INVESTIGATIONS ON THE USE OF THE FUNGUS, *BEAUVERIA BASSIANA* (HYPHOMYCETES: MONILIALES) FOR CONTROL OF THE SENEGALESE GRASSHOPPER, *OEDALEUS SENEGALENSIS* (ORTHOPTERA: ACRIDIDAE)

by

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INVESTIGATIONS ON THE USE OF THE FUNGUS, BEAUVERIA BASSIANA (HYPHOMYCETES: MONILIALES) FOR CONTROL OF THE SENEGALESE GRASSHOPPER, OEDALEUS SENEGALENSIS (ORTHOPTERA: ACRIDIDAE)

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ABSTRACT

The Senegalese grasshopper, *Oedaleus senegalensis* (Orthoptera: Acrididae), is a major pest of crops in the sahelian zone of West Africa. Ten new isolates of *Beauveria bassiana* (Hyphomycetes: Moniliales) were obtained from soil and grasshoppers collected in Burkina Faso, from zones of significant grasshopper and locust activity. The effects of temperature on conidial germination and pathogenicity toward *O. senegalensis* were investigated under laboratory conditions.

Germination was studied at temperatures of 20°, 25°, 30° and 35^oC. The most favorable and the most adverse temperatures were 25° and 35^oC, respectively. Temperature response curves for the germination rate and the time to 95% germination were adequately described by quadratic functions. Intra-specific variations in the components of germination were observed.

Pathogenicity was assessed after topical application of 10^5 conidia onto third-instar nymphs. All isolates were found to be pathogenic to *O. senegalensis*. Significant differences in virulence were observed among isolates, and the median lethal times varied between 3.5 and 5 days. High virulence appeared to correlate with high conidial germination.

The effects of oral and topical applications of conidia on third-instar nymphs were compared. The fungus was pathogenic to *O. senegalensis* regardless of the route of exposure. In addition, there was no significant difference in virulence between the two methods of application.

These studies show that 1) *B. bassiana* has potential use for control of grasshoppers, 2) influence of temperature on germination should be considered when selecting isolates to develop as a mycopesticide in the Sahel, 3) selection for virulent isolates is necessary, even among isolates collected from the same geographical location,

and 4) conidia could be used in baits or droplets in a similar way as chemical pesticides for control of *O. senegalensis*.

RESUME

Le criquet sénégalais, *Oedaleus senegalensis* (Krauss) (Orthoptera: Acrididae), est un important ravageur des cultures dans la zone saheliennne en Afrique de L'Ouest. Dix nouvelles souches de *Beauveria bassiana* (Hyphomycètes: Moniliales) ont été isolées à partir d'échantillons de sol et de criquets recoltés au Burkina Faso dans des zones d'intenses activités acridiennes. Les effets de la temperature sur la germination des spores et la susceptibilité de *Oedaleus senegalensis* ont été étudiés dans les conditions de laboratoire.

La germination a été testée à 20, 25, 30 et 35°C. La temperature la plus favorable et la plus adverse ont été respectivement 25 et 35°C. Les effets de la température sur le taux ainsi que le temps de germination de 95% des spores sont adéquatement décrites par des fonctions polynomiales de second degré. Des variations intra-spécifiques des composantes de germnation ont été observées.

La pathogenicité a été évaluée sur des larves de 3e stade. A la dose d'application de 10^5 spores sur le tégument, toutes les souches se sont avérées pathogeniques à *Oedaleus senegalensis*. Des différences significatives ont été observées dans la mortalité causée par les différentes souches et les temps létaux medians ont varié entre 3.5 et 5 jours. La virulence apparaît correler avec la germination.

L'efficacité entre l'application des spores sur le tégument et celle par voie orale a été comparée sur des larves de 3e stade. *Oedaleus senegalensis* a été susceptible à l'agent pathogène indépendemment de la voie d'infection. De plus, à la dose de 10^5 spores/larve, il n'y avait aucune différence significative de mortalité entre les deux méthodes d'applications.

Cette étude a montré que 1) *B. bassiana* possède une utilisation potentielle dans la lutte anti-acridienne, 2) l'influence de la température devrait être prise en compte dans la sélection des souches à promouvoir dans le Sahel, 3) la sélection de souches virulentes reste nécessaire même parmi des souches de même provenance geographique et 4) les

spores pourraient etre formulés dans des appâts ou sous forme de gouttellettes de la même manière que les produits chimiques pour lutter contre *O. senegalensis*.

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CHAPTER 1

GENERAL INTRODUCTION

The Senegalese grasshopper, *Oedaleus senegalensis* (Krauss) (Orthoptera: Acrididae), is a major pest of cultivated and rangeland crops in the Sahelian zone of West Africa (Batten 1969; Cheke *et al.* 1980a, 1980b; Launois 1979; Lecoq 1978). To date, control programs have relied on the use of synthetic insecticides (Cheke 1990). For instance, in 1986 alone, about 1.2 million litres and 5,000 tonnes of insecticides were used in six Sahelian countries for control of grasshoppers and locusts (Appendix 1). However, intensive use of chemical pesticides has raised concerns about possible impact on the environment (Berger 1991; Everts 1990; Matteson 1992). While detailed studies of the environmental impact of chemical pesticides have not been conducted, there is agreement that control methods need to be reassessed (Greathead 1992; Prior *et al.* 1992).

Entomopathogenic fungi represent an alternative method of controlling insects (Ferron 1978; Hall and Papierok 1982). There are numerous records of fungi infecting acridids in a field environment (Balfour-Browne 1960; Chapman and Page 1979; Erlandson *et al.* 1988; Milner 1978; Roffey 1968; Schaefer 1936; Steinhaus 1949; Uvarov 1928). Prior and Greathead (1989) suggested that the Deuteromycetes *Beauveria spp.* and *Metarhizium spp.* are among the most promising candidates for control of locusts and grasshoppers, for several reasons. First, these fungi may include host-specific strains that are virulent to grasshoppers but are not hazardous to non-target organisms. Second, fungal spores may be formulated and applied in a similar way as chemical pesticides and therefore benefit from existing technology; this includes oil-based formulations and ultra-low volume applications which are very popular in the Sahel. Third, they are cheap to produce and may provide low-cost control.

The Senegalese grasshopper, *Oedaleus senegalensis*

Oedaleus senegalensis belongs to the subfamily Oedipodinae. Adults measure 2.5 to 3 cm in length, with variable coloration of green, grey, beige and maroon. The genus is distinguishable from related genera by an X-marking composed of four separate pale spots on the dorsum of the pronotum (Ritchie 1983). Within the genus, *O. senegalensis* is distinguished by the broadly rounded posterior margin of the pronotum and the black fascia on the hind wings which extend forward to the anterior margin (Batten 1969).

Oedaleus senegalensis occurs in Africa, the Middle East and India (Batten 1969; Boys 1978). In West Africa, the species is found between 11° and 20° N, within the isohyets 250 and 1000 mm; this zone corresponds to the Sudan savanna in the south and the Sahel in the north of West Africa (Cheke 1990; Duranton *et al.* 1987; Fishpool and Cheke 1983). The species prefers sandy soils for egg-laying (Cheke *et al.* 1980b) and passes the dry season in egg diapause, embryonic development recommencing after the onset of the first rains in May or June (Fishpool 1983; Launois 1978). The nymphs develop through five instars and become adults after about three weeks (Duranton *et al.* 1987). Females may lay one or two egg pods, each containing 30 to 40 eggs (Batten 1969). Under suitable conditions, a generation (egg to egg) takes about two months to complete. The rates of development at the various stages of the life cycle are influenced by many factors, including temperature and moisture; optimal conditions are 22.5°C to 27°C and precipitation of 25 mm per month (Duranton *et al.* 1987). Two to four generations may occur per year depending upon environmental conditions. Favorable conditions accelerate the rates of development and contribute to the increase of the number of generations within the reproductive season. This may play an important role in outbreaks (Cheke *et al.* 1980b).

