassiana*, *B. brongniartii*, *B. velata*, *B. vermiconia* and an

undescribed taxon close to *B. amorpha* but morphologically and biochemically distinct. However, *B. bassiana* and *B. brongniartii* are the only insect pathogens from this genus for which about 500 host species are known.

Other well-studied entomopathogenic Deuteromycetes include *Nomuraea rileyi,* mainly parasitizing lepidopterous pests (Ignoffo, 1981), the genus *Paecilomyces,* common parasite of several insect orders, *Hirsutella*, and *Verticillium lecanii*, a pathogen of aphids, scales and whitefly (Hall, 1982).

2.4. Mechanism of fungal infection

Entomopathogenic fungi invade their hosts by direct penetration of the insect cuticle (Charnley, 1992). The cuticle has two layers, the outer epicuticle and the procuticle. The outer most epicuticular layer of locust is composed of lipid, containing approximately 65% alkanes, 25% secondary alcohol wax esters with lower amounts of normal and sterol wax esters, triglycerides, alcohols, sterols and free fatty acids (Bidochka *et al.*, 1997). The ability to utilize alkanes and other lipids as a nutrient source may be an important adaptation (Bidochka *et al.*, 1997).

The conidia and insect cuticle are hydrophobic which contributes for the initial non-specific adsorption of conidia onto the cuticle surface (Boucias *et al.*, 1988). Following spore attachment, germination begins (Hegedus and Khachatourians, 1996) and subsequent interactions between the fungus and insect result appressorium formation and extrusion of an adhesive mucus layer (Bidochka *et al.*, 1997). Studies conducted on *M. anisopliae* conidia showed that low levels of complex nitrogen compounds, surface topography and some catabolites stimulate differentiation into appressorium (Clarkson and Charnley, 1996). Then,

the appressorium penetrates through the host cuticle and eventually emerge into the insect's haemocoel. Deviations have also been noted in some Entomophthorales and *B. bassiana* that can penetrate the host cuticle without appressorium formation (Hall and Papierok, 1992).

Proteins are the major constituents of acridid cuticle (61.4% w/w) (Bidochka and Khachatourians, 1992), and observations of high production of extracellular proteases by entomogenous fungi gives the impression that this enzymes are involved in cuticle hydrolysis (Bidochka *et al.*, 1997). Mechanical pressures exerted by appressorium have also been suggested to aid in penetration (Clarkson and Charnley, 1996).

The reason why oral ingestion of fungi does not cause infection among insects was explained by the fact that the gut transit time is shorter than the germination time. This coupled with antifungal toxins produced by indigenous gut microflora, which inhibit conidial germination, results failure of infection through the gut (Veen, 1966; Dillon and Charnley, 1986).

Once the cuticle has been breached, the penetrating hyphae bud and develop as blastospores within the haemocoel (Bidochka *et al.*, 1997). Inside the haemocoel, the fungus proliferates rapidly and spreads throughout the body by means of the open circulatory system (Hegedus and Khachatourians, 1996). The fungus also produces secondary metabolites such as beauveracin (by *B. bassiana*) and destruxins (by *M. anisopliae*) which are toxins (Bidochka *et al.*, 1997). It has been found that lepidopteran larvae injected with destruxins exhibit sustained muscle contraction followed by flaccid paralysis (Charnley, 1992).

In conclusion, it is speculated that mechanical damage, nutrient deprivation and toxic

metabolites produced by the fungi are involved in the death of the locust (Bidochka *et al.*, 1997). Following death of the insect, hyphae eventually emerge from the cadaver producing aerial spores capable of initiating the cycle once again (Hegedus and Khachatourians, 1996).

2.5. Host specificity

Some insect-pathogenic fungi have restricted host range, while others such as *Beauveria bassiana* and *Metarhizium anisopliae* have a wide array of insect hosts (Ferron, 1981). Studies have indicated that host specificity may first be manifested at the level of host cuticle. Brobyn and wilding (1977) showed that *Nomuraea fresenii* conidia germinated on the cuticle of susceptible and resistant aphid hosts but infected only the former. Similarly, Goettel *et al.* (1990) suggested that host cuticle is of major importance in host specificity, because differences in host specificity are not usually present when the fungi are injected into the host's haemocoel.

St. Leger *et al.* (1991) have shown that enzymes located at the surface of spores are involved in the prepenetration events. Rath *et al.* (1995), on the other hand, have suggested that the spore surface antigens are the primary determinant of host specificity. They have hypothesized that the spore surface antigens are a primary factor determining pathogenecity of isolates, and that other pathogenecity determinants such as enzyme production during penetration or toxin production of the fungus within the host are secondary.

In general, mechanisms of pathogenecity and specificity appear to be complex and successful penetration can be said to depend upon three major factors: the ability of spores to adhere to the cuticle, to germinate and to penetrate enzymatically (Hall and Papierok, 1992).

2.6. Host defense

Successful virulent entomopathogenic fungi must be able to adhere, penetrate the cuticle and overcome resistance mechanism of the locust inside the haemocoel (Bidochka *et al.*, 1997).

The primary barrier of the host insect to penetration of the fungus is the integument or the cuticle (Hall and Papierok, 1992). Phenoloxidases, an enzyme that is localized in the cuticle and haemocoel, have been implicated in wound healing and defense reactions against the pathogen (Bidochka *et al.*, 1989). It has also been implicated in melanic patches on wounded cuticles or fungal infection sites (Bidochka *et al.*, 1997). Melanin granules in the insect cuticle also appear to impede the penetration of *M. anisopliae* (Goettel *et al.*, 1989).

Fungi that crossed successfully the integument are challenged by cellular and humoral immune responses (Hall and Papierok, 1992). Cellular defense mechanisms involve cellular encapsulation and phagocytosis of the pathogen (Bidochka *et al.*, 1997). It has been found that the *B*-1, 3-glucan components of the fungal cell wall are recognized as non-self by the haemocytes and trigger the insect's immune response such as cellular encapsulation (Bidochka *et al.*, 1997).

Despite the insect's cellular defense, the fungi have also developed evading mechanisms. One method is assuming of different morphology in the form of blastospores, hyphal bodies or protoplasts that enable the disease to be disseminated to other tissues (Hall and Papierok, 1992). The growth rate of the fungi inside the haemocoel also helps to out compete the insect's immune response (Bidochka *et al.*, 1997). Toxins, such as destruxins produced by *M. anisopliae* may assist in establishing and extending infection by interfering with the immune response (Clarkson and Charnley, 1996).

Humoral defense reaction, on the other hand, does not involve the participation of free cells. It includes haemagglutinins, which play an important role in insect defense reaction by opsonizing the invading fungi (Wheeler *et al.*, 1993). Agglutinins purified from *Melanophus differentialis* haemolymph have been shown to enhance the *in vitro* association of *B. bassiana* blastospores with haemocytes. It appears that agglutinins are involved in the recognition of invading fungi (Bidochka *et al.*, 1997).

Although humoral encapsulation plays an important role in prevention of mycoses due to several fungi, Gotz and Vey (1974) have shown that *B. bassiana* could break out of the encapsulation.

2.7. Mass production

There are several methods available for mass production of entomopathogenic fungi. These are *in vivo*, submerged and surface culture. The method used depends largely on the growth requirements of the fungus in question and the end products desired (Goettel and Roberts, 1992).

2.7.1. Production *in vivo*

Many obligate entomopathogenic fungi, such as *Entomophaga grylli* (Fresenius), have complex nutritional requirements and are difficult to grow them in artificial culture. Therefore, the remaining alternative is to grow them *in vivo* (Ignoffo and Hink, 1971). However, *in vivo* production is relatively expensive and is questionable if this method can be used for mass production of a fungus for use as a microbial pesticide.

2.7.2. Submerged culture

Industrial fermentation provides the simplest and most economical way to produce large numbers of fungal spores for the existing equipment can be used without modification (Auld, 1992). However, it may also have limitation in that most hyphomycete fungi grow as mycelium and produce blastospores in submerged liquid culture while conidia are the desired end product (Jaronski and Goettel, 1997; Lomer *et al.*, 1997).

Blastospores are unpigmented, hydrophilic and compared to conidia less resistant towards environmental stress (Van Winkelhof and McCoy, 1984). Despite this, blastospores are infective and can be produced easily in deep tank fermentors, and hence may have potential use in biological control (Kleespies and Zimmerman, 1992).

Few entomopathogenic fungi produce conidia in submerged culture. An example is *B. bassiana*, which produce conidia, as opposed to blastospores, in submerged culture (Thomas *et al.*, 1987). Studies carried out by Hegedus *et al.* (1992) on aerial conidia, submerged conidia and blastospores of *B. bassiana* showed that blastospores were more virulent than both conidia types. Aerial and submerged conidia, on the other hand, have greater viability during storage than blastospores.

Production of submerged conidia by *M. flavoviride* was also possible in liquid medium containing sucrose and brewers yeast with limited nitrogen and excess carbon (Jenkins and Prior, 1993). Bioassay and semi-field trials against *S. gregaria* also indicated that both aerial and submerged conidia have comparable virulence (Jenkins and Thomas, 1996). However, submerged conidia are hydrophilic as blastospores and that affects formulation in oils for ultra low volume (ULV) application (Moore and Caudwell, 1997).

Some fungi that only produce mycelium in submerged culture can be dried and applied as fragments or pellets in the field (Pereira and Roberts, 1990). When introduced in the field, particles of dry mycelium rapidly produce infectious conidia following rehydration from dew, rain, or soil moisture. This method has been used for many entomopathogenic fungi, among them is *Metarhizium anisopliae* commercially produced by Bayer, Munich, Germany, as BIO 1020 (Pereira and Roberts, 1990).

Media for growth of the biocontrol organism should be as simple as possible, utilizing a standard set of inorganic salts and sources of carbon and nitrogen (Auld, 1992). Animal manure and waste products could be suitable as nitrogen sources and are efficient nutrients for mass production of fungi. Waste products such as molasses and Naturpur™ may be used to produce blastospores of *M. flavoviride* in high concentrations. The mycelial production is reduced. Therefore, a separation of blastospores from the mycelium is not necessary and the processing of blastospores is simplified (Stephan *et al.*, 1995).

2.7.3. Solid substrate

Most entomopathogenic fungi can grow profusely on solid substrates and produce aerial conidia (Goettel and Roberts, 1992). The solid media used could be either nutritive such as rice, wheat or grains or non-nutritive such as sponge and cloth (Jenkins and Goettel, 1997). Solid substrate offers large surface area that enable to increase spore yield (Jenkins and Goettel, 1997). However, particle size, moisture content and temperature need to be controlled for successful production (Auld, 1992). Equipment used may be bags, trays or big flasks. Accordingly, production on solid substrate may be less costly provided that suitable raw material is cheaply available (Auld, 1992).

2.7.4. Diphasic liquid-solid fermentation

Diphasic fermentation system has been developed that combines both liquid and solid fermentation; blastospores and mycelia produced in submerged culture are transferred to surface culture for production of hydrophobic conidia (Goettel and Roberts, 1992). Currently, this method is being used to produce *M. flavoviride* by LUBILOSA program (Lomer *et al.*, 1997).

In general, it may be necessary to screen isolates for spore productivity under fermentation conditions. The most virulent isolates may not necessarily have high spore productivity *in vitro*. It is also essential to store the original single spore isolate to overcome problems of loss of virulence through sub-culturing in continued use of large scale production (Auld, 1992). Following recovery of spores from a production process it is usually necessary to dry them for long term storage. This must be done immediately under clean conditions to prevent bacterial contamination (Auld, 1992).

2.8. Formulation

Successful use of fungal pathogens in agricultural pest control requires appropriate formulation that is stable during storage and compatible with the existing application technology (Prior *et al.*, 1992). Therefore, development of improved methods to formulate and apply fungal pathogens is a prerequisite to their economic use. Good formulation usually can help improve the shelf life, persistence and efficacy (Goettel and Roberts, 1992). The formulation may incorporate additives such as wetters, humectants, UV protectants, and thickening agents to adjust the viscosity of the pesticide (Goettel and Roberts, 1992; Auld,

1992).

Fungal pathogens for locust and grasshopper control are usually applied using controlled droplet application (CDA) at ultra low volume (ULV) rates in an oil diluents or baits (Moore and Caudwell, 1997). They can also be applied as dry material or formulated as wettable powder that may contain dispersing, suspending, or wetting agents as well as inert fillers (Auld, 1992).

Most often oil formulations are preferred for application in dry condition. This is particularly important to reduce evaporation during ULV application (Moore and Caudwell, 1997). Moreover, the oil helps the lipophilic cell wall of conidia to adhere to the insect cuticle and spread to inter-segmental membranes facilitating penetration (Prior *et al.*, 1988).

Normally, fungal conidia require a high relative humidity (r. h.) to germinate and infect insects. However, studies conducted by Bateman *et al.* (1993) showed that *Schistocerca gregaria* can be killed at very low environmental humidities (30-40% r. h.) using oil based formulation.

Conidia and blastospores produced in submerged culture are hydrophilic and not suitable for standard ULV application with oil diluents (Moore and Caudwell, 1997). Studies carried out by Stephan *et al.* (1995), however, demonstrated that formulation of *M. anisopliae* blastospores in 20% molasses and 80% water has been found effective even in the field situation. Semi-field trials showed that spray-dried blastospores formulated in a water based formulation (20% molasses + 80% water) were able to kill desert locust under arid conditions. Molasses based formulation is advantageous for it is cheap, easily available and safe to the environment. Moreover it can also be used as an adhesive (Stephan *et al.*, 1995).

Incorporation of fungal mycelium in alginate pellets was found to offer major advantages compared with conidial suspensions. Pellets can be stored dry and then reactivated by rehydration, and alginate may protect the fungus from UV radiation and other environmental factors that could reduce its efficacy in the field (Knudsen *et al.*, 1990). *B. bassiana* pellets formulated had excellent shelf life, consistently exhibiting 100% viability over several months. It can also be stored dry at room temperature under non-sterile conditions (Knudsen *et al.*, 1990). Field work with alginate formulations of dry mycelium of *B. bassiana* and *M. anisopliae* showed that they were more resistant to artificial solar radiation and heat than are pure mycelium formulations (Pereira and Roberts, 1990).