Oedaleus senegalensis may form nymphal bands and adult swarms (Batten 1969; Popov 1989). In the Sahelian zone of West Africa, the species engages in long-range, nocturnal and mass flight with favorable winds; there are reports of flights oriented towards the limits of the Inter-Tropical Convergence Zone (Riley and Reynolds 1979; 1983). The climatic conditions in this zone are most favorable to the species, and migrations are northwards at the beginning of the rainy season in June and July and southwards at the end of August and in September (Cheke 1990; Launois 1978, 1979).

Both nymphs and adults may damage crops. Early in the wet season, seedlings are very susceptible and attack on seedlings may force farmers to re-sow (Cheke 1990). Cereal plants are also susceptible at the milky stage of the grain formation. Two strategies are used in the Sahel to contain the pest: 1) elimination of nymphal populations before the grasshopper numbers build up excessively and long-distance movement occurs (Cheke 1990); and 2) suppression of adults to prevent egg-laying, regardless of crop damage.

The control of *Oedaleus senegalensis* in Burkina Faso

Grasshopper and locust outbreaks are considered a public threat in Burkina Faso. Control campaigns mobilize all available organizations and public services in the country; the crop protection service is responsible for organization and coordination of spraying operations.

The control campaign against *O. senegalensis* is conducted in two phases. The first phase, from May to July, corresponds to the beginning of the wet season. During this period, *O. senegalensis* damages seedlings of cereal and leguminous crops, including *Pennisetum spp.*, *Sorghum spp.*, *Arachis hypogea,* and *Vigna unguiculata*, as it migrates from South to North with the monsoon. Initial infestations are usually limited to small areas at egg-laying sites. Nymphs at this stage of development are targeted before adults

disperse and migrate. During this period, the participation of farmers with hand-held sprayers for ultra-low volume (ULV) applications is encouraged. The second phase lasts from September to October and corresponds to the end of the wet season, when adults have commenced their southwards migration. Large areas are usually infested during this period, and the most damage occurs on cereal grains at the milky stage. Because control must be rapidly conducted over large areas, aerial application is usually employed rather than ground application, which is limited by the inaccessibility of certain sites during this period.

Based on information about egg-laying activities during the previous season, pesticides and spraying materials are placed close to sites with the most likelihood of hatching and pest activity. These sites are surveyed and populations monitored frequently, so that spraying operations can be started whenever control is needed. During the most recent outbreak (1986-1989), large areas with population densities in excess of 50 individuals per m^2 were recorded. Control consisted of ground and aerial applications of the insecticides fenitrothion, malathion, propoxur, deltamethrin, diazinon and lindane. Although various formulations were used, the most common application method was ULV sprays.

The pathogen, *Beauveria bassiana*

Beauveria bassiana is a hyphomycete fungus in the subdivision Deuteromycotina. A disease induced by *B. bassiana* was first described in 1763, causing white muscardine of the silkworm, *Bombyx mori* (Steinhaus 1949). It was not until 1835 that the fungal nature of the disease was established (Benham and Miranda 1953). The genus *Beauveria* was described in 1912 by Vuillemin; further investigations on the morphology and taxonomy of the genus have been conducted by Benham and Miranda (1953) and Macleod (1954). The genus is characterized by the formation of sympodial spores on

short, globose or flask-shaped conidiogenous cells with an apical denticulate rachis. Conidia are one-celled, thin-walled and globose to ellipsoidal. *Beauveria bassiana* is distinguished from other species of the genus by having *ca.* 50% of globose spores.

Beauveria bassiana occurs worldwide (Steinhaus 1949; Macleod 1954), with a host range of over 700 species (Li 1987) including many insects of agricultural and medical importance (Hall and Papierok 1982). Infection occurs when hosts come in contact with the infectious propagules, usually the conidia (Allee *et al.* 1990; Ferron 1978; Pekrul and Grula 1979; Fargues and Vey 1974). Conidia adhere to the host's integument (Boucias *et al.* 1988), germinate and then penetrate the host. Both mechanical pressure and enzymatic lysis of the cuticle are involved in host penetration (Vey and Fargues 1977). The endoprotease, PR1, produced by most Deuteromycete fungi is suspected, in combination with other enzymes, to play a key role in the penetration process (Charnley and St Leger 1991; Goettel *et al.* 1989; St Leger *et al.* 1987, 1988). However, the exact role of the enzymes and their sequential appearance during the process are not well understood. Host infection via the mouth parts (Bao and Yendol 1971; Miranpuri and Khachatourians 1991; Siebeneicher *et al.* 1992; Yaganita 1987), the alimentary tract during the consumption of contaminated food (Broome *et al.* 1976; Gabriel 1959), the anus after passage through the gut (Allee *et al.* 1990) and the respiratory system (Clark *et al.* 1968) have also been reported. After the fungus has entered the body cavity, it invades the haemolymph, producing short filaments or hyphal bodies (blastospores). The invasion is sometimes hampered by defense mechanisms (Charnley 1992), including phagocytosis and/or encapsulation (Roberts and Humber 1981) which involve the formation of hemocytic aggregates or nodules (Bidochka and Khachatourians 1987; Dunn 1986; Hou and Chang 1985). However, these defenses are usually overcome by the fungus, possibly because *B. bassiana* grows more quickly than the insect defenses can respond (Hou and Chang 1985). An alternative explanation is that toxins (Roberts 1981) and extra cellular proteases (Bidochka and Khachatourians 1987)

lead to lysis and/or disintegration of the hemocytic nodules. The toxins would incite a progressive degeneration of host tissues, leading to a physiological perturbation. Similar results could also be obtained as a result of asphyxiation or obstruction of haemolymph circulation due to the fungal growth. In the later stages of infection, the haemolymph becomes pasty, which slows and then stops circulation (Madelin 1963). Death occurs and the fungus proceeds to grow saprophytically through virtually all tissues of the insect. In the presence of high relative humidity, the hyphae emerge from the cadaver's intersegmental areas and produce conidia (Ferron 1978). The latter are readily infectious to a susceptible host. Conidia remain on the cadaver or disperse in soil or other substrates where they may persist for years, depending on environmental conditions (Doane 1959; Schabel 1982).

As with other fungi, *B. bassiana* is limited by abiotic and biotic factors. Abiotic factors affecting *B. bassiana* include temperature, humidity and light (Fargues 1972; Ferron 1977; Lane *et al.* 1991; Lingg and Donaldson 1981; Schaerffenberg 1964). Free water or relative humidity of over 90% has been considered for many years as the most serious constraint for germination and growth. By contrast, a number of studies indicated that infection occurs independently of relative humidity (Ferron 1977; Marcandier and Khachatourians 1987; Ramoska 1984; Schaerffenberg 1964). High relative humidity is only required for fungal sporulation, which is necessary for horizontal transmission from cadavers (Ferron 1978; Schaerffenberg 1964). Humidity also affects the survival of conidia (Lingg and Donaldson 1981), with long survival at low relative humidity. Temperature affects both germination and mycelial growth. For most strains, the optimal temperatures are thought to fall between 23°C and 25°C (Ferron 1978; Hall and Papierok 1982). Temperatures above or below the optimum range prolong the lag phase and decrease the germination rate (Hywel-Jones and Gillespie 1990). In this case, infection may be inhibited and mycosis retarded (Carruthers *et al.* 1985; Fargues 1972; Walstad *et al.* 1970). Exposure to sunlight, especially to the ultraviolet portion of the spectrum,

adversely affects the survival of conidia (Daoust and Pereira 1986a, 1986b; Inglis *et al.* 1993). Biotic factors influencing *B. bassiana* include microbial antagonists on host integument, leaf surface or in soil (Groden and Lockwood 1991; Lingg and Donaldson 1981; Markova 1991), host susceptibility (Fargues 1972; Feng *et al.* 1985; Quintela *et al.* 1990) and strain virulence (Fargues 1976; Feng and Johnson 1990; Lai *et al.* 1982). Physiological condition, temperature, nutritional stress and age may predispose insects to infection (Donegan and Lightheart 1989; Ferron 1978; Hall and Papierok 1982); young developmental stages are usually most susceptible (Feng *et al.* 1985; Quintela *et al.* 1990). Insects may escape infection during molts (Fargues 1972; Vey and Fargues 1977) or because of some protective structures such as the chorion and elytra that prevent fungal penetration (Hunt *et al.* 1984; Vey *et al.* 1982).