Overall, in the past problems of formulation, storage and application have been limiting factors with the use of entomopathogens. However, recent advances and strong emphasis on development of improved formulation may open avenues for large-scale use of entomopathogens.

2.9. Storage

One essential quality of a biopreparation is longer shelf life. According to Couch and Ignoffo, (1981), the shelf life of biopreparations has to be at least 18 months. For practical use long term storage at higher temperatures without loss of viability is needed.

The moisture content of conidia and temperature of storage condition can affect the viability (Stathers *et al.*, 1993). Conidia for locust control can be kept dry as pure conidia or with dry diluents or carriers such as clays (Moore and Caudwell, 1997). It is suggested that clay carriers protect the pathogen during storage against the effects of its own metabolism and

resultant by-products, and also from adverse environmental factors (Moore and Caudwell, 1997).

Dried conidia of *M. anisopliae* stored under deep frozen condition were able to maintain 50-70% viability following one to two years' storage (Moore and Caudwell, 1997).

Storage in oils has also been indicated to reduce loss of viability. Both vegetable and mineral oils have been noted to maintain viability of conidia for some time. Part of their value as diluents for storage depend on their sensitivity to oxidation (Moore and Caudwell, 1997). Therefore, it is recommended to avoid contact between oil and air (oxygen) and high temperature (Moore and Caudwell, 1997).

Blastospores stored in different water-based formulations at room temperature have only a relatively short shelf life (Stephan *et al.*, 1997). Studies carried out by Stephan *et al.* (1997) showed that the germination rate of spray-dried blastospores stored at 5 and 20°C for 52 weeks under dry and oxygen-reduced conditions was 73.1 and 68%, respectively. The result has shown that storage at 5 and 20°C is possible for at least one year.

Recently, a new method has been developed that help to determine viability of spores following a long-term storage. Jimenez and Gillespie (1990) have shown that viable conidia of *B. bassiana* and *M. anisopliae* fluoresced only weakly after staining in bi-striazinyl amino stilbene (0.5% Tinopal BOPT) and exposure to UV light. By contrast, non-viable conidia fluoresced brightly and could be distinguished from viable spores.

2.10. Application

The aim of biopesticide application is to kill the maximum number of target pests by direct contact, as is the case with non-persistent chemical pesticides, rather than to create epizootics (Bateman *et al.*, 1993). It has been found that topical application of conidia to some insects including grasshoppers may proceed independently of ambient relative humidity (r. h.) (Ferron, 1978). This indicates that epizootics in the field are limited by the inhibiting effect of the low r. h. on sporulation rather than infection. It also implies that if conidia can be delivered to the target pest, they may infect even under ambient conditions too dry for their *in situ* production (Bateman *et al.*, 1993). Therefore, the concern is how to deliver the inoculum to the target and assure the infectivity of this inoculum under conditions of low ambient humidity (Bateman *et al.*, 1993).

The active ingredient of a microbial insecticide is a living organism accordingly; application aspects must be closely linked to those of production, formulation, storage and delivery to the site of treatment (Moore and Caudwell, 1997). In addition, methods for microbial agents should be as compatible as possible with existing methods, especially with a standard ultra low volume (ULV) application techniques (Bateman, 1997).

Currently, the strategy followed for application of mycoinsecticide is to formulate the spores in vegetable oils with increased efficacy and less reliance on conditions of high humidity (Prior *et al.*, 1992). This is not a problem for aerially produced conidia of *Metarhizium* spp. and *B. bassiana* that can easily be suspended in oil (Moore and Caudwell, 1997).

Viscosity of the formulation is important for ULV application. Accordingly, the high viscosity of vegetable oils must be reduced by mixing with lighter mineral oils (Bateman,

1992). It is also essential to add combinations of additives which help protect the pathogen from UV radiation, temperature extremes, microbial degradation, desiccation and wind (Moore and Caudwell, 1997).

However, conidia produced in submerged culture have hydrophilic cell wall that is not suitable for ULV application with oil diluents. Stephan *et al.* (1997) have demonstrated that blastospores of *M. flavoviride* could be suspended in both water and oil-based formulation following a spray drying. Results of semi-field trials showed that spray-dried blastospores formulated in water-based formulation (20% molasses: 80% water) killed desert locust under arid conditions. Investigation of the efficacy of blastospores formulated in oil, however, resulted in lower mortality (Stephan *et al.*, 1997).

Use of bait formulation have been suggested to provide environmental advantages such as efficacy at reduced dosages, elimination of spray drifts, reduced microbial degradation and UV radiation (Ewen and Mukerji, 1987). Although fungi do not cause infection upon oral ingestion, during eating of baits, locusts are likely to pick a dose that could be effective as penetration can occur through the mouth parts (Moore and Caudwell, 1997).

At present, the most suitable application strategy of entomopathogenic fungi (*Metarhizium* and *Beauveria* spp.) to locusts is augmentation (Prior and Street, 1997). This involves a massive addition of the pathogen that is naturally present, but normally exists at levels too low to achieve useful population reduction. This method has the additional advantage of using indigenous agent that reduces quarantine restrictions on importation (Prior and Street, 1997). An exotic agent could also be used provided quarantine requirements are satisfied.

Currently, fungi are being used for pest control on a moderate scale in China, Russia and

Brazil, and several European and North American companies are selling small amounts of mycoinsecticides based on isolates of *V. lecanii*, *M. anisopliae* and *Beauveria* species (St Leger *et al.*, 1990). Entomopathogens have often been applied singly against the desert locust. Recently, application in combination with insect growth regulators has also resulted promising results (Prior and Streett, 1997).

3. MATERIALS AND METHODS

3.1. Rearing of *S. gregaria*

Eggs of *Schistocerca gregaria* were obtained from the Desert Locust Control Organization (DLCO EA) Addis Ababa and the colony maintained in the Biology Department, Addis Ababa University. The eggs were incubated in wooden box at 31 ± 1°C for about 12 -14 days. The temperature of the box was maintained by placing in a 60-watt lamp for 24 hours. Every other day, few drops of water were added to the egg tubes to maintain the moisture of the sand at the margin of 70-80% relative humidity.

Fig. 2. Locally made rearing cage.

When eggs hatched, the first instars, also called "vermiform larvae", were transferred into metallic cages of size 38 cm x 38 cm x 52 cm (l x w x h) adopted from the standard designs by National Research Institute, NRI (Fig. 2). A 60 watt light bulb was used to maintain an inside ambient temperature of 32 - 37°C. The light was kept on for 12 hours. The humidity of the cages was also in the range of 35 - 65%. The insects were fed cabbage leaves; barley and wheat seedlings supplemented with wheat bran.

3.1.1. Oviposition

The desert locust under natural conditions oviposits into sands. In the present work, the sand was sieved, washed and sterilized in an oven at 110°C for 24 hours. A humidity of 70 - 80% was maintained by adding drops of water into cylindrical egg tubes of diameter 3.5cm and length 10cm prepared by fusing two plastic photograph film cases. The egg tubes were then placed in plywood of the cage with circular holes for the insect to lay their eggs in and were replaced every two days.

3.2. Collection of pathogens

Roadside survey was made in different locations in the regions of Tigrie and Wollo recognized as locust breeding areas. Searching for dead insects was made under trees, bushes and grasslands paying particular attention to humid habitats that could allow sporulation of cadavers. Accordingly, several dead insects with external sporulation and soil samples were collected in sterile glass vials and brought to the laboratory.

3.3. Isolation

3.3.1. Isolation of fungi from insect

Spores were directly transferred from the external portion of the cadaver using a fine sterile needle onto sabouraud dextrose agar media. The cultures were incubated at 28°C in the dark for seven days.

3.3.2. Isolation of fungi from soil

Attempts were made to isolate entomopathogenic genera *Metarhizium* or *Beauveria* spp. from the soil. Soil samples collected from each site were grounded manually and sieved using 0.63µm size mesh. Then one gram of the fine soil was suspended in 10ml of sterile water. The suspension was further diluted in sterile water.

A selective media was prepared using sabouraud dextrose agar by adding 0.4g/l of cychohexamide and 0.5g/l of chloroamphinicol (Veen and Ferron, 1966). An aliquot of 0.5 ml of each soil suspension was then transferred onto plates using a Pasteur pipette. The plates were then incubated at 28°C in the dark for 7 days.

3.4. Storage of pathogens

Cadavers of insects were placed in glass vials and kept at 4°C. Slants of sabouraud dextrose agar were prepared in glass tubes having a screw cap. Pure cultures of each fungal isolate were given codes and maintained at 4°C in the refrigerator (Fig. 3).

3.5. Preparation of media

3.5.1. *Water agar medium (WA)*

Agar	15.0 g
Distilled water	1 l

The agar was boiled until dissolved. Then it was sterilized at 121°C for 15 min.

3.5.2. *Oatmeal agar (OA)*

Oatmeal	1.0 l
Agar	30.0 g

The oatmeal was prepared by boiling 30 g oat flakes, bought from local market, in 1 liter of tap water and filtering it through gauze.

3.5.3. *Sabouraud dextrose agar (SDA)*

Mycological peptone	10.0 g
Glucose	40.0 g
Agar	15.0 g
Distilled water	1.0 l

About 65 grams of the ready-mixed powdered SDA was suspended in 1 liter of distilled water, boiled and autoclaved at 121°C for 15 minutes.

3.5.4. *Malt extract agar (MEA)*

Malt extract	30.0 g
Mycological peptone	5.0 g
Agar	15.0 g

About 50 grams of the ready-mixed powdered MEA was suspended in 1 liter of distilled water and boiled to dissolve. It was then sterilized by autoclaving at 121°C for 15 minutes.

3.6. Culturing of fungi

The fungi were cultured on SDA or MEA and incubated at 28°C in the dark. Virulence of the isolates was maintained by re-isolation from the host insect every third passage on appropriate culture media.

3.7. Preparation of oil formulation

For each fungal isolate, the conidia were harvested 15 days after inoculation by pouring 10 ml of good quality oil (pure peanuts oil or extra refined noug oil) onto the fungal cultures in plates. A spatula was used to brush the conidia into the oil. The suspension was then sieved through fine cotton to remove the hyphal fragments and agitated in test tube shaker for six minutes to avoid clumping of spores.

The conidial concentration of each isolate was adjusted using haemocytometer. For screening the new isolates, a concentration of 5×10^7 conidia/ml was used in the assay (LUBILOSA, 1996).

3.8. Insect bioassay

For each new isolate 20 fifth instar *Schistocerca. gregaria* in duplicates were used in the assay. Each insect was kept in individual plastic beakers (400ml capacity) covered with fine mosquito net. The insects were inoculated with a dose of 2µl of 5 x 10^7 conidia/ml (LUBILOSA, 1996) under the pronotum using a micropipette. Twenty control insects in duplicates were also treated with 2 µl of blank oil. For comparison, *Metarhizium anisopliae* isolate ICIPE 30 supplied by International Center for Insect Physiology and Ecology (ICIPE), Nairobi was formulated in peanut oil similar to the native isolates and used in the assay.

Treated insects were kept in a room at 28°C and 39% r. h. This was possible by raising the room temperature with a heater. The insects were fed and cages cleaned daily as described in section 3.1.

Insect mortality was recorded daily for 21 days beginning from the second day following treatment. All cadavers in all treatments were removed daily and kept at -20°C in the refrigerator until used (see section 3.9.).

Further bioassays were conducted on two native isolates that showed better pathogenecity and compared with *M. anisopliae* ICIPE 30. Two oil formulations of peanut (*Arachis hypogaea*) and noug (*Guizotia abyssinica*) were used in the assay.

Serial dilutions of each oil formulation were made to obtain four different conidial suspensions ($1x10^5$, $1x10^6$, $1x10^7$, $1x10^8$). Twenty insects per replicate were used for each treatment.

The response of different instars of *S. gregaria* to the most potent native isolate was examined by applying a uniform 2 µl of $1x10^8$ conidia/ml formulated in peanuts oil. For each stage twenty insects in duplicates were used and controls of each instar were treated with blank peanuts oil.

3.9. Examination of sporulating cadavers

Confirmation of fungal caused mortality was made by allowing cadavers to sporulate. Dead insects following inoculation were surface-sterilized by dipping in 70% ethanol for three seconds and 5% sodium hypochlorite for two minutes (Odindo, 1994). Then, the insects were rinsed in sterile water and transferred onto water agar medium. Following incubation at 28°C in the dark for seven days, cadavers were checked for external sporulation. Spores recovered from dead locusts were also used to maintain isolate virulence following every third passage of the culture.

3.10. Characterization

MEA cultures of 15 days old were used to study the distinctive morphological features of the isolates. Identification of the isolates was done according to Domsch *et al.* (1980). Conidial size was measured under light microscope fitted with a micrometer.

3.11. Growth optimization

3.11.1. Growth rate

Three media, oatmeal agar (OA), malt extract agar (MEA) and sabouraud dextrose agar (SDA) were compared for their effect on the growth rates of the fungal isolates. Circular agar

discs, 9 mm in diameter were transferred from 15 days old SDA cultures to the media. The cultures were incubated for ten days at 28°C in the dark and colony diameter was measured on the tenth day.

3.11.2. Spore yield

The spores from each culture media (section 3.11.1) after ten days of incubation, were harvested by flooding 10 ml of sterile water onto the plates. The plates were then scraped carefully using a spatula to recover the spores. The spore suspension was then filtered through cotton and diluted (1:10) in sterile water. The suspension was vortexed for six minutes to avoid clamping of the spores. Then, the spore density was determined using haemocytometer.

3.12. Germination test

For development of most entomopathogenic fungi, temperature should be optimized. The aim of the study was to determine the germination rates of the isolates in different temperature ranges.

3.12.1. Effect of temperature on spore germination

The germination behavior of two potent isolates was studied at different temperature ranges. A spore suspension was prepared with sterile water adjusting the number of spores to be about 5×10^5 spores/ml using haemocytometer. Two agar discs of about 2 mm thick were placed on sterile glass slide, replicating twice. Then drop of water containing suspended spores of the respective isolate was placed on each agar disc. The slides were incubated in the dark at 4, 24, 28, 37 and 45°C and the percent germination was determined after 24 hours. Germination

was considered when a germ tube equal to the minimum diameter of the spore was achieved.