Beauveria bassiana grows and sporulates profusely on various natural substrates, including bran, whole grains, potatoes, hay and straw (Fogal *et al.* 1986). Synthetic media used to stimulate sporulation contain either inorganic or organic nitrogen (Barnes *et al.* 1975; Kucera 1971). Various methods of liquid culture (submerged fermentation), solid culture (surface fermentation) or a combination of both (two phase systems) have been used for mass production (Roberts and Humber 1981; Samsináková *et al.* 1981). *Beauveria bassiana* produces blastospores and conidia in liquid and solid culture, respectively. Both propagules are infectious and can be formulated and applied like chemical pesticides (Auld 1992; Goettel 1992). Commercial formulations of *B. bassiana* such as Biotrol BB and Boverin are available (Ignoffo *et al.* 1979; McCoy *et al.* 1985). Also, large scale applications of the fungus have been reported in the former USSR and China against the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) and the pine caterpillar, *Dendrolimus punctatus* (Lepidoptera: Lasiocampidae) (Ferron 1981).

Field infections of acridids by *B. bassiana* have been reported (Macleod 1954; Moore and Erlandson 1988; Schaefer 1936). Experimental studies have shown that the

fungus is pathogenic to grasshoppers (Goettel and Johnson 1992, Johnson and Goettel 1993; Johnson *et al.* 1988a; Marcandier and Khachatourians 1987; Moore and Erlandson 1988), including several African species (Johnson *et al.* 1992; Lobo Lima *et al.* 1992). Some evidence also suggests that infection can occur in semi-arid conditions (Marcandier and Khachatourians 1987) although application of *B. bassiana* in Mali resulted in limited efficacy (Johnson *et al.* 1992).

Objectives

While there is evidence that *O. senegalensis* becomes infected during field applications of *B. bassiana* (Lobo Lima *et al.* 1992), detailed pathogenicity studies are lacking. Therefore, the main objectives of this study were 1) to collect isolates of *B. bassiana* from Burkina Faso and 2) to evaluate their pathogenicity towards *O. senegalensis*. To achieve these objectives four experiments were conducted. The first experiment consisted of the isolation of *B. bassiana* from soil and grasshoppers within the breeding areas of the species in Burkina Faso (West Africa). Because temperatures in the Sahel are variable and may play an important role on fungal development, the second experiment determined the effects of temperature on the germination of isolates. To assess pathogenicity, a bioassay with *O. senegalensis* was conducted in a third experiment. A fourth experiment compared the efficacy of conidia applied to integument and conidia applied on leaves.

CHAPTER 2

GENERAL MATERIALS AND METHODS

Rearing of *Oedaleus senegalensis*

A colony of *Oedaleus senegalensis* was established from egg pods collected in Niger (West Africa) by G. Popov and initially established and reared by D. Johnson (*pers. comm.*). Hatching was induced by allowing egg pods to dry and then burying them in moist sand and vermiculite (about 50 egg pods in 750 ml plastic containers). Nymphs and adults were maintained in 40 x 40 x 40 cm cages, fed Iceberg lettuce and Lancer Spring wheat leaves and bran. To facilitate oviposition, females were provided with containers containing sterile sand; moisture was provided by adding 70 ml water /250ml container. Egg pods were collected weekly and reincubated for colony growth or stored at 5°C. Temperature for development was maintained at 25 to 27°C, with a 12L:12D photoperiod and relative humidity ranging between 35 and 45%. Development was controlled by altering the temperature, using 25 to 60 watt incandescent bulbs.

Inoculum preparation

Potato dextrose agar (PDA) was used to culture *Beauveria bassiana*. Conidia were suspended in sterile water containing 0.05% Tween 80, polyoxyethylene sorbitan mono-oleate (Sigma Chemical Co.). Aliquots of 0.5 ml of the suspension for each isolate were spread onto the surface of PDA in 10 x 1.5 cm Petri dishes. Plates were incubated at 25°C. Sporulation occurred within six to nine days. Conidia were harvested using a sterile rubber blade and stored at 4°C until use. Unless otherwise stated, fifteen-day old cultures were used in experiments.

Conidial suspensions were aseptically prepared with sterile water containing 0.05% Tween 80 or sunflower oil (Safflo® - Culinar Foods Inc., Toronto). Preparations were thoroughly mixed with a blender to suspend conidia evenly. Suspensions in water required a longer time to mix as conidia are hydrophobic. Sometimes filtration using a Whatman® # 1 filter paper was necessary to reduce clumping. Concentrations were calibrated using a Neubauer haemocytometer (Appendix 2) under a phase contrast microscope.

Bioassays

The bioassay method used in this study was adapted from Goettel and Johnson (1992). Grasshoppers were inoculated using two methods, topical and oral applications. Topical application involved a direct application of conidia (water- or oil-based suspensions) onto the posterior dorsum of the pronotum. For oral application, conidia were deposited on the surface of 5 mm diameter lettuce disks which were then fed to the grasshoppers.

For each experiment, the number of conidia were adjusted to the desired volume of application. An accurate delivery rate of inoculum was obtained using a micro-applicator (Instrumentations Specialties Co.), equipped with a syringe and a foot-operated pedal. After grasshoppers were inoculated (oral or topical) they were placed individually in 500 ml plastic containers. Holes made in the container lids provided aeration. The experiments were run in controlled environment growth chambers. Every day, during the observation period, grasshoppers were fed fresh wheat, and the frass was removed from containers. Dead grasshoppers were removed daily and checked for mycosis. To avoid saprophytic growth of *B. bassiana* from the integument, cadavers were surface-sterilized before incubation. Surface sterilization ensured that fungal sporulation resulted from hyphae emerging from the body cavity, providing evidence of infection. The surface

sterilization procedure consisted of immersing grasshoppers in 1% sodium hypochlorite for 3 min and then rinsing them twice in sterile water (Bao and Yendol 1971; Wright and Chandler 1991). Incubation was conducted under high relative humidity, necessary for fungal sporulation. High relative humidity was provided by placing insects in sealed Petri dishes containing a piece of wet cotton. Plates were maintained at room temperature (22 to 25°C), and the number of cadavers exhibiting growth of *B. bassiana* after 48 hours was recorded. Haemolymph extracted from the femur of grasshoppers that had not died by the end of the experiment was examined under a phase contrast microscope for the presence of blastospores.

Grasshoppers used in each experiment were collected at the desired developmental stage using the morphological criteria described by Ritchie (1983). During this study, grasshoppers from the 15 th to the 17 th generation of the colony were used.

CHAPTER 3

IDENTIFICATION OF NEW ISOLATES OF *BEAUVERIA BASSIANA*

Introduction

Beauveria bassiana has been recovered from insects, soil or plant materials inhabited by insects (Doberski and Tribe 1980; Inglis *et al.* 1993; Macleod 1954; Majchrowicz and Yendol 1973; Moore and Erlandson 1988; Quinn and Hower 1985). For isolation purposes, samples can be collected at sites where the pest occurs. Methods used for sampling include the survey of sites via a network. Baiting the fungus from the soil using a susceptible species has been also suggested (Zimmermann 1986). Since samples collected in the field are usually contaminated with bacteria and various other fungi, it is necessary to use selective culture media that act against bacterial growth and minimize the development of other fungi (Bååth 1991; Beilharz *et al.* 1982; Chase *et al.* 1986; Doberski and Tribe 1980; Veen and Ferron 1966).

For the control of locusts and grasshoppers, much emphasis has been placed on the search for suitable genotypes in the environment where the insect occurs (Prior and Greathead 1989). Isolates found in these areas may combine virulence with the necessary ecological adaptations. In the Sahel, tolerance to high temperatures and high levels of ultraviolet radiation would be necessary. It is also hoped that the use of such isolates would be subject to fewer regulatory constraints in comparison to those from elsewhere.