3.12.2. Effect of oil on spore germination

The effect of peanuts and noug oil on spore germination was studied using conidial concentration of 5 x 10^5 spores/ml. The slide cultures prepared were incubated at 28°C in the dark for 24 hours. The germinating spores were counted and percent germination in the respective oil was computed and compared.

3.13. Statistical analysis

The median lethal time was estimated using the MELTIMOR program. For the analysis, cumulative percentage mortality data was subjected to one-way analysis of variance using SPSS program. Two-way analysis of variance was used to see the interaction between dose and efficacy of the isolates. Duncan's multiple range test was used to separate the means. Finney's probit analysis program was used to estimate the LC_{50} values of the different concentrations.

4. RESULTS

4.1. Collection of pathogens

During the survey, we failed to obtain dead Orthopteran insects. Nearly all the fungal isolates found belong to the order Coleoptera and were discovered as infected cadavers (Table 1).

Table 1. Fungal isolates tested against *S. gregaria*, their origin and Order of host insects.

Local code	IITA Code	Genus	Location	Stage of insect	Host insect Order	Appearance of sporulation	Altitude m a.s.l.	Crop
AA	21	*Paecilomyces*	Korem	Adult	Coleoptera	White muscardine	2450	Grass
BB	19	*Paecilomyces*	Gusquam	"	C. Arachinida	"	2050	"
CC	22	*Beauveria*	Woldiya	"	Coleoptera	"	1950	Millet
DD	23	*Beauveria*	Debremarkos	"	"	"	2030	Tef
EE	20	*Metarhizium*	Alamata	"	?Crustacean	Green muscardine	1500	Grass
FF	18	*Beauveria*	Ashengie	"	Coleoptera	White muscardine	2400	Wheat
GG	17	*Beauveria*	"	"	"	"	2400	"
HH	16	*Beauveria*	"	"	"	"	2450	"
II		?	Woldiya	"	"	"	1950	Millet
AK		?	Tikurinchini	Larvae	*M. melolontha*	"	2225	Barley

4.2. Isolation

Isolation of fungi from insect cadavers was carried out as described in section 3.3.1. Ten fungal isolates were recovered from insects as shown in Table 1. All the spores recovered from the cadavers were viable, and a pure culture of each isolate was prepared.

An attempt to isolate *Metarhizium* and *Beauveria* spp. from the soil using selective media (Veen and Ferron, 1966) was not successful. Several types of molds grew even in the presence of cyclohexamide, which might have affected the growth of slow developing entomogenous fungi.

4.3. Storage of pathogens

Strains of entomopathogenic fungi were kept on agar slants (Fig. 3) for the bioassay study.

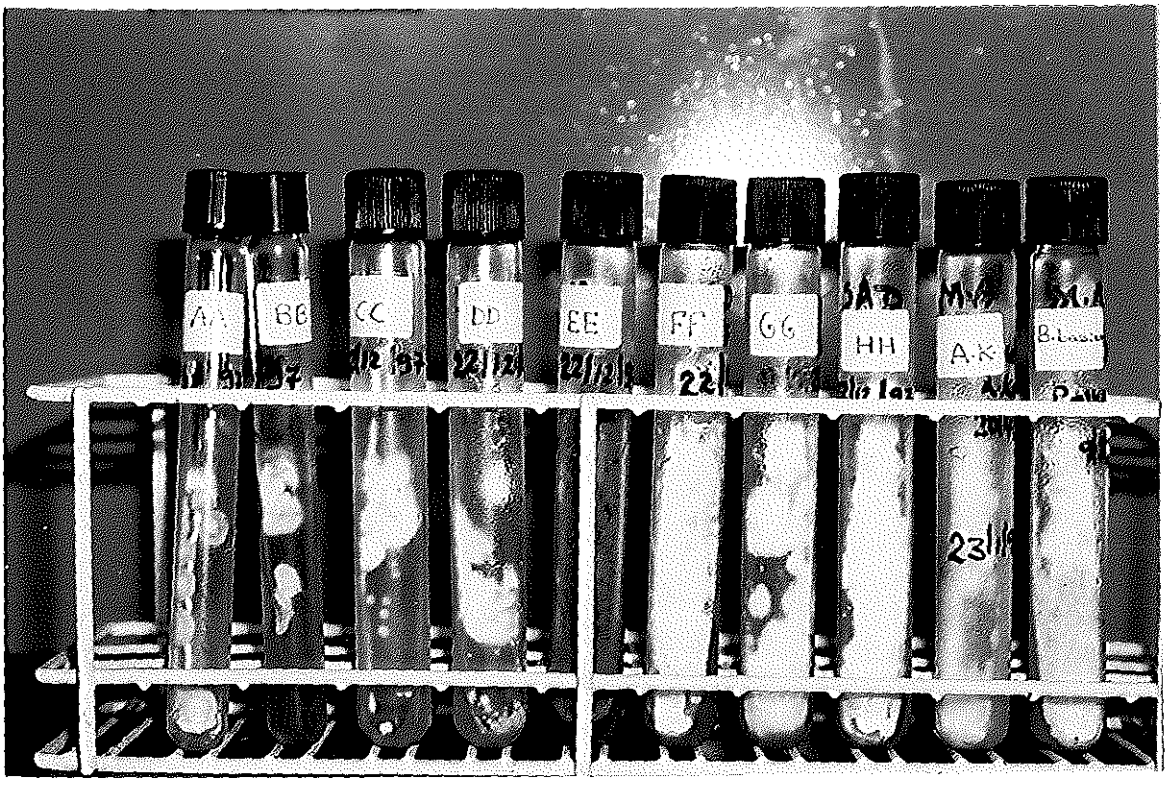


Fig. 3. Slants of pure culture of each isolate.

4.4. Insect bioassay

Among ten isolates indicated in Table 1, eight were selected on the basis of spore production. All the isolates tested showed pathogenecity of different degree to fifth instar *Schistocerca gregaria.* Percentage cumulative mortality and the median lethal time (Bateman, 1994) for each isolate against fifth instar *S. gregaria* are presented in Table 2. The results showed that under the conditions of the present work there were highly ($P<0.001$) significant differences between the isolates with respect to mortality. Depending on the preliminary bioassay, the isolates could be categorized as low, intermediate, moderately high and highly virulent.

Isolates FF (*Beauveria* spp.), EE (*Metarhizium* spp.) and ICIPE30 (*M. anisopliae*) caused high mortality (90-100%) in 11 days after inoculation. FF treated insects began to die three days after inoculation and took 6.71 and 11 days to achieve 50% and 100% cumulative mortality, respectively. The *Metarhizium* isolates EE and ICIPE 30, each resulted in 90% cumulative mortality within 11 days after inoculation. In the preliminary screening, however, ICIPE 30 was faster in kill than isolate EE with a median lethal time of 6.67 days. Isolate EE took eight days to cause 50% mortality. Death of treated insects with ICIPE 30 and EE started on the fifth and sixth days after inoculation, respectively (Fig. 4).

The *Beauveria* spp. isolate GG, A.K and HH showed moderately high virulence resulting in a cumulative mortality of 83.3%, 80% and 72.2%, and median lethal time of 8.60, 8.00 and 8.40 days, respectively (Table 2)

Isolates AA, *Paecilomyces* spp., and CC, *Beauveria* spp., appeared to have intermediate virulence with cumulative mortality of 55.6% and 33.3%, respectively at 11 days after inoculation. Mortality caused by the two isolates raised gradually resulting 75% and 80% by

day 21 (Fig. 4).

The *Beauveria* isolate DD may be considered as a weak pathogen on the ground that the corrected cumulative mortality recorded at day 11, 15 and 21 were 0%, 22.2% and 47.1%, respectively (Fig. 4).

The time by which the treated insects started dying of mycosis varied considerably. The earliest death recorded was on day three for the *Beauveria* isolate FF at $5x10^7$ conidia/ml and *Metarhizium* isolates EE and ICIPE 30 at $1x10^8$ conidia/ml (Figs. 4, 5 and 9). With less virulent isolates, the first death was recorded on the ninth day by which time, however, 50% mortality was recorded with six of the isolates (Table 2).

Cumulative mortality increased for all isolates as the number of observation days increased (Fig. 4). Death increased more rapidly with more virulent isolates, while it was slower for less virulent ones as expected.

Controls treated with blank peanut oil showed low (15%) mortality throughout the experiment. However, in few instances, failure to successfully molt was noted.

The main objective of the first phase of the bioassays was to screen isolates on the basis of speed of kill for further studies. Accordingly, isolates EE (*Metarhizium* spp.) and FF (*Beauveria* spp.), were finally selected and compared with ICIPE 30 (*M. anisopliae*). The efficacy of isolates EE and FF was first investigated in peanut and noug oil formulations against fifth instar *S. gregaria*.

Peanut oil based formulation of EE caused 36.8-94.7% mortality at all concentrations tested in

eight days after application (Table 3). The estimated time in days to cause 50% mortality with the various concentrations are indicated in Table 4 and the cumulative daily mortality data in Fig. 5. The median lethal time for the highest ($1x10^8$ conidia/ml) and lowest ($1x10^5$ conidia/ml) concentrations ranged between 5 and 8.83 days, respectively.

The noug oil based formulation appeared to reduce efficacy of EE at all doses ($P<0.05$) (Table 3). Particularly at lower doses ($1x10^5$ and 10^6 conidia/ml) 50% mortality was not achieved even until 17 days after inoculation (Table 4). The cumulative mortality at eight days after inoculation ranged 5-60% for all doses examined. As the duration increased, however, the cumulative mortality gradually raised. At high dose ($1x10^8$ conidia/ml), 100% mortality was achieved in 14 days after inoculation and took 7.33 days of median lethal time (Fig. 6 and Table 4). Control mortality was high only after 14 days of inoculation.

There was a significant ($P<0.001$) difference between various treatments of the *Beauveria* isolate FF, formulated in peanut oil, and low mortality of the controls. FF in peanut oil took a median lethal time of 4.2 days (Table 4) and caused 100% mortality within seven days at a dose of $1x10^8$ conidia/ml (Fig. 7). The lowest dose ($1x10^5$ conidia/ml) took 8.63 days to cause 50% kill. Concentrations above $1x10^5$ conidia/ml were found highly effective causing 100% morality in 13 days after inoculation (Fig. 7).

There was no significant difference in the efficacy of noug and peanut oil formulation of FF ($P<0.05$) (Table 3). The highest dose ($1x10^8$ conidia/ml) caused 100% mortality in nine days (Fig. 8) after inoculations and the median lethal time taken was five days (Table 4). As can be shown in Fig. 8, there was a distinct dose-response pattern. However, treatment of noug oil without conidia also resulted in higher mortalities toward the end of the experiment. The median lethal time for the doses $1x10^5$, 10^6 and 10^7 ranged between 8.75 to 7.5 days.

The peanut oil formulation of *M. anisopliae* ICIPE 30 tested at doses of $1x10^5$ to 10^8 conidia/ml gave 42.1 to 94.7% mortality within eight days after inoculation (Table 3). Strong dose-response patterns were evident as shown in Fig. 9. At $1x10^8$ conidia/ml, 5.5 days were the median lethal time and nine days to achieve 100% mortality. Mortality of locusts treated with ICIPE 30 was significantly greater ($P<0.001$) than the controls during the whole experimental days.

The resulting LC_{50} value of each isolate is shown with acceptable x^2 values ($P<0.05$) in Table 5. Among the tested isolates, FF in peanut oil formulation had the lowest LC_{50} values ($2.02x10^5$ conidia/ml). The *Metarhizium* isolates EE and ICIPE 30 in peanut oil and FF, *Beauveria* spp., in noug oil formulation showed less virulence to *S. gregaria* as compared to peanut oil formulation of FF. EE in noug oil formulation showed significantly ($P<0.05$) lower virulence yielding the LC_{50} of $3.66x10^7$ conidia/ml (Table 5).

Among the different stages of *S. gregaria* tested with $1x10^8$ conidia/ml, the fourth instars were highly susceptible to infection by the indigenous isolate FF. Locust mortality was 100% against the fourth instars by day five, and seven days for fifth instars while it took nine days for the adults (Fig. 10). Control mortality of fourth instars during the first four days was about 25%. By day five, however, mortality resulting from treatment with oil alone was reduced. In all the treatments, there was a significant ($P<0.001$) mortality in treated insects than the controls. The median lethal time taken to fourth, fifth and adults treated *S. gregaria* were 3.47, 4.14 and 4.83 days, respectively (Table 6).

Table 2. Mean corrected percentage cumulative mortality by day 11, of fifth instar *S. gregaria* treated with various local fungal isolates in the laboratory at a dose of $5x10^7$ conidia/ml in peanut oil.

Isolate tested		% mortality (corrected)	Median lethal time ± s.d.
Beauveria spp.	FF	100^{a}	6.71±2.32
M. anisopliae	ICIPE 30	$90\ (89.5)^{ab}$	6.67±3.44
Metarhizium spp.	EE	90^{ab}	8.00±2.37
Beauveria spp.	GG	$85\ (83.3)^{bc}$	8.60±3.46
.	A.K.	80^{bc}	8.00±4.04
Beauveria spp.	HH	$75\ (72.2)^{bc}$	8.40±3.04
Paecilomyces spp.	AA	$60\ (55.6)^{d}$	9.00±6.36
Beauveria spp.	CC	$40\ (33.3)^{e}$	13.5±4.77
Beauveria spp.	DD	$10\ (0)^{f}$	*****

Values followed by the same letter are not significantly different using Duncan's multiple range test [$P<0.001$].

Table 3. Mean percentage cumulative mortality by day eight of fifth instar *Schistocerca gregaria* verses conidial concentration.

Isolate	Cumulative (%) mortality (corrected)			
	10^5	10^6	10^7	10^8
FF, peanut oil	$40\ (36.8)^{fg}$	$75\ (73.7)^{cd}$	$80\ (78.9)^{b}$	100^{a}
FF, noug oil	35^{g}	60^{e}	60^{e}	95^{a}
EE, peanut oil	$40\ (36.8)^{fg}$	$60\ (57.9)^{e}$	$85\ (84.2)^{bc}$	$95\ (94.7)^{a}$
EE, noug oil	5^{h}	10^{h}	40^{fg}	60^{e}
ICIPE30 peanut oil	$45\ (42.1)^{f}$	$45\ (42.1)^{f}$	$70\ (68.4)^{d}$	$95\ (94.7)^{a}$

Means associated with the same letter are not significantly different (Duncan's multiple range test {$P<0.05$}).