The objective of this experiment was to isolate natural genotypes of *B. bassiana* from soil and grasshoppers within the breeding zone of *Oedaleus senegalensis* in the Sahel.

Materials and Methods

1) Collection of samples

Soil and grasshoppers were collected from grasshopper and locust breeding grounds in Burkina Faso. Two methods were adopted to collect the samples. The first involved a network of technicians from the crop protection service (Direction de la Protection des Végétaux et du Conditionnement), the extension agents from the regional service of agriculture (Centres Regionaux de Promotion Agro pastorale) and farmers via village brigades (Cellule d'Intervention Phytosanitaire). The second method was a points survey that consisted of taking samples, at 15 km intervals, along public roads. Grasshopper samples were composed of diseased or dead grasshoppers and locusts found in sites free from pesticide application. The survey emphasized sites with a history of high and/or diverse populations of grasshoppers or locusts. Because it was not easy to differentiate diseased from healthy insects in the field, the criterion of lethargy was used. Soil samples consisted of soil from sites where dead grasshoppers were found and/or sites with a suspected decline of grasshopper populations. These criteria were not used in the points survey which consisted of taking samples at each point. Soil samples were collected under shrubs and grasses, at a depth of *ca.* 5 cm below the soil surface, where the temperature was more favorable to the survival of the fungus than at the soil surface. Sub-samples within a site were mixed together to represent the site sample. Samples were air dried, placed in plastic bags and stored at 4°C if they could not be processed immediately. Survey and sampling were conducted during the wet seasons from June to October of 1991 and 1992.

2) Isolation of *Beauveria bassiana*

A selective medium adapted from Chase *et al.* (1986) was used to isolate *B. bassiana*. The medium was composed of oatmeal agar, dodine, crystal violet, an

antibiotic solution (penicillin and streptomycin sulfate) and distilled water (Appendix 3). Fifteen grams from each soil sample were added to 135 ml of sterile water and mixed thoroughly in a blender. Suspensions were then diluted four times in a 10-fold dilution series in 0.05% Tween 80 sterile water. Aliquots of 0.1 ml from each dilution were spread onto the selective medium. Grasshoppers were ground individually and homogenates were spread onto oatmeal-dodine as described above. Cultures were stored in the dark at room temperature or at 22° to 25°C in incubators. Four to five days after the incubation, colonies characteristic of *B. bassiana* were subcultured on potato dextrose agar (PDA). To obtain pure cultures, conidia were streaked on PDA. If necessary, that process was repeated. Slide cultures were used for identification on the basis of morphological criteria (Macleod 1954). Isolates identified as *B. bassiana* were subcultured on agar slopes and maintained at 4°C.

Results

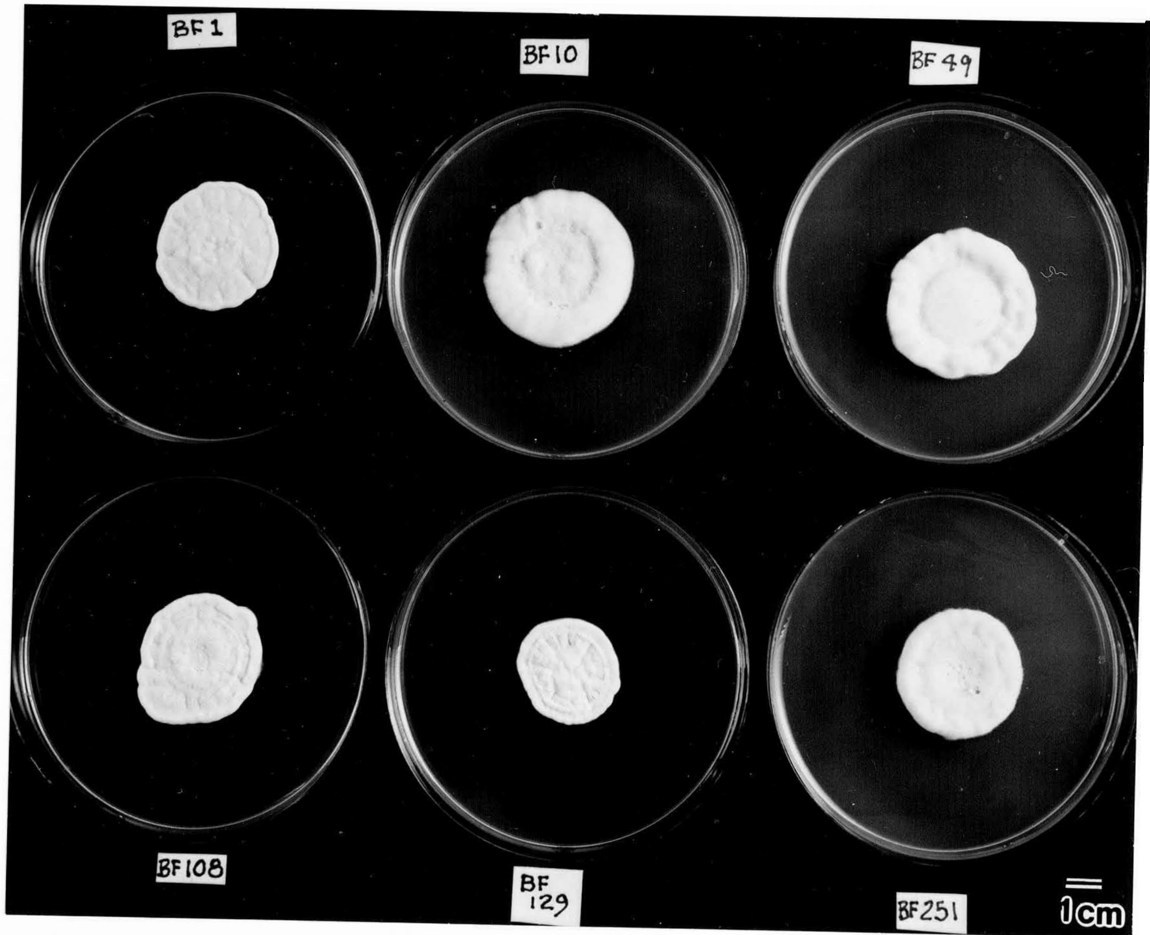
A total of 451 and 84 soil and grasshopper samples, respectively, was collected. From these samples, 245 were obtained from the network. Among the grasshopper samples, 43 dead grasshoppers were collected. From all samples, ten isolates of *B. bassiana* were recovered (Table 3.1); one and nine isolates were recovered from respectively *O. senegalensis* and the soil. Four isolates did not survive storage at 4°C. The remaining six isolates (Figure 3.1) were evaluated for germination and pathogenicity towards *O. senegalensis*.

Table 3.1. Isolates of *Beauveria bassiana* recovered from soil and grasshoppers collected in Burkina Faso in 1991 and 1992.

Isolate	Collection site	Province	Source
BF 1	Berebella	Seno	soil
BF 10	Pobe Mengao	Soum	soil
BF 25*	Amsia	Seno	soil
BF 32*	Moussoua	Seno	soil
BF 49	Koubel Alpha	Soum	soil
BF 58*	Dakiri	Gnagna	soil
BF 69*	MadiaHollo	Soum	soil
BF 108	Gosseye	Oudalan	soil
BF 129	Moussoua	Seno	grasshopper
BF 251	Kongoussi	Bam	soil

* Did not survive storage at 4°C for six months.

Figure 3.1. Fifteen-day old cultures on potato dextrose agar of six new isolates of *Beauveria bassiana* recovered from Burkina Faso. A drop of 50 µl sterile water with 0.05% Tween 80 containing 10^5 conidia was deposited in the center of each plate, which was then placed in the dark at 25°C.


BF 1
BF 10
BF 49
BF 108
BF 129
BF 251
1cm

Discussion

Beauveria bassiana was, for the first time, isolated from Burkina Faso and from the Senegalese grasshopper *O. senegalensis*. While most samples were collected in zones with intensive grasshopper activity, very few diseased or dead grasshopper samples were observed during the survey. Since no epizootic was reported during the sampling period, it is likely that the few insects that died during this enzootic phase were removed by predators, including birds and ants. The low recovery level suggests that either *B. bassiana* was not abundant in the Sahel or the sampling methods were inefficient. A sampling method concentrating on historically known sites of high and/or diverse populations possibly might have yielded more isolates, but the random sample collection by the network, and the systematic selection of points in the second collection method, provide probably realistic assessment of the occurrence of *B. bassiana* in Burkina Faso.

The survival of *B. bassiana* in storage is affected by storage temperature (Hall and Papierok 1982; Walstad *et al.* 1970). The four isolates from the Sahel that were lost during storage at 4°C may not have been cold tolerant. Differences in colony morphology between isolates of *B. bassiana* have been reported (Feng and Johnson 1990; Macleod 1954). Variation in growth, sporulation, germination and virulence may also occur within an isolate due to the type of media and the subculturing history (Macleod 1954). However, because the six isolates were recovered from different geographical locations and because they differ morphologically (Figure 3.1), it is likely that genomic variation exists among them (McCoy *et al.* 1985). To measure genetic variation, immunochemical, biochemical or molecular analyses are necessary (Duriez-Vaucell *et al.* 1981; Fargues *et al.* 1981; Kosir *et al.* 1991; Shimizu and Aizawa 1988; Tan and Ekramoddoullah 1991).

CHAPTER 4

EFFECTS OF TEMPERATURE ON GERMINATION OF ISOLATES OF *BEAUVERIA BASSIANA*

Introduction

The germination of conidia is important in the infection process because only those conidia that germinate will represent the infecting propagules; often, only a fraction of conidia succeed in germinating. Conidia from isolates that germinate rapidly and synchronously have a good chance of infecting a host. It has been found that a high germination rate is correlated with high pathogenicity (Al-Aidroos and Roberts 1978; Samuels *et al.* 1989). Isolates with good germination characteristics are likely to play a major role in the development of disease. In the study of fungal germination, three components are usually considered. The first component is the lag phase, *i.e.* the time required to initiate germination. The second component is the rate of germination. The third component is the total proportion of germinated conidia after a given period of time. Successful germination is usually recorded when the germ tube achieves some arbitrarily determined length.

Two methods are commonly used to study fungal germination (Milner *et al.* 1991). The more common method is to spread conidia onto the surface of either solidified water- or nutrient-agar. The second method consists of inoculating conidia into a liquid medium and removing samples periodically for microscopic examination. In both methods the rapid growth of germ tubes of conidia that germinate first tend to obscure later-germinating conidia, making accurate assessment of germination difficult (Milner *et al.* 1991). Moreover, in a liquid medium some germinated conidia adhere to the walls of the flask and are not counted. In a study with *Metarhizium spp.*, Milner *et al.* (1991) found that, at low concentrations, the fungicide benomyl can be used to

improve the assessment of germination, because it inhibits the growth of germ tubes (Hall 1979) without adversely affecting germination. Benomyl was also used at low doses to recover *B. bassiana* and *M. anisopliae* from selective media (Chase *et al.* 1986).

Differences in germination occur between isolates and even within isolates, depending upon environmental conditions (Fargues 1972; Ferron 1978; Groden and Lockwood 1991; Hall and Papierok 1982; Lingg and Donaldson 1981; Stoy *et al.* 1988), particularly temperature (Hywel-Jones and Gillespie 1990; Vanniasingham and Gilligan 1988). Most isolates of *B. bassiana* can germinate over a relatively wide range of temperatures, but the optimum falls between 23° and 25°C (Ferron 1978; Hall and Papierok 1982). In the Sahel, temperatures often rise above 35°C during the day and decline to about 20°C at night. Therefore, it appeared relevant to determine the range of temperature over which the new isolates may perform well.

Materials and Methods

Seven isolates of *B. bassiana* were evaluated, the six isolates from Burkina Faso (Table 3.1) and one isolate, GHA, which was originally recovered from the migratory grasshopper, *Melanoplus sanguinipes* near Three Forks, Montana (USA) (provided by Mycotech Corporation, Butte, Montana). For each isolate, 5 ml of water-based suspension, containing 10^{5} conidia/ml was prepared (Chapter 2). Aliquots of 0.05 ml were spread onto the surface of potato dextrose agar supplemented with 0.0005% benomyl, in 5 x 1.5 cm Petri dishes. Preliminary tests showed that germination of *B. bassiana* was not inhibited by 0.0005% benomyl. Plates were incubated in the dark at 20°, 25°, 30° and 35°C. For each combination of isolate and temperature, representing a total of 28 treatments, 21 plates were prepared. At 6, 12, 18, 24, 30 and 36 h post-inoculation, three plates were chosen from each treatment. One drop of lactophenol was

placed in the center of each plate and covered with a 22 x 30 mm cover glass and stored at 5°C. This technique halted germination and allowed accurate counts of germinated conidia to be made over time. Plates were examined at 200x under a phase contrast microscope. In each plate, at least 500 conidia were scored in four to five randomly selected fields of view and the numbers of germinated and non-germinated conidia were counted. A spore was considered as germinated if the germ tube was longer than the width of the spore in accordance with the method of Hywel-Jones and Gillespie (1990).

The percentage of germination was calculated for each plate. For each treatment, the mean percentages were transformed to their Logit values to obtain a straight-line relationship between germination and time (Ashton 1972; SAS Institute, Inc. 1989). In treatments in which the mean percentage of germination approached the asymptote, only the first record of the asymptotic value was included, so as not to distort the linearization of the curve (Vanniasingham and Gilligan 1988). The rate and the maximum germination were estimated from the transformed curve as its slope and the time to 95% of conidia to germinate, respectively. Curves were fitted to these values (© Systat Inc., 1989). The analysis of variance was performed followed by the Tukey HSD test ($P < 0.05$) if indicated, on the mean percent germinations at 6 h post-inoculation.

Results

The use of benomyl limited the growth of germ tubes and allowed accurate counts to be made throughout the duration of an experiment (Figure 4.1). Germination occurred within 6 h in all combinations of isolates and temperatures, with the exception of isolates BF49, BF 251 and GHA at 35°C. There were significant differences in percent germination among the isolates at all temperatures (Table 4.1). Germination of conidia in all isolates but BF1 was considerably reduced at 30°C, and at 35°C no isolate achieved better than 20% (Figure 4.2). Because germination approached the asymptote after 24 h, data beyond this point were omitted in the analysis of germination curves (Figure 4.2).

The relationship between the germination rate and the temperature was adequately described by quadratic functions (Figure 4.3). The germination rates were greater at 25°C than at 20°, 30° and 35°C. Germination rates were similar at 20° and 30°C and lowest at 35°C. Quadratic functions also adequately described the effect of temperature on the time taken for 95% of conidia to germinate (Figure 4. 4). Germination was fastest at 25°C, with the length of time required for 95% of germination ranging between 11 and 12 h, with the exception of *ca* 17 h for isolate BF251. By contrast, duration until 95% germination rose sharply at 20°, 30° and 35°C. At all temperatures, BF1 and BF129 appeared to be among the best isolates (Figure 4.4). The response of GHA was similar to that of the other isolates.

Figure 4.1. Effects of benomyl (B) on growth of *Beauveria bassiana*. Sterile water (50 µl) 0.05% Tween 80 containing 10^3 conidia was spread on potato dextrose agar, supplemented with 0.0005% benomyl and incubated at 25°C for 24 h.