Table 4. $LT_{50's}$ of three isolates against *S. gregaria* at levels of concentration from 10^5 to 10^8 conidia/ml

Isolate	LT_{50} ± s.d.			
	10^5	10^6	10^7	10^8
FF, peanut oil	8.63±5.91	7.17±2.18	7.00±1.75	4.2±1.22
FF, noug oil	8.75±3.73	7.6±1.49	7.5±3.01	5.0±1.17
EE, peanut oil	8.83±3.77	7.39±4.08	7.23±1.96	5.00±1.79
EE, noug oil	-	-	8.33±2.89	7.33±2.46
ICIPE30, peanut	9.5±4.47	8.75±2.14	7.21±2.79	5.5±1.72

Table 5. Probit analysis of mortality data of EE (*Metarhizium* spp.), FF (*Beauveria* spp.) and ICIPE 30 (*M. anisopliae*).

Isolate	Upper	LC_{50}	Lower	X^{2a}	Relative potency[b]
FF, peanut oil	$6.39x10^5$	$2.02x10^5$	$2.08x10^4$	1.7289	246.5
EE, peanut oil	$9.39x10^5$	$2.93x10^5$	$3.32x10^4$	0.1513	169.9
ICIPE30, peanut oil	$1.84x10^6$	$4.98x10^5$	$4.28x10^4$	2.7805	100
FF, noug oil	$2.20x10^6$	$6.13x10^5$	$6.52x10^4$	3.0460	81.24
EE, noug oil	$2.85x10^8$	$3.66x10^7$	$1.20x10^7$	0.6280	1.36

Analysis was based on data taken eight days after inoculation

[a] $P<0.05$, $df=2$

[b] Relative potency calculated using the following formula (Moorehouse *et al.*, 1988):

$$\text{Activity of tested isolate} = \frac{LC_{50} \text{ of standard ICIPE30 (conidia/ml)}}{LC_{50} \text{ of tested isolate}} \times 100$$

Table 6. The LT_{50} values of FF (*Beauveria* spp.) formulated in peanut oil at a dose of $1x10^8$ conidia/ml to the different stages of *S. gregaria.*

Isolate	LT_{50} ± s.d.		
	4^{th} instar	5^{th} instar	Adult
FF	3.47±0.83 days	4.14±1.27 days	4.83±1.72 days

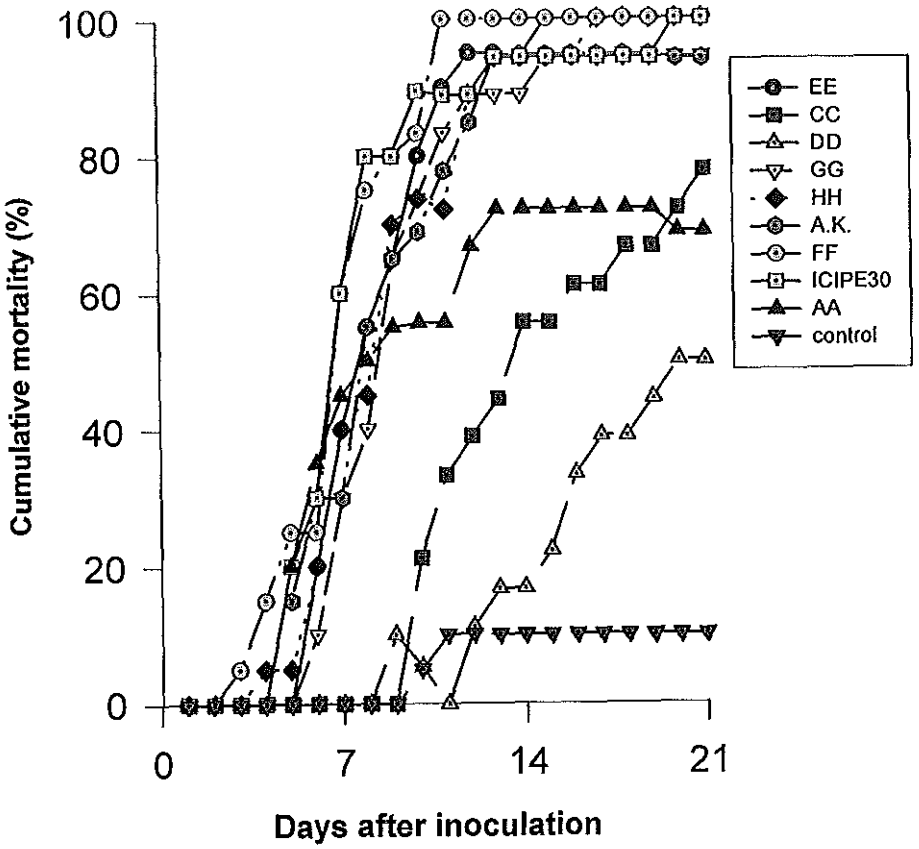


Fig. 4. Cumulative mortality of fifth instar *S. gregaria* treated with nine fungal isolates at a dose of $5x10^7$ conidia/ml in peanut oil formulation.

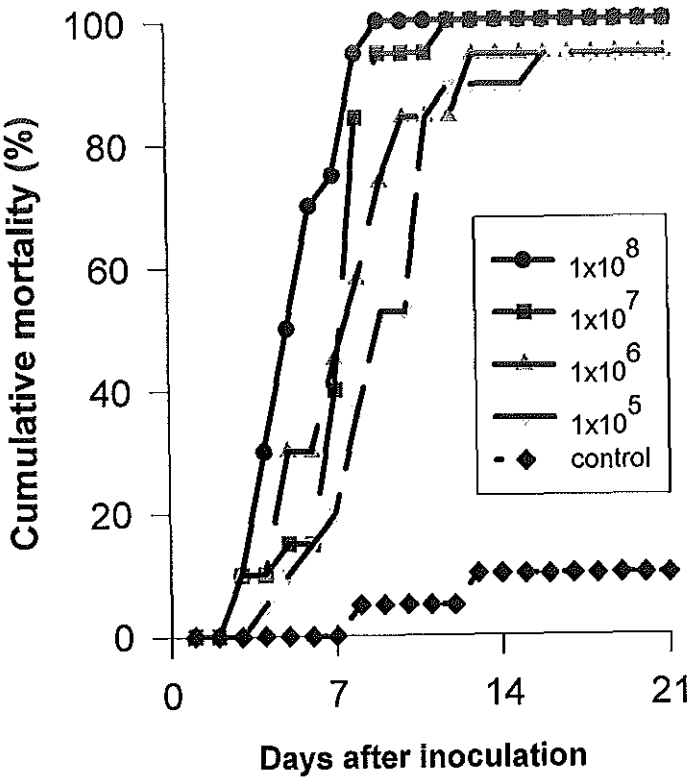


Fig. 5. Cumulative mortality of fifth instar *S. gregaria* after treatment with *Metarhizium* isolate EE formulated in peanut oil.

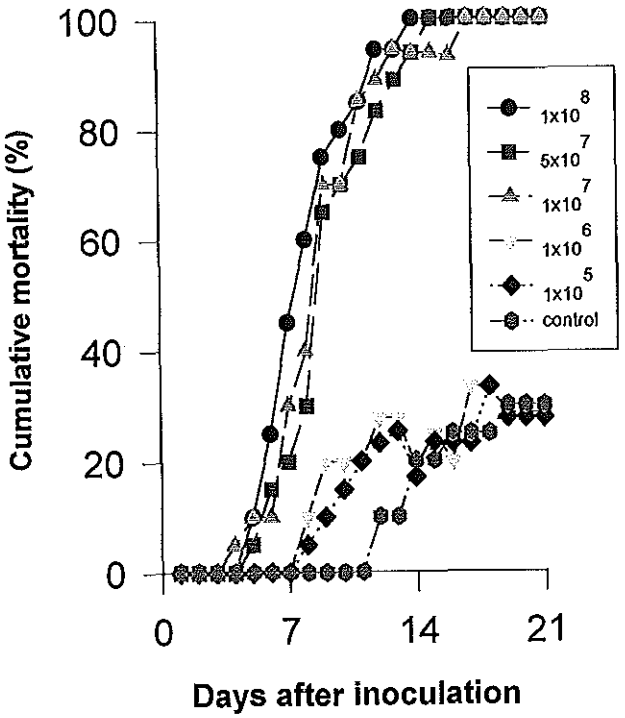


Fig. 6. Cumulative mortality of fifth instar *S. gregaria* treated with *Metarhizium* isolate EE formulated in noug oil

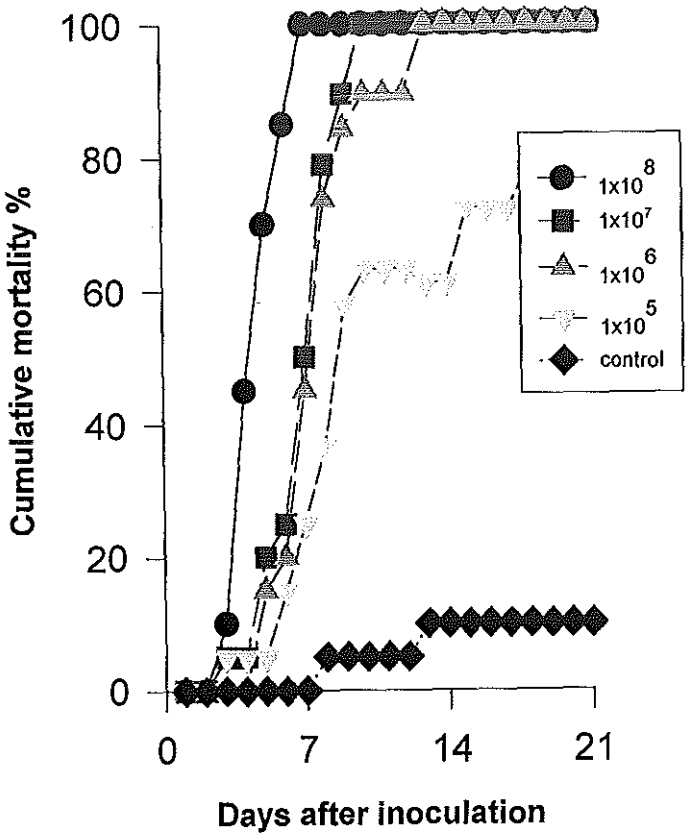


Fig. 7. Cumulative mortality of fifth instar *S. gregaria* after treatment with *Beauveria* isolate FF formulated in peanut oil.

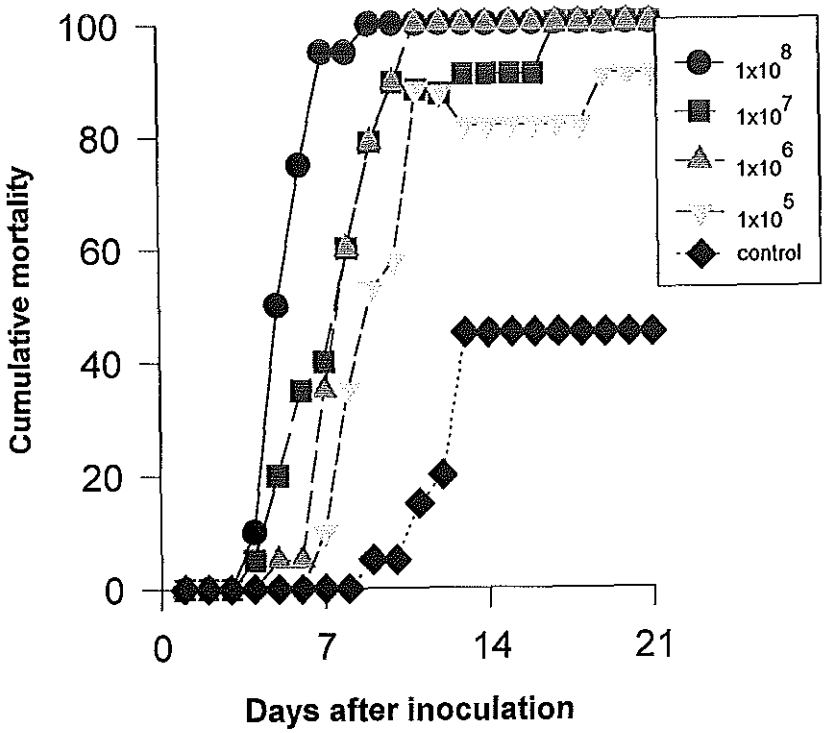


Fig. 8. Cumulative mortality of fifth instar *S. gregaria* after treatment with *Beauveria* isolate FF formulated in noug oil

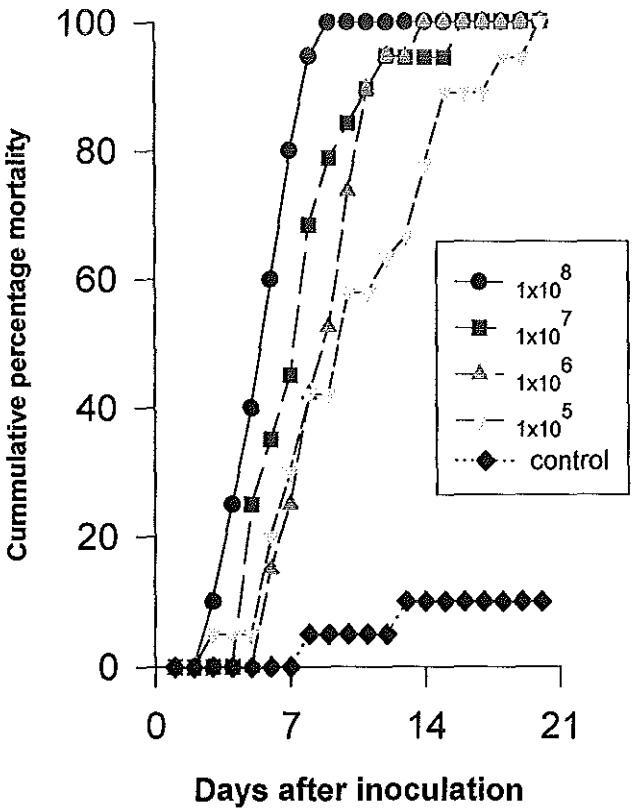


Fig. 9. Cumulative mortality of fifth instar *S. gregaria* after treatment with *M. anisopliae* ICIPE30 in peanut oil formulation.

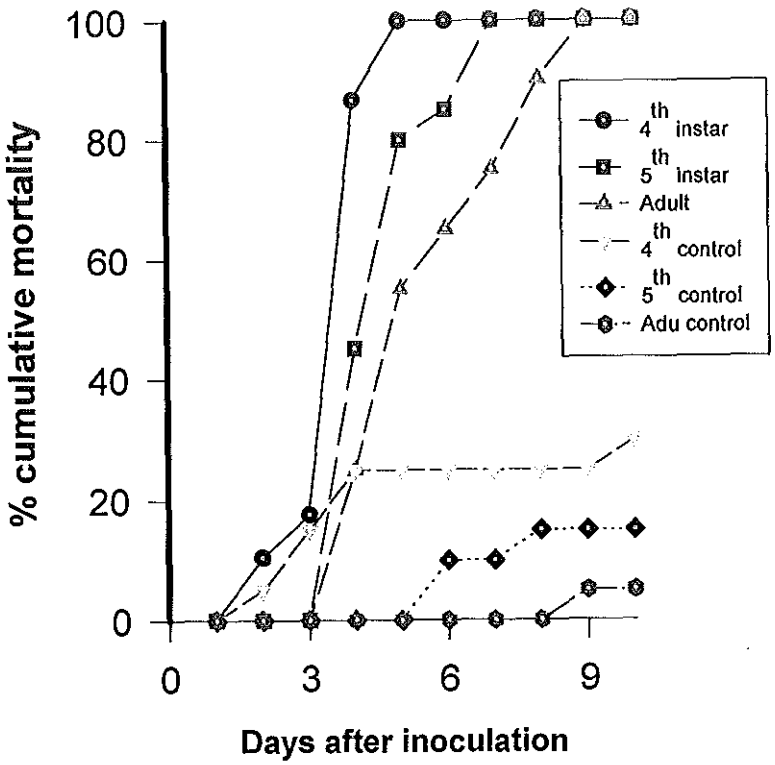


Fig. 10. Cumulative mortality of different stages of *S. gregaria* after treatment with *Beauveria* isolate FF ($1x10^8$ conidia/ml) in peanut oil diluents.

4.5. Examination of sporulating cadavers

Dead insects were allowed to sporulate as described in section 3.9. Insects died as the result of infection by the *Beauveria* isolate FF produced white powdery mass of spores on the external surface (Fig. 11). Infected insects also developed red pigmentation upon death (Fig. 12). The *Metarhizium* isolates EE and ICIPE 30 formed green crust-like velvet on the walls of the cuticle (Fig. 13 and 14).

4.6. Identification and characterization

The fungal samples were identified and coded at International Institute of Tropical Agriculture (IITA).

Characterization was made for the *Beauveria* isolate FF and *Metarhizium* EE. Conidia of EE are cylindrical in shape and have a size (l x w) of 6.57-7.89 x 2.63-2.89 µm. Colonies on OA showed white mycelial margins with clumps of conidiophores that became colored in the course of spore development, varying from green to olivacious green. The spores were released in chains (Fig. 15a). Conidia of FF are elliptical in shape, 2.63-5.26 µm long and 2.63-2.89 µm in width. Colonies on OA were generally white, at the edge becoming creamy. Conidiophores of FF arose from vegetative hyphae, bearing groups of clustered conidiogenous cells, which are globular in shape with a well-developed rachis (stalk) (Fig. 15b).

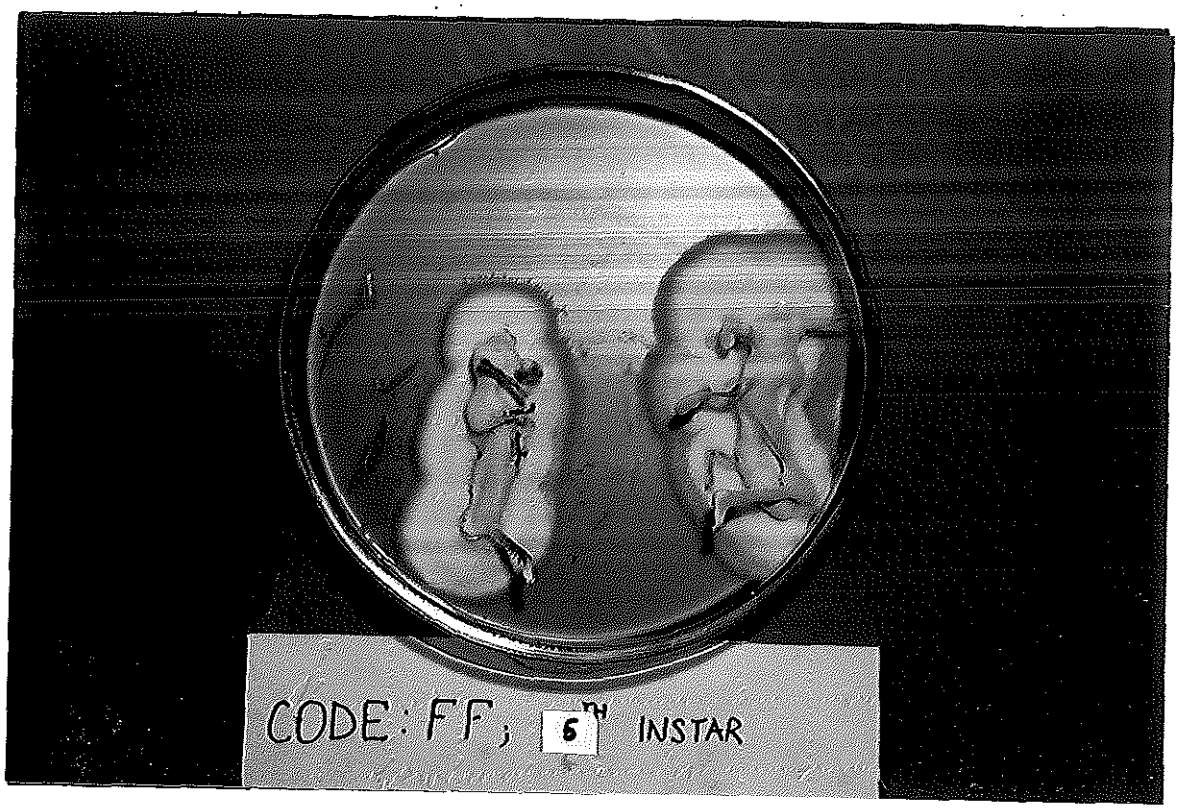


Fig. 11. White mass of spores on the surface of fifth instar *S. gregaria* killed by isolate FF.

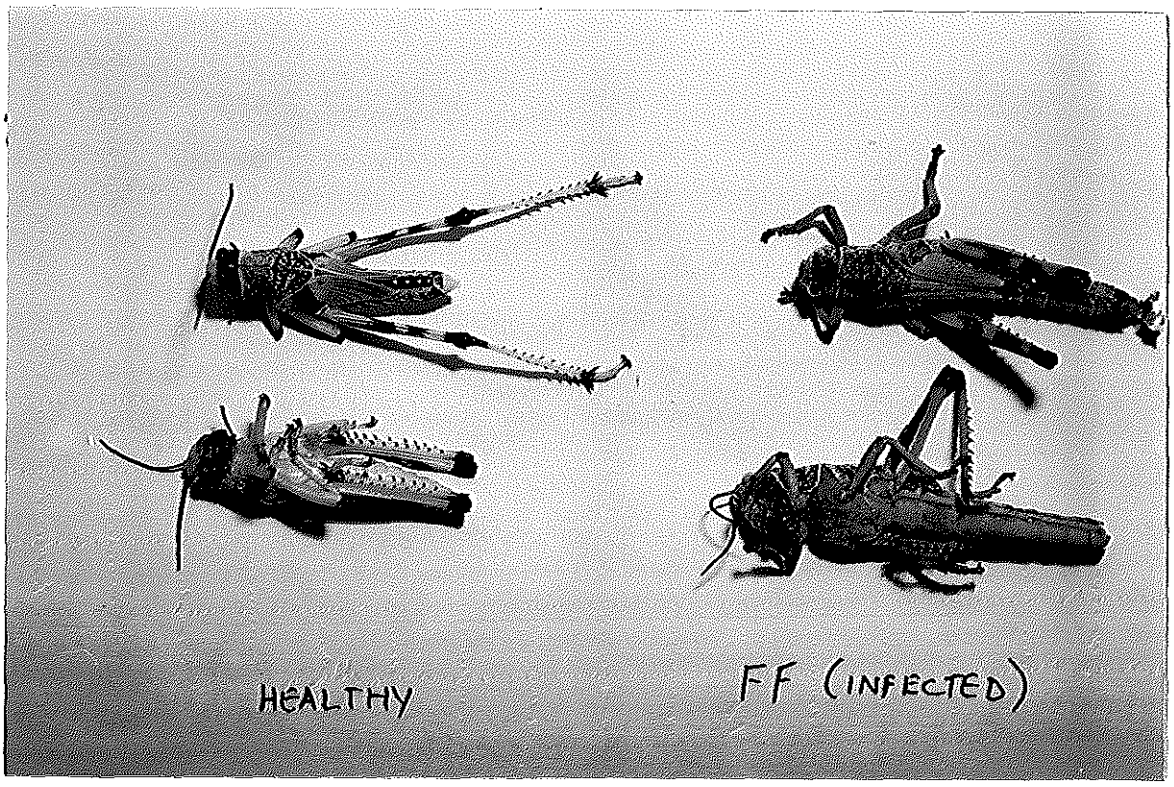


Fig. 12. Fifth instar *S. gregaria* killed by isolate FF. Infected insects develop red pigmentation.

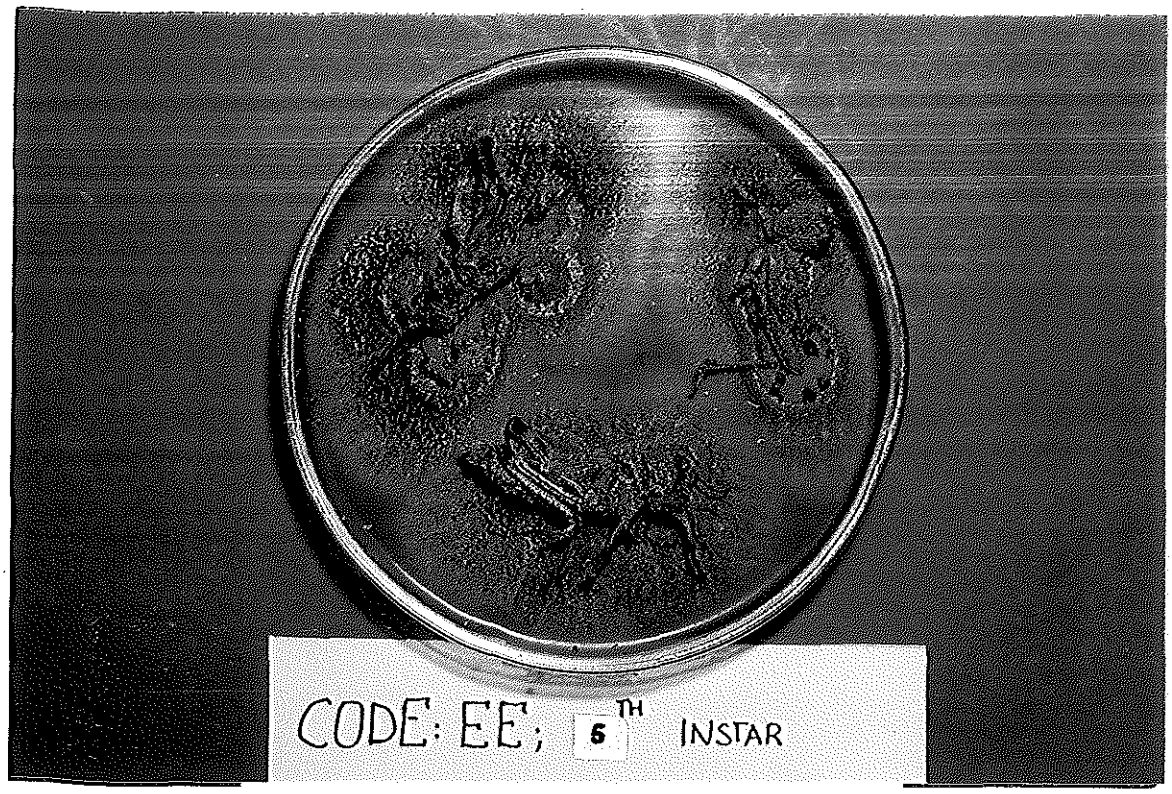


Fig. 13. Fifth instar *S. gregaria* killed by isolate EE.