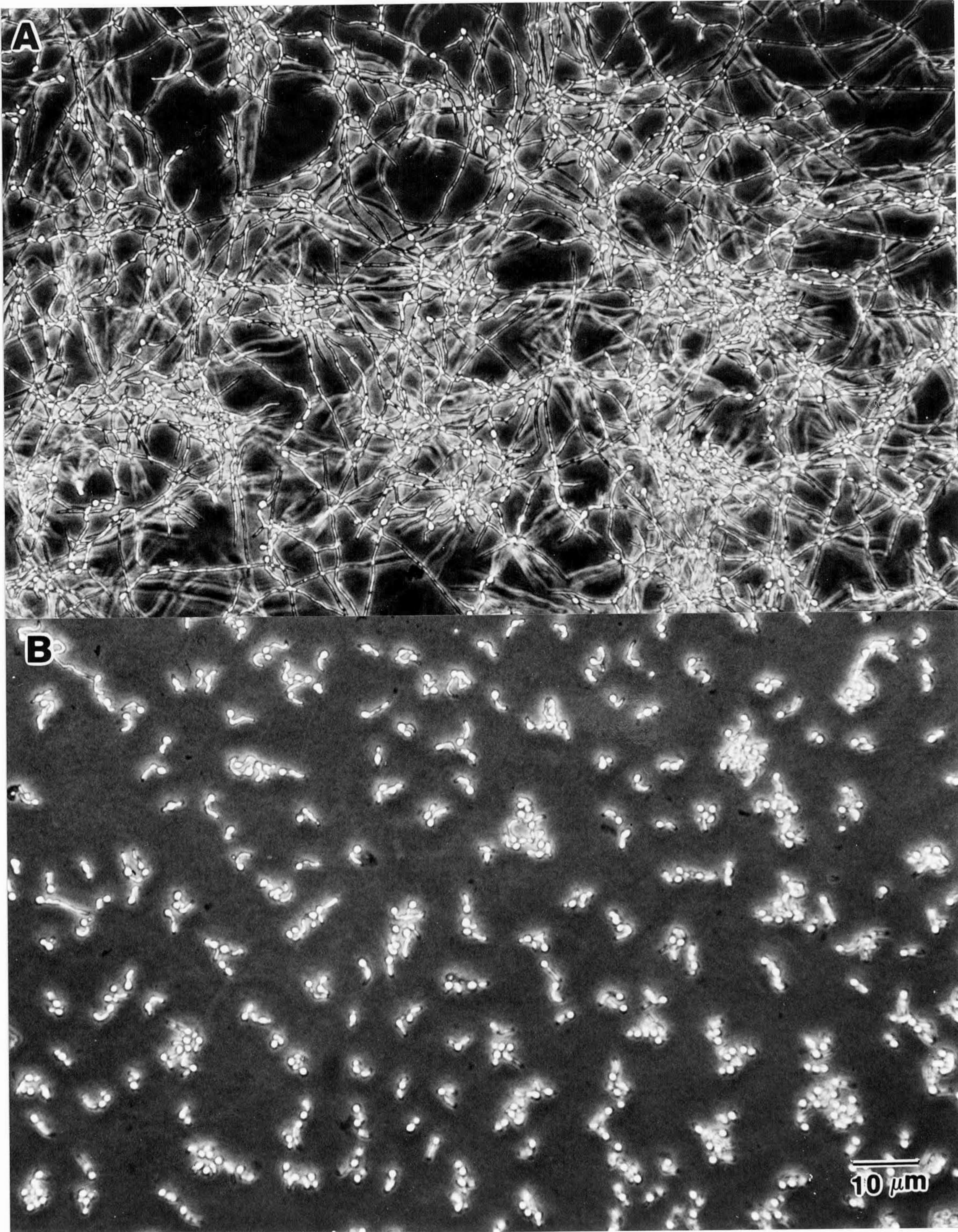

A
B
10 μm

Table 4.1. Comparisons of mean percentages of germination among 7 isolates of *Beauveria bassiana* at 6 h post-incubation at 20°C , 25°C, 30°C and 35°C.

	Mean percent germination[1]			
	20°C	25°C	30°C	35°C
Isolate	Mean (SE)[1]	Mean (SE)	Mean (SE)	Mean (SE)
BF1	26.0 (2.25) a	35.5 (1.01) ab	16.5 (2.36) a	1.3 (0.32) a
BF10	24.1 (2.68) a	41.0 (2.15) a	8.0 (0.91) b	0.3 (0.33) ab
BF49	8.1 (0.54) b	19.4 (1.55) c	4.5 (0.88) bc	0.0 b
BF108	5.7 (0.43) c	12.7 (1.70) d	2.7 (0.12) c	0.6 (0.33) ab
BF129	20.1 (4.62) a	32.1 (0.86) b	3.1 (0.66) c	0.8 (0.15) a
BF251	0.2 (0.03) c	1.2 (0.73) e	0.1 (0.03) c	0.0 b
GHA	7.1 (0.14) bc	26.9 (0.92) b	11.5 (1.27) a	0.0 b

[1] Means sharing the same letter within a column are not significantly different; Tukey's HSD test ($P < 0.05$). In all cases ANOVA, $P < 0.001$.

Figure 4.2. Cumulative mean percentage of germination over time of 7 isolates of *Beauveria bassiana*, BF1, BF10, BF49, BF108, BF129, BF251 and GHA, at 4 temperatures.

20^0C (———); 25^0C (—o—);30^0C (--------); 35^0C (—Δ—)