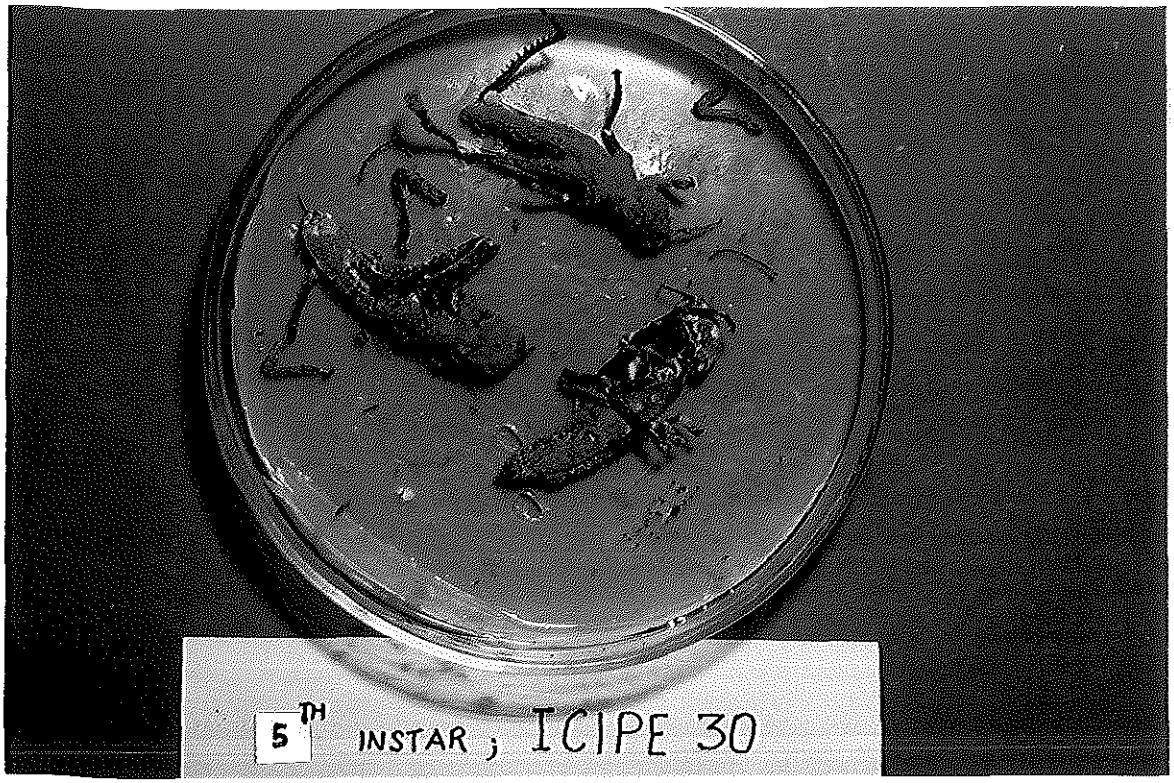
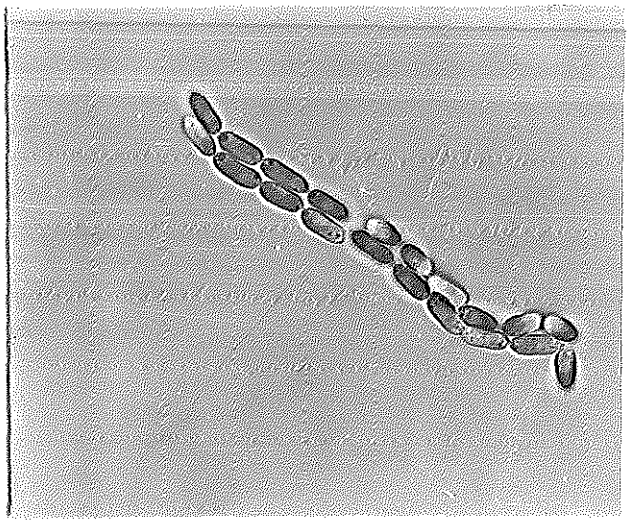
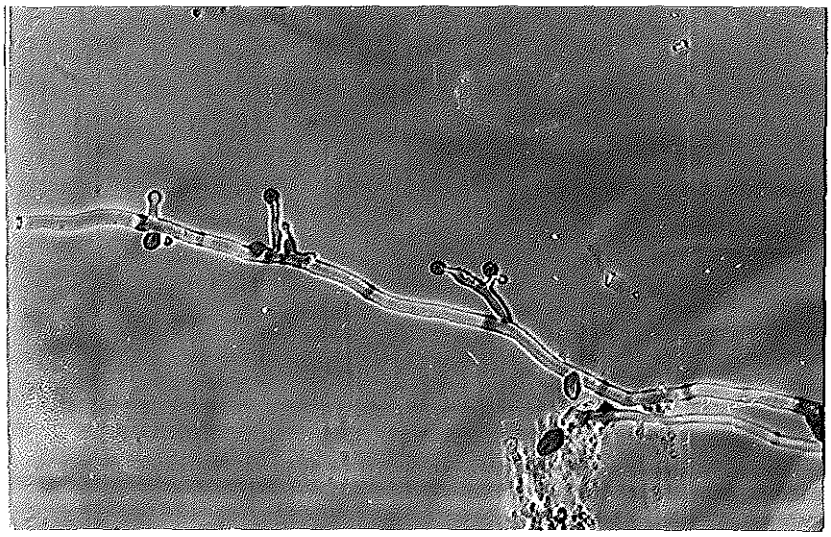


Fig. 14. Fifth instar *S. gregaria* killed by ICIPE 30



a)



b)

Fig. 15. a) Conidia of the *Metarhizium* isolate EE. b) Conidiogenous cells of the *Beauveria* isolate FF.

4.7. Effect of media on growth and sporulation

Variation in colony growth was noted among the isolates over ten days of incubation period (Fig. 16). Colonies of EE and FF showed similar colony growth rates on oatmeal agar. On malt extract agar, EE grew at a faster rate with a diameter of 3.2 cm as compared with that of FF (2.35 cm). The spore yields, however, were comparable (Fig. 17). Sabouraud dextrose agar appeared to favor growth and sporulation of EE.

4.8. Effect of temperature on spore germination

Isolate EE (*Metarhizium* spp.) and isolate FF (*Beauveria* spp.) germinated at temperature ranges of 24°C-37°C. Both isolates failed to germinate at 4°C and 45°C (Fig. 18). The peaks in germination of FF occurred at 24°C (90.8%) and 28°C (92%), which was significantly ($P<0.05$) higher than at 37°C (65.4%). Isolate EE attained peak germination at 28°C (40.3%) and was significantly ($P<0.05$) higher than at 24°C (27.9%) and 37°C (30.4%).

4.9. Effect of oil type on spore germination

Comparison of spore germination of EE and FF indicated that significantly ($P<0.05$) higher germination occurred in peanut oil than noug oil formulation. This was particularly evident with FF in which only 61.2% of the spores germinated in noug oil compared with 94.8% in peanuts oil (Fig. 19).

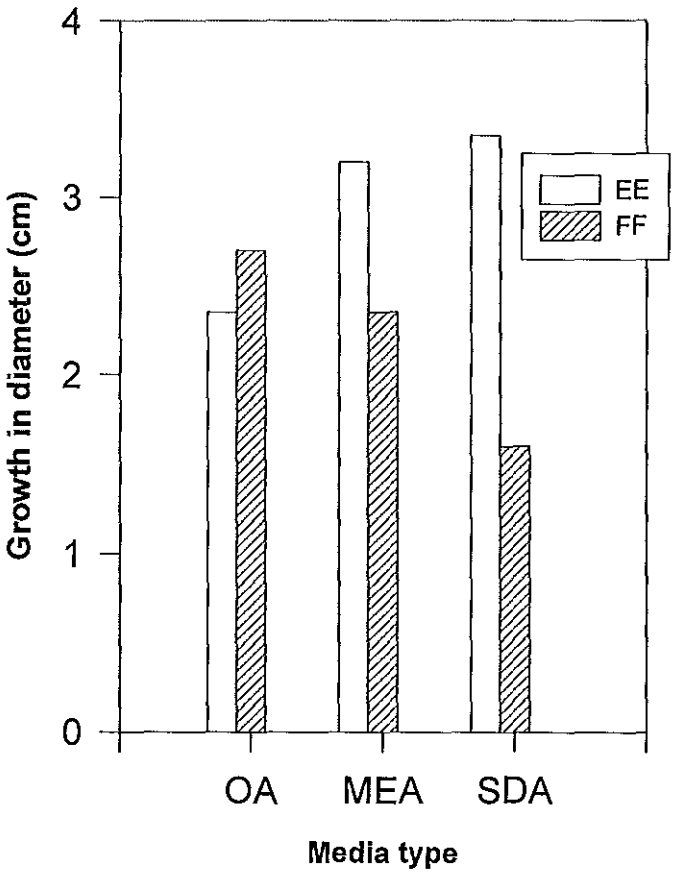


Fig. 16. Effect of medium on growth rate

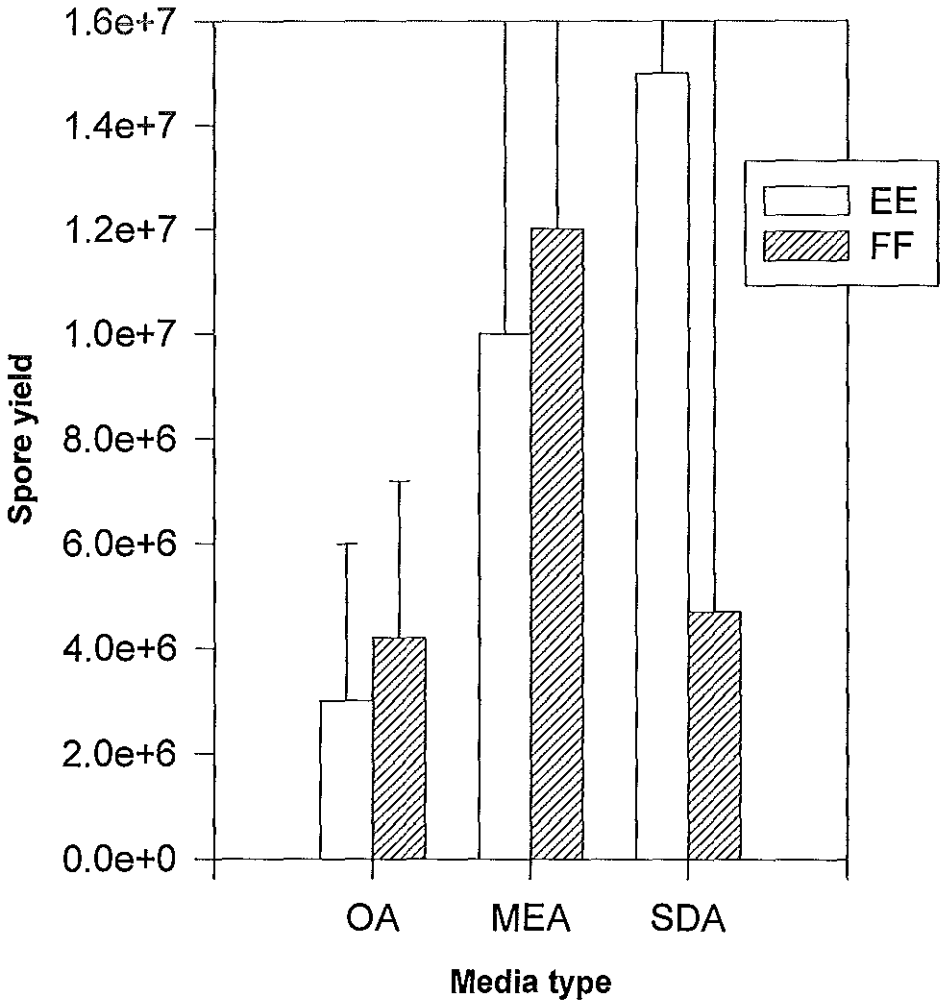


Fig. 17. Effect of media type on spore yield

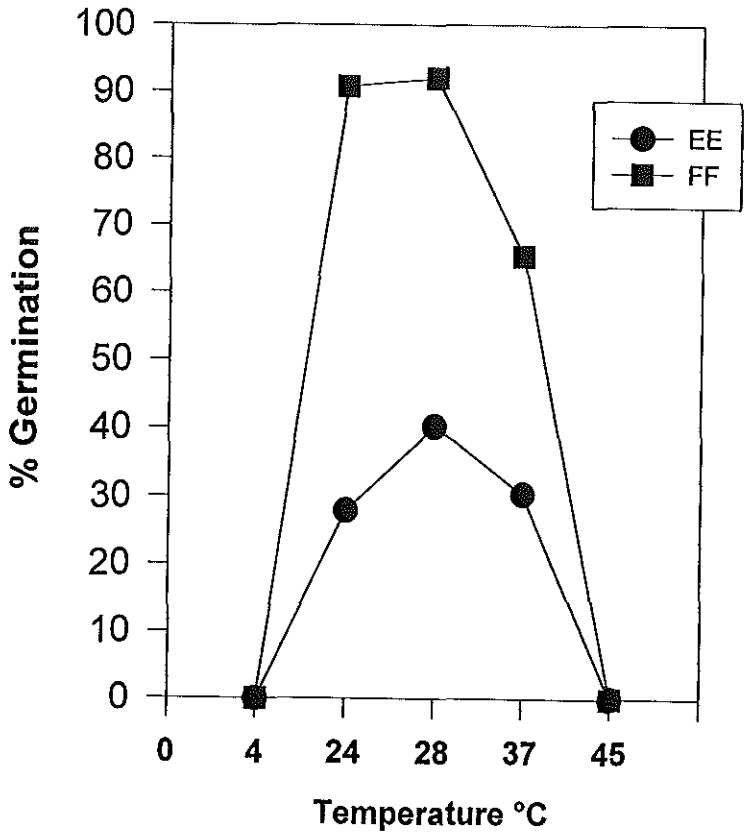


Fig. 18. Effect of temperature on spore germination

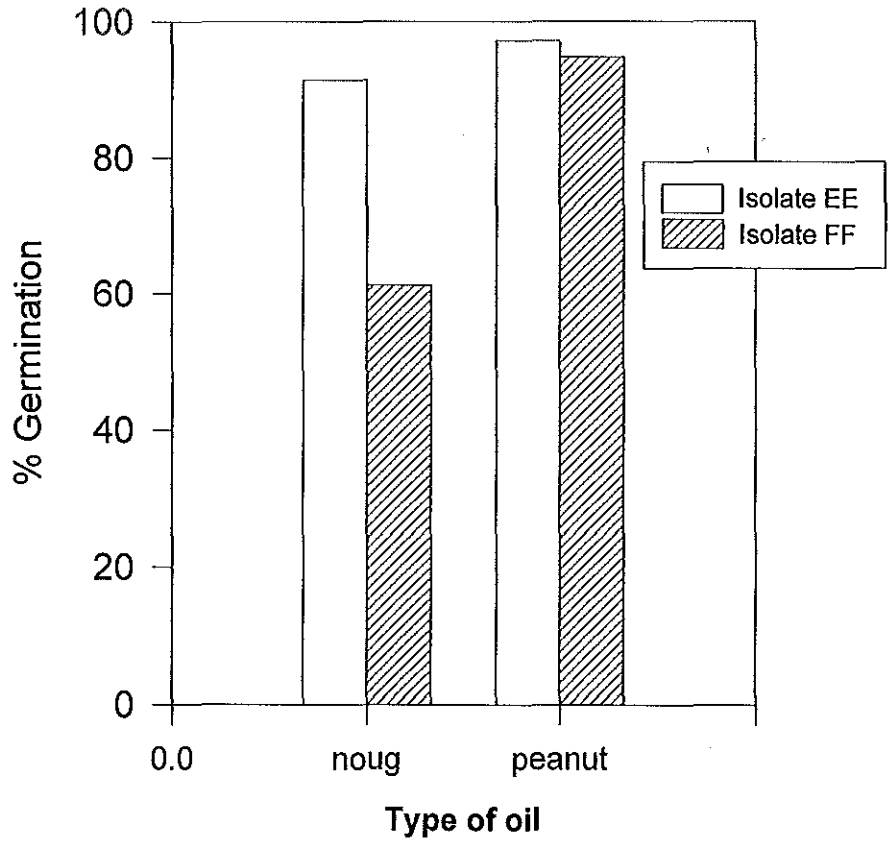


Fig. 19. Effect of oil type on spore germination

5. DISCUSSION

Establishment of efficient mass rearing system is an essential step to sufficiently supply insects for bioassay. Accordingly, a healthy colony of *Schistocerca gregaria* was established using locally produced cages as shown in Fig. 2. The different stages of the insects were maintained in separate cages and assays were conducted on the fifth instars. The fifth instars were selected during the assay considering their sufficiently large size as compared to the first four instars. This eases controlled droplet application under the pronotum. At this stage control mortality due to oil application is also low. Furthermore, the fifth instars are highly voracious and can cause economic yield loss and develop into the adult stage, which is capable of flight. Therefore, this stage could be an appropriate target for application of mycopesticides. In addition, the fifth instars undergo molting to become adults. An advantage of this was taken to see if molting could affect the progress of infection.

A total of five *Beauveria,* two *Paecilomyces* and a *Metarhizium* spp. isolates have been collected and identified through the present work for the first time in this country as opposed to many collections and worldwide records. From practical perspective also, the development of mycopesticides with indigenous fungi is encouraged for it eases the process of quarantine restrictions. Moreover, indigenous entomopathogenic fungi are better adapted to survive in the natural environment where control must be achieved, and hence are suitable for inundative augmentation (Prior and Street, 1997).

Observation in this work showed that entomopathogenic fungi belonging to the genera *Paecilomyces*, *Metarhizium* and *Beauveria* occur naturally in Ethiopia. However, their contribution to the control of the desert locust and other insect pests under natural conditions is not well studied yet.

Fungal isolates usually show variation in terms of virulence, spore production and other characters. Therefore, possible optimum growth conditions of the potent isolates were studied following the efficacy screening.

From the collected ten native fungal isolates, two, namely, BB and II, showed weak sporulation and hence their efficacy was not evaluated. All native isolates tested showed pathogenecity of different magnitude to *S. gregaria*. A preliminary screening at a dose of $5x10^7$ conidia/ml revealed a continuous gradation of virulence ranging between 100% mortality within 11 days to no mycosis within the period.

The Coleopteran-origin isolate FF in peanut oil suspension was the most virulent with LC_{50} value of $2.02x10^5$ conidia/ml, which is equivalent to 406 conidia/insect. This study was also in accord with that of Prior's (1992) argument that virulent isolates may originate from a taxonomic group unrelated to the target pest. This suggests that either the host of origin or the phylogenetic relationship between potential hosts may not be a reliable indicator of the probable virulence of a specific fungal isolate to a specific host (Feng and Johnson, 1990).

Following the preliminary screening, two native isolates with better performance were further assayed and compared with a known entomopathogen, *M. anisopliae* ICIPE 30. Comparison of virulence showed that the two native isolates, EE and FF, have superior performance over ICIPE 30 (Table 5). A virulent fungus is considered to have evolved biochemical, morphological, and tropic mechanisms to attach and adhere to cuticle; solubilize, penetrate, and utilize the cuticle; and overcome the resistance mechanisms in the haemocoel (Bidochka *et al.*, 1997). Therefore, further studies are required to determine which of the factors for virulence attribute to the high efficacy of FF and EE compared to the ICIPE 30.

It is believed that the estimates of time to kill by each fungal isolate in the present work could serve as a starting point for further investigations. The time to kill by a fungal pathogen is proportional to the initial dose, and for virulent isolates even very low dose treatments eventually could cause 100% mortality. This is because fungi can multiply inside the host insect following penetration unlike chemical insecticides where the insect dies or recovers.

It was observed that few infected fifth instar locusts molted and died as adults. This indicated that once the fungi penetrate the locust's cuticle, molting has no effect on mycosis. Among the different life stages tested, fourth instars were highly susceptible to infection by FF than higher stages. The reason might be that at younger stages locusts have soft cuticle, less weight and weak resistance than the higher instars, all of which could enhance susceptibility to mycosis.

All *S. gregaria* killed by the fungal isolates became tough and rigid upon death. The *Beauveria* isolate FF commenced white sporulation gradually covering the insect body when cadavers were placed on water agar medium (Fig. 11). Isolates EE and ICIPE 30 both exhibited a characteristic green sporulation on cadavers (Figs. 13 and 14). Insects killed by FF also showed a characteristic red pigmentation on their cuticle (Fig. 12). This happened less frequently in isolates EE and ICIPE 30 than was in FF. Bidochka *et al.* (1997) have indicated that insect phenoloxidases are implicated in melanic patches on wounded acridid cuticle and melanization of *B. bassiana* within the haemocoel to impede penetration.

Comparison of oil formulation between peanut and noug oil indicated that conidia in peanut oil yielded significantly higher germination rate and better efficacy. Probit analysis of the mortality data indicated that peanut oil formulation of FF and EE resulted in lower LC_{50} and

LT_{50} values compared with noug oil (Tables 4 and 5). Controls treated with blank noug oil showed higher mortality from 12 days after treatment. Noug oil is known to contain a high amount (54-73%) of linoleic acid (Dagne and Jonsson, 1994) that may have lethal effect on insects.

The germination rate of FF was particularly retarded in noug oil diluents. Although no significant difference in cumulative mortality was found in peanut and noug oil formulations of FF, delays in mortality were noted (Table 4).

The germination rate and efficacy of EE in noug oil suspension were significantly lower compared to peanut oil formulation. About a five days delay was noted in noug oil to achieve similar mortality in peanut oil. The low efficacy recorded in noug oil might be due to its effect on virulence factors. The germ tubes, appressoria and penetration structures were formed in the presence of an oil film. Accordingly, even though the germination rate was sufficiently high, suspension in noug oil seemed to impede infection for a reason that was not clear.

The experiment was carried out at 28°C and about 39% r.h. which indicated that the tested isolates, when formulated in oil suspensions, could infect *S. gregaria* and cause mycoses in dry conditions. The result also supports the fact that infection post application is not dependent on high humidity (Marcandier and Khachatourians, 1987). Field trials in West Africa with the arid environmental condition (15-80% r.h.) showed that oil formulations applied at ULV rates caused about 90% mortality to *S. gregaria* in less than 10 days (Bateman, 1994).

The germination rates of isolates EE and FF showed that they are capable of germinating in a

wide range of temperatures (24-37°C). Germination rate of isolate EE in water diluent was lower than that in oil formulation. High germination (97.2%) attained in peanut oil formulation could be due to the lipophilic layer of the conidia that enhanced germination in oil than water.

The peak germination rate attained with EE was at 28°C (40.3%). Whereas both 24°C (90.8%) and 28°C (92%) provided non-significant peak germination rates for FF. Germination rate of FF at 37°C was unusually high (65%). Although, the virulence of the isolates was not examined, the germination potential at different temperatures may imply their possible application in the above temperature ranges. Moreover, the tested isolates may behave differently at various temperatures. Hence, it is suggested that further bioassays be carried out in different temperature conditions in the future.

The most virulent isolate FF had high sporulation and intermediate growth on OA and MEA. Isolate EE showed rapid growth and sporulation on SDA than did FF, which exhibited intermediate sporulation rate and colony growth. Therefore, MEA and SDA could be used to obtain high sporulation and colony growth of isolates FF and EE, respectively.

It was observed that the activity of infected *S. gregaria* decreased from approximately three days after inoculation. This included uncoordinated movement, and lack of response to external stimuli at times. Moribund insects which ceased feeding were also noted.

The bioassay results obtained in the laboratory were generated under controlled conditions, which might have made the insects more stressed or favored the process of mycosis. Therefore, the results may not be exactly reproducible under field situations where sub-optimal conditions for the growth of the pathogen and adverse weather conditions prevail.

Furthermore, although, interactions between insects and entomogenous fungi have been the subject of considerable investigation, little is known with any precision about the insect-pathogen interactions in the field.

Under field situations, the ability of a pathogen to spread from diseased insects is important. Saprophytic development of the fungi on the host is always essential for completion of the fungal life cycle (Moorehouse *et al.*, 1994). But, usually conidial production is limited by low r.h. that also affects the spread of fungal diseases in a population. Therefore, artificial application of the inoculum at larger scale through spraying could help occurrence of infection even under dry condition.

Overall, the result we obtained in the laboratory condition is satisfactory. However, further studies should be done to see if there are detrimental effects on non-target organisms, toxigenecity to non-target organisms and allergenicity. Moreover, the effect of combinations of other control approaches with fungi should be evaluated with the ultimate aim of developing an integrated pest management system (IPM).

This preliminary investigation showed that several potential entomogenous fungi are found in nature that have potentials in controlling locusts. Therefore, extensive surveys to collect entomogenous fungi and bioassays should be a continuing process to exploit their potential.

6. REFERENCES

Auld, B. A. (1992). Mass production, formulation and application of fungi as biocontrol agents. pp. 219-229. **In:** Lomer, C. J. and Prior, C., (eds), *Biological control of locusts and grasshoppers*. CAB International, UK. 394 pp.

Baker, G. L. and Capinera, J. L. (1997). Nematodes and nematomorphs as control agents of grasshoppers and locusts. pp. 157-211. **In**: Goettel, M. S. and Johnson, D. L. (eds), Microbial control of grasshoppers and locusts. *Memoirs of the Entomological Society of Canada,* **171**: 400 pp.

Baron, S. (1972). *The Desert Locust*. Eyre Methuen. London. Pp. 28-43.

Barrass, R. (1980). *The locust: a laboratory guide* (3rd edn), Heinman Educational Book Ltd. London. Pp. 1-9.

Bateman, R. P. (1992). Controlled droplet application of mycoinsecticides to locusts. pp. 249-254. **In:** Lomer, C. J. and Prior, C., (eds), *Biological control of locusts and grasshoppers*. CAB International, UK. 394 pp.

Bateman, R. P. (1994). Physical properties and atomization of ULV formulations of myco-insecticides. pp. 222-225. **In:** Proceedings of the IOBC/WPRS Meeting, Zurich, September 1993.

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

Bateman, R. P. (1997). Methods of application of microbial pesticide formulations for the control of grasshoppers and locusts. pp. 69-81. **In**: Goettel, M. S. and Johnson, D. L. (eds), Microbial control of grasshoppers and locusts. *Memoirs of the Entomological Society of Canada,* **171:** 400 pp.

Bidochka, M. J., Gillespie, J. P. and Khachatourians, G. G. (1989). Phenoloxidase activity of acridid grasshoppers from the subfamilies Melanoplinae and Oedipodinae. *Comparative Biochemistry and Physiology,* **94:** 117-124.

Bidochka, M. J. and Khachatourians, G. G. (1992). Growth of the entomopathogenic fungus *Beauveria bassiana* on cuticular components from the migratory grasshopper, *Melanoplus sanguinipes*. *Journal of Invertebrate Pathol*ogy, **59:** 165-173.

Bidochka, M. J, St. Leger, R. and Roberts, D. W. (1997). Mechanisms of deuteromycete fungal infections in grasshoppers and locusts: An overview. pp. 213-224. **In**: Goettel, M. S. and Johnson, D. L. (eds), Microbial control of grasshoppers and locusts. *Memoirs of the Entomological Society of Canada,* **171**: 400 pp.

Boucias, D. J., Pendland, J. C. and Latge, J. P. (1988). Nonspecific factors involved in the attachment of entomopathogenic deuteromycetes to host insect cuticle. *Applied Environmental Microbiology*, **54**: 1795-1805.

Brader, L. (1988). Control of grasshoppers and migratory locusts. *Brighton Crop Protection Conference: Pests and Diseases*. Pp. 283-288.

Brobyn, P. J. and Wilding, N. (1977). Invasive and developmental processes of *Entomophtora* speicies infecting aphids. *Transactions of the British Mycological Society,* **69:** 349-66.

Charnley, A. K. (1992). Mechanism of fungal pathogenesis in insects with particular reference to locusts. pp. 181-189. **In:** Lomer, C. J. and Prior, C., (eds), *Biological control of locusts and grasshoppers*. CAB International, UK. 394 pp.

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

Conley, R. A. M. (1969). Locusts: "Teeth of the wind". *National Geographic,* **136:** Pp. 202-226.

Couch, T. L. and Ignoffo, C. M. (1981). Formulation of insect pathogens. pp 621-634. **In**: Burges, H. D. (ed). *Microbial control of pests and diseases 1970-1980.* New York: Academic Press.

Dagne, K. and Jonsson, A. (1994). Seed oil content and fatty acid composition of *Guizotia Cass.* (Compositae). (Manuscript). pp. 1-6. **In:** Cytology, phylogeny and oil quality of *Guizotia Cass.* (Compositae). Ph.D. Thesis.

David, W. A. L. (1975). The status of viruses pathogenic for insects and mites. *Annual Review of Entomology,* **20**: 97-111.

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

Domsch, K. H., Gams, W. and Anderson, T. H. (1980). *Compendium of soil fungi.* Volume I and II. Academic Press.