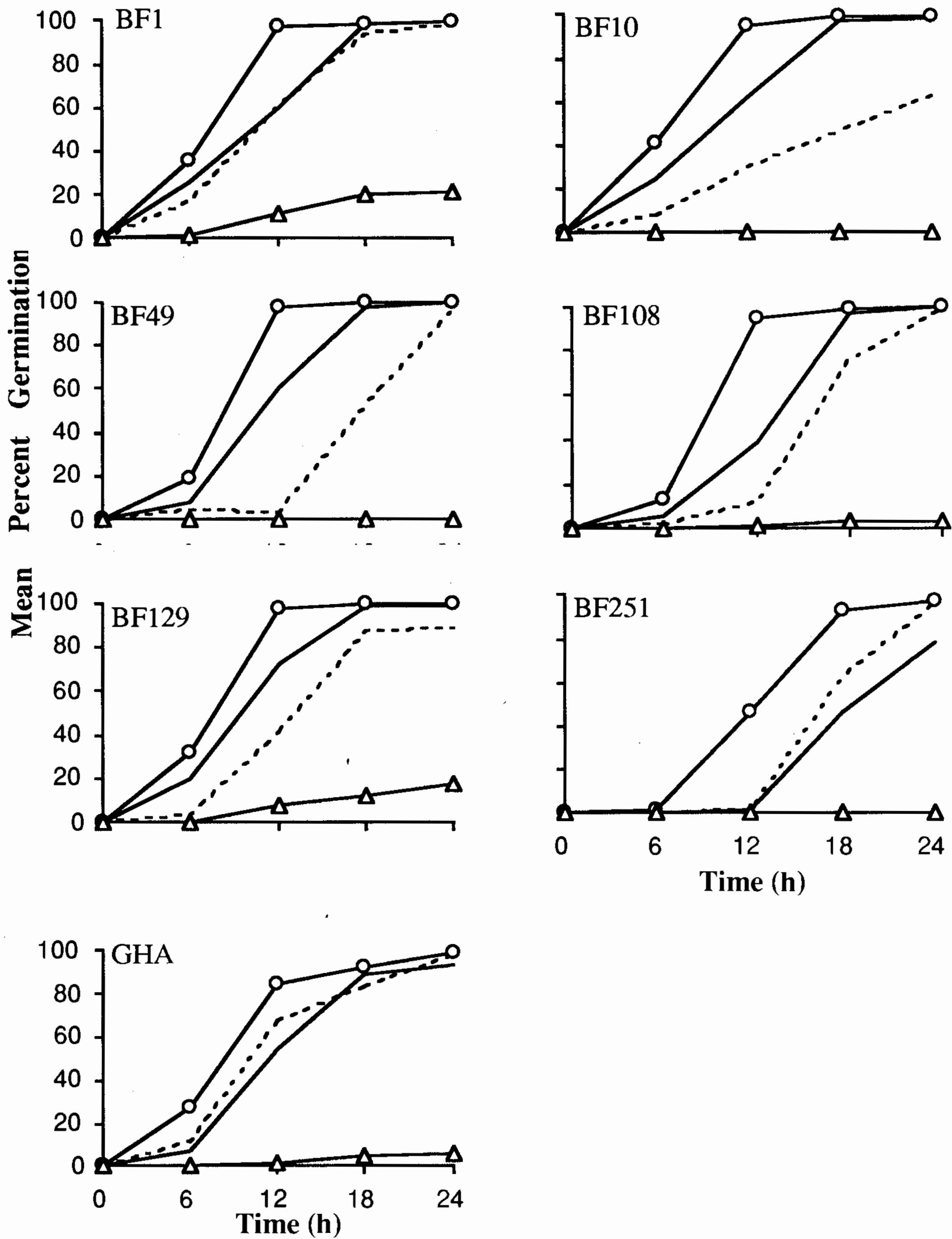


Figure 4.3. Effect of temperature on the germination rate of 7 isolates of *Beauveria bassiana*, BF1, BF10, BF49, BF108, BF129, BF251 and GHA.
S = Germination rate, T = Temperature.

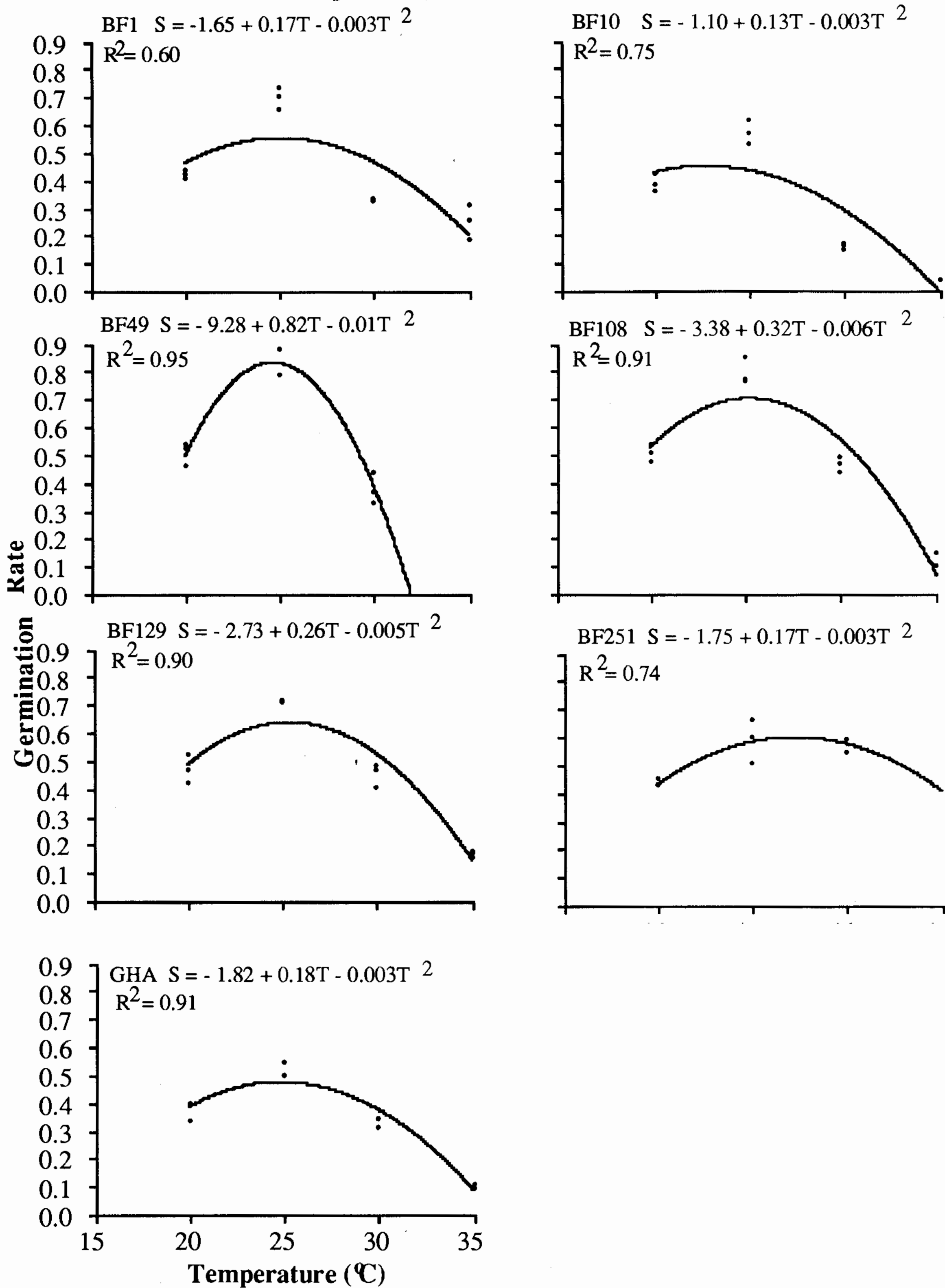
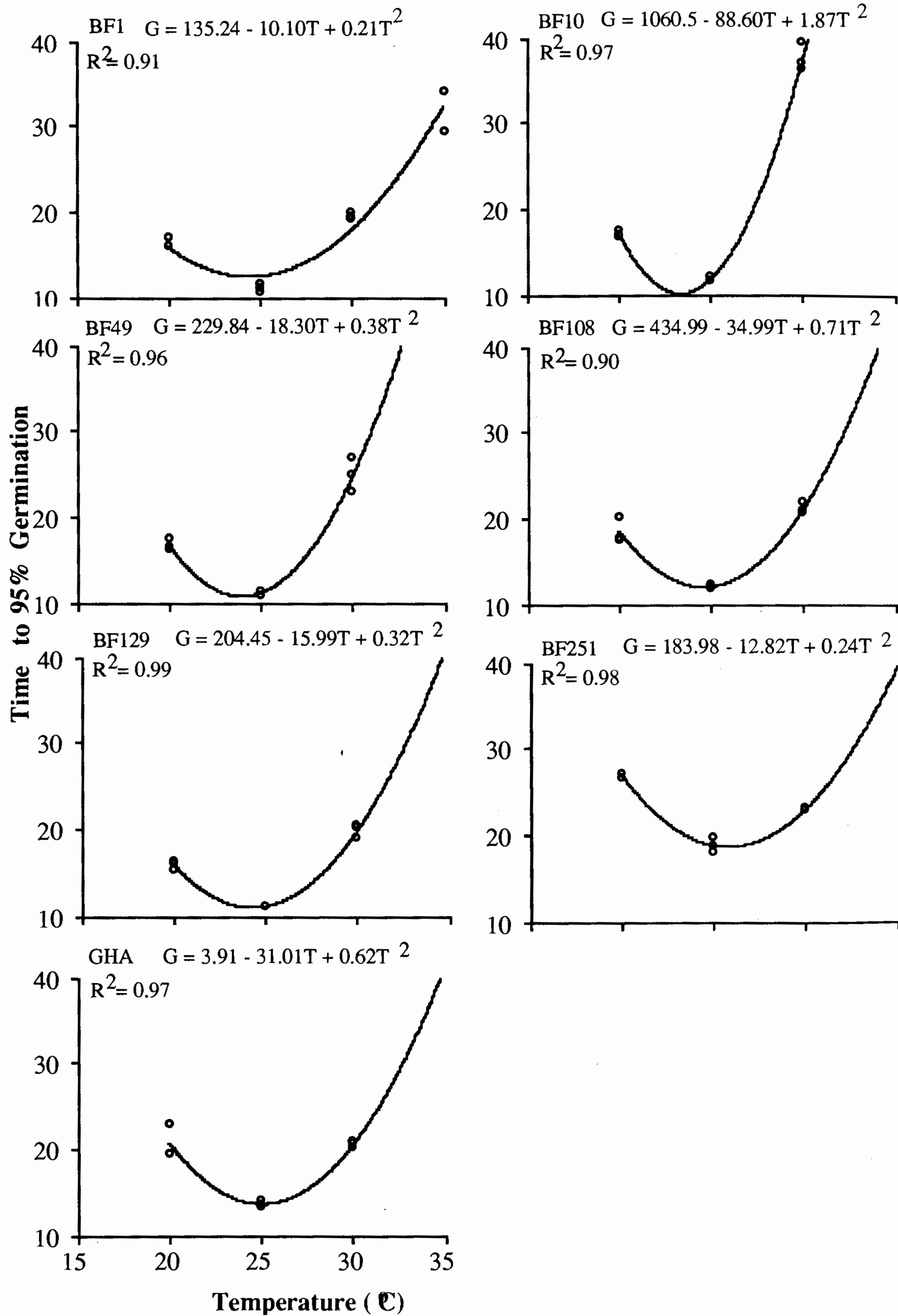


Figure 4.4. Effect of temperature on the time for 95% conidia to germinate of 7 isolates of *Beauveria bassiana*. G = Time to 95% germination, T = Temperature.



Discussion

The germination curves (Figure 4.2) provide a relatively good quantification of the effects of temperature on germination. The use of Logit-transformation also provided satisfactory estimates of the germination rate and the time to reach 95% germination (Figure 4.3, 4.4). However, because the use of zero was precluded, sufficient data were not obtained for these estimates with isolates BF49 and BF251 at 35°C. The lag phase was not estimated, as the first record at 6 h post-inoculation appeared to be well beyond the onset of germination.

The effects of temperature on the germination of the isolates were similar to those affecting germination of pycnidiospores of *Leptosphaeria maculans* (Vanniasingham and Gilligan 1988) and conidia of the Deuteromycetes *M. anisopliae* and *B. bassiana* (Hywel-Jones and Gillespie 1990). At 6 h post-inoculation, the percent germination was already adversely affected at 35°C. A temperature of 25°C appeared to be the most favorable regardless of the isolate. Differences in germination also occurred among isolates, consistent with intraspecific variation reported by Hywel-Jones and Gillespie (1990). In the hot climate of the Sahel, the temperature favorable for germination can be limited to a few hours so fast germinating conidia would be most useful.

The quadratic functions relating temperature with germination rate and time to 95% of conidia to germinate confirmed that 25°C and 35°C provided respectively the best and the worst conditions among the tested temperatures (Figure 4.3, 4.4). As field conditions are not always optimal, it is desirable to find isolates tolerant to a wide range of temperatures. In this regard, isolates BF1 and BF129, which displayed relatively good germination at all temperatures would be most preferred under Sahelian conditions. By contrast, isolates BF49 and BF251, which did not germinate at 35°C would be least desirable for practical use in the Sahel.

It is possible that germination success of the seven isolates may differ on the host integument or under field conditions from the results in Figure 4.2, because of the influence of other environmental factors including the cuticular antagonists and the ultraviolet radiation (Lane *et al.* 1991; Woods and Grula 1984). However, my results provide a temperature range over which the new isolates should be tested.

CHAPTER 5

EVALUATION OF THE PATHOGENICITY OF NEW ISOLATES OF *BEAUVERIA BASSIANA*

Introduction

Pathogenicity is the ability of a pathogen to cause disease; virulence is the quantitative measurement of this ability. The factors that govern pathogenicity and the differences in virulence between isolates of entomopathogenic fungi are not well understood. There are suggestions that enzymatic production at the cuticle level, secretion of toxins or rapid mycelial growth within the host might play a role (Charnley 1992; Bidochka and Khachatourians 1987, 1990; Dunn 1986; Hou and Chang 1985; Samuels *et al.* 1988a, 1988b; St Leger *et al.* 1987, 1988). Some isolates are host-specific, a fact suggesting that the *in vitro* assessment of these parameters cannot be used to select isolate. Pathogenicity bioassays are the only reliable and simple way of determining the activity of new isolates (Hall and Papierok 1982; Milner 1992; Moorhouse *et al.* 1993a). Also, with such bioassays, Koch's postulates can be fully tested.

In the quest of new isolates for insect control, Milner (1992) suggested a three-tiered strategy. The first tier is a rapid assessment of virulence using a relatively high dose of inoculum; under these conditions, "good" isolates are expected to kill 100% of the targeted organisms in a relatively short time. Isolates that show high virulence are used in the second tier consisting of a quantitative bioassay, involving different doses of inoculum. Only isolates with short lethal times are selected for testing in the third tier, wherein isolates are assayed under simulated field conditions of temperature, light and relative humidity.

The selection criteria in bioassays include the number of hosts that die from the infection and the length of time before death or the cessation of feeding. There have been

few reports on isolate selection in *B. bassiana* (Feng and Johnson 1990; Ferron and Robert 1975; Lai *et al.* 1982; McCoy *et al.* 1985). Experiments with grasshoppers evaluated only one isolate at a time (Johnson *et al.* 1988a; Johnson and Goettel 1993; Marcandier and Khachatourians 1987; Moore and Erlandson 1988). The bioassay methods used in the inoculation of grasshoppers included: 1) dipping in an inoculum preparation of known concentration (Marcandier and Khachatourians 1987); 2) injecting the inoculum into the gut (Moore and Erlandson 1988); and 3) depositing or spraying a spore preparation onto the integument or food (Goettel and Johnson 1992; Johnson *et al.* 1988a; Moore and Erlandson 1988). Water- or oil-based conidia suspensions and conidia mixed with bran were used. *Beauveria bassiana* infected hosts, regardless of the bioassay method or conidial formulation. However, oil-based formulations were most infective, possibly because oil provides good adherence of the inoculum to the hydrophobic cuticle of grasshoppers (Bateman *et al.* 1993; Prior *et al.* 1988). In addition, oil-based formulations facilitate the use of ultra-low volume and controlled-droplet applications (Bateman 1992; Bateman *et al.* 1992, 1993) that are suitable in the Sahel. Based on studies using *Melanoplus bivittatus* and *M. sanguinipes* (Appendix 4, 5; Goettel and Johnson 1992; Moore and Erlandson 1988), *B. bassiana* is pathogenic at doses ranging from 10^2 to 10^6 conidia per grasshopper; at a given dose, the pathogenicity varied with the method of inoculation, formulation and age of the test insects.

The objective of this experiment was to conduct a preliminary evaluation of the pathogenicity of new isolates of *B. bassiana* on the Senegalese grasshopper, *Oedaleus senegalensis* .

Materials and Methods

Seven isolates of *B. bassiana,* BF1, BF10, BF49, BF108, BF129, BF251 and GHA (Table 3.1; Chapter 4)) were tested using third instars of *O. senegalensis*. Prior to the test, the germination capacity of each isolate was determined to ensure that conidia were viable. Conidia were suspended in oil (Safflo® - Culinar Foods Inc., Toronto) at a concentration of 10^8 conidia per ml (Chapter 2). Grasshoppers were topically inoculated with 1 µl of spore suspension (10^5 conidia per grasshopper) using a micro-applicator (Chapter 2). There were eight treatments (seven isolates and one oil control). Each treatment consisted of 20 to 22 grasshoppers randomly selected from the colony. The experiment was repeated three times, for a total of 484 grasshoppers. The three replicates were run on separate days. The sex ratio was kept close to 1:1 in each treatment, depending on insect availability. The treated insects were maintained under a day:night regime of 25:20°C, 12:12 h light:dark and a relative humidity of 45 to 55%. Grasshoppers were fed fresh wheat leaves, the frass was removed and the number of dead insects was recorded daily for 10 days. Mycosis was checked on cadavers that were surface-sterilized (Chapter 2). The grasshoppers that survived were examined for the presence of blastospores in the haemolymph.

Observed percentages of mortality were corrected for control mortality by Abbott's formula. Rank transformations were used on percent mortality followed by Tukey's HSD test ($P < 0.05$)(Conover