Ewen, A. B. and Mukerji, M. K. (1987). Field evaluation of carbofuran bait against grasshopper (Orthoptera: Acrididae) populations in Saskatchewan. *The Canadian Journal of Entomology,* **119:** 537-540.

Feng, MG. And Johnson, J. B. (1990). Relative virulence of six isolates of *Beauveria bassiana* on *Diuraphis noxia* (Homoptera: Aphididae). *Environmental Entomology,* **19:** 785-790.

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

Ferron, P. (1981). Pest control by fungi *Beauveria and Metarhizium.* pp. 465-482. **In**: Burges, H. D. (ed). *Microbial control of pests and plant diseases 1970-1980.* Academic Press. London.

Geddes, A. M. W. (1990). The relative importance of crop pests in sub-Saharan Africa. *Natural Resources Institute Bulletin* no., **36:** 69pp.

Goettel, M. S. (1992). Fungal agents for biocontrol. pp. 123-131. **In:** Lomer, C. J. and Prior, C., (eds), *Biological control of locusts and grasshoppers*. CAB International, UK. 394 pp.

Goettel, M. S., Poprawski, T. J. Vanderberg, J. D., Li, Z. and Roberts, D. W. (1990). Safety to non-target invertebrates of fungal biocontrol agents. pp. 209-232. **In:** Laird, M., Lacey, L. A. and Davidson, E. W. (eds), *Safety of microbial insecticides*. CRC Press, Boca Raton, FL. 259 pp.

Goettel, M. S. and Roberts, D. W. (1992). Mass production, formulation and field application of entomopathogenic fungi. pp. 230-238. **In:** Lomer, C. J. and Prior, C., (eds), *Biological control of locusts and grasshoppers*. CAB International, UK. 394 pp.

Goettel, M. S., St. Leger, R. J. Rizzo, N. W. Staples, R. C. and Roberts, D. W. (1989). Ultrastructural localization of a cuticle-degrading protease produced by the entomopathogenic fungus *Metarhizium anisopliae* during penetration of host (*Manduca sexta*) cuticle. *Journal of General Microbiology,* **135:** 2233-2239.

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

Greathead, D. J. (1992). Keynote address Biological control as a potential tool for locust and grasshopper control. pp. 5-7. **In:** Lomer, C. J. and Prior, C., (eds), *Biological control of locusts and grasshoppers*. CAB International, UK. 394 pp.

Hall, R. A. (1982). Control of whitefly, *Trialeurodes vaporariorum* and cotton aphid, *Aphis gossypii* in glasshouse by two isolates of the fungus *Verticillium lecanii*. *Annals of Applied Biology,* **101:** 1-11.

Hall, R. A. and Papierok, B. (1992). Fungi as a biological control agents of arthropods of agricultural and medical importance. *Parasitology,* **84:** 205-240.

Hegedus, D. D. , Bidochka, M. J., Miranpuri, G. S. and Khachatourians, G. G. (1992). A comparison of the virulence, stability and cell-wall-surface characteristics of three spore types produced by the entomopathogenic fungus *Beauveria bassiana*. *Applied Microbiology and Biotechnology,* **36:** 785-789.

Hegedus, D. D. and Khachatourians, G. G. (1996). Analysis of cellular defense reactions of the migratory grasshopper, *Melanoplus sanguinipes*, infected with heat-sensitive mutants of *Beauveria bassiana*. *Journal of Invertebrate Pathology,* **68:** 166-169.

Ignoffo, C. M. (1981). The genus *Nomuraea rileyi* as a microbial insecticide. pp. 513-538. **In**: Burges, H. D. (ed), *Microbial control of pests and plant diseases, 1970-1980* Academic Press. London.

Ignoffo, C. M. and Hink, W. F. (1971). Propagation of arthropod pathogens in living system. pp. 541-580. **In**: Burges, H. D. and Hussey, N. W. (eds), *Microbial control of insects and mites.* Academic Press, New York.

Jaronski, S. T. and Goettel, M. S. (1997). Development of Beauveria bassiana for control of grasshoppers and locusts. pp. 225-237. **In**: Goettel, M. S. and Johnson, D. L. (eds), Microbial control of grasshoppers and locusts. *Memoirs of the Entomological Society of Canada,* **171:** 400 pp.

Jenkins, N. E. and Goettel, M. S. (1997). Methods for mass-production of microbial control agents of grasshoppers and locusts. pp. 37-48. **In**: Goettel, M. S. and Johnson, D. L. (eds), Microbial control of grasshoppers and locusts. *Memoirs of the Entomological Society of Canada,* **171:** 400 pp.

Jenkins, N. E. and Prior, C. (1993). Growth and formation of true conidia *by Metarhizium flavoviride* in a simple liquid medium. *Mycological Research,* **97:** 1489-1494.

Jenkins, N. E. and Thomas, M. B. (1996). Effect of formulation and application method on the efficacy of aerial and submerged conidia of *Metarhizium flavoviride* for locust and grasshopper control. *Pesticide Science,* **46:** 229-306.

Jimenez, J. and Gillespie. (1990). Use of optical brightener Tinopal BOPT for the rapid determination of conidial viabilities in entomogenous deuteromycetes. *Mycological Research,* **94:** 279-283.

Johnson, D. L. (1997). Nosematidae and other protozoa as agents for control of grasshoppers and locusts: Current status and prospects. pp. 375-389. **In**: Goettel, M. S. and Johnson, D. L. (eds), Microbial control of grasshoppers and locusts. *Memoirs of the Entomological Society of Canada,* **171:** 400 pp.

Kaiser, H. (1991). Terrestrial and semi-terrestrial mermithidae. pp 899-960. **In**: Nickle, W. R. (ed). *Manual of agricultural nematology*, Mercel Dekker, Inc., New York.

Kaya, H. K. (1987). Diseases caused by nematodes. pp. 454-465. **In:** Fuxa, J. R. and Tanada, Y. (eds). *Epizootiology of insect diseases*, A wiley-Interscience Publication. John and Wiley and Sons, Newyork.

Kleespies, R. G. and Zimmerman, G. (1992). Production of blastospores by three strains of *Metarhizium anisopliae* (Metch.) Sorokin in submerged culture. *Biocontrol Science and Technology*, **2:** 127-235.

Knudsen, G. R., Johnson, J. B. and Eschen, D. J. (1990). Alginate pellet formulation of *Beauveria bassiana* (Fungi: Hyphomycetes) isolate pathogenic to cereal aphids. *Journal of Economic Entomology,* **83:** 2225-2228.

Kremer, A. R. (1993). Pests and donors in Mali, 1985-1990. *Disasters,* **16:** 207-216.

Lomer, C. J., Prior, C., Herren, H., Paraiso, A., Kooyman, C. and Smit, J. J. (1992). The IIBC/IITA/ DFPV collaborative research program on the biological control of locusts and grasshoppers. pp. 9-18. **In:** Lomer, C. J. and Prior, C., (eds), *Biological control of locusts and grasshoppers.* CAB International, UK. 394 pp.

Lomer, C., Prior, C. and Kooyman, C. (1997). Development of *Metarhizium* sp. For the control of grasshoppers and locusts. pp. 265-286. **In:** Goettel, M. S. and Johnson, D. L. (eds), Microbial control of grasshoppers and locusts. *Memoirs of the Entomological Society of Canada,* **171:** 400 pp.

LUBILOSA. (1996). *Technical bulletin.*

Maddox, J. V. (1987). Protozoan diseases. pp. 418-444. **In:** Fuxa, J. R. and Tanada, Y. (eds). *Epizootiology of insect diseases.* A wiley Interscience Publication, John and Wily and Sons, New York.

Marcandier, S. and Khachatourians, G. G. (1987). Susceptibility of the migratory grasshopper, *Melanoplus sanguinipes* to *Beauveria bassiana*: Influence of relative humidity. *The Canadian Journal of Entomology,* **119:** 901-907.

Moore, D. and Caudwell. (1997). Formulation of entomopathogen for the control of grasshoppers and locusts. pp. 49-67. **In:** Goettel, M. S. and Johnson, D. L. (eds), Microbial control of grasshoppers and locusts. *Memoirs of the Entomological Society of Canada,* **171:** 400 pp.

Moorehouse, E. R., Gillespie, A. T. and Charnley, A. K. (1994). The influenceof temperature on the susceptibility of vine weevil, *Otiorhynchus sulcatus* (Fabricius) (Coleoptera: Curculionidae), larvae to *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes). *Annals of Applied Biology,* **124:** 185-193.

Mugnai, L., Bridge, P. and Evans, H. C. (1989). A chemotaxonomic evaluation of the genus *Beauveria*. *Mycological Research,* **92:** 199-209.

Nasseh, O., Freres, T., Wilps, H., Kirkilionis, E. and Krall, S. (1992). Field cage trials on the effects of enriched neem oil, insect growth regulators and the pathogens *Beauveria bassiana* and *Nosema locustae* on desert locusts in the Republic of Niger. pp. 311-320. **In:** Lomer, C. J. and Prior, C., (eds), *Biological control of locusts and grasshoppers.* CAB International, UK. 394 pp.

Odindo, M. O. (1994). Identification of insect pathogens common in the insectary. pp. 261-264. **In:** Ochieng-Odero (ed). *Techniques of insect rearing for the development of integrated pest and vector management strategies.*

OTA (Office of Technology Assessment). (1990). *A plague of locusts special report* OTA-F-450. US Government Printing Office, Washington, DC.

Pereira, R. M. and Roberts, D. W. (1990). Dry mycelium preparations of entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*. *Journal of Invertebrate Pathology,* **56:** 39-46.

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

Prior, C. (1992). Discovery and characterization of fungal pathogens for locust and grasshopper control. pp. 159-180. **In:** Lomer, C. J. and Prior, C., (eds), *Biological control of locusts and grasshoppers.* CAB International, UK. 394 pp.

Prior, C., Lomer, C. J., Herren, H., Paraiso, A. Kooyman, C. and Smit, J. J. (1992). The IIBC/IITA/DFPV collaborative research program on the biological control of locusts and grasshoppers. pp. 8-18. **In:** Lomer, C. J. and Prior, C., (eds), *Biological control of locusts and grasshoppers.* CAB International, UK. 394 pp.

Prior, C. and Street, D. A. (1997). Strategies for use of entomopathogens in the control of the desert locust and other acridoid pests. **pp.** 5- 25. **In**: Goettel, M. S. and Johnson, D. L. (eds), Microbial control of grasshoppers and locusts. *Memoirs of the Entomological Society of Canada,* **171:** 400 pp.

Rath, A. C. Guy, P. L., and Webb, W. R. (1995). *Metarhizium* spore surface antigens are correlated with pathogenecity. *Mycological Research,* **100:** 57-62.

Samson, R. A. (1981). Identification: entomopathogenic Deuteromycetes. pp. 93-106. **In**: Burges, H. D. (ed), *Microbial control of pests and plant diseases 1970-1980.* Academic Press.

Sekizawa, J. M. Miyamoto, E. J. and Matsuo. (1992). Fenitrothion. *Environmental Health Criteria* **133:** 184pp. World Health Organization, Geneva.

Stathers, TE. Moore, D. Prior, C. (1993). The effect of different temperatures on the viability of *Metarhizium flavoviride* conidia stored in vegetable and mineral oils. *Journal of Invertebrate Pathology,* **62:** 111-115.

Steedman, A. (1990). *Locust handbook.* (3rd edn). Chatham. Natural Resource Institute, U. K. Pp. 14-22.

Stephan *et al.*, (1995). Locust control with *Metarhizium flavoviride*: formulation and application of blastospores. pp. 232-235. **In**: *Proceedings of the first joint meeting on "Insect pathogens and insect parasitic nematodes". Poznan (Poland), 27 August – 1 September 1995.*

Stephan, D. Welling, M. and Zimmerman, G. (1997). Locust control with *Metarhizium flavoviride*: New approaches in the development of a biopreparation based on blastospores. pp. 151-158. **In:** Krall, R. *et al* (eds). *New Strategies in Locust Control.* Birkhuser Verlag Basel/Switzerland.

St Leger, R. J. Roberts, D. W. and Staples, R. C. (1990). Protein kinases in the entomopathogenic fungus *Metarhizium anisopliae. Journal of General Microbiology,* **136:** 1401-1411.

St Leger, R. J., Goettel, M., Roberts, D. W. and Staples, R. C. (1991). Prepenetration events during infection of host cuticle by *Metarhizium anisopliae*. *Journal of Invertebrate Pathology,* **58:** 168-179.

Street, D. A., Woods, S. A. and Erlandson, M. A. (1997). Entomopoxviruses of grasshoppers and locusts: Biology and biological control potential. pp. 115-130. **In**: Goettel, M. S. and Johnson, D. L. (eds), Microbial control of grasshoppers and locusts. *Memoirs of the Entomological Society of Canada,* **171:** 400 pp.

Thomas, K. C., Khachatourians, G. G. and Ingledew, W. M. (1987). Production and properties of *Beauveria bassiana* conidia cultivated in submerged culture. *Canadian Journal of Microbiology,* **33:** 12-20.

Tulloch, M. (1976). The genus *Metarhizium*. *Transactions of the British Mycological Society,* **66:** 407-11.

Van Winkelhof, A. J. and Mc Coy, C. W. (1984). Conidiation of *Hirsutella thompsonii var. synnematosa* in submerged culture. *Journal of Invertebrate Pathology,* **43:** 59-68.

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

Veen, K. H. and Ferron, P. (1966). A selective medium for the isolation of Beauveria bassiana and of Metarhizium anisopliae. *Journal of Invertebrate Pathology,* **8:** 268-268.

Wheeler, M. B., Stuart, G. S. and Hapner, K. D. (1993). Agglutinin mediated opsonization of fungal blastospores in *Melanoplus differentialis* (Insecta). *Journal of Insect Physiology,* **39:** 477-483.

Zelazny, B., Goettel, M. S. and Keller, B. (1997). The potential of bacteria for microbial control of grasshoppers and locusts. pp. 147-156. **In**: Goettel, M. S. and Johnson, D. L. (eds), Microbial control of grasshoppers and locusts. *Memoirs of the Entomological Society of Canada,* **171:** 400 pp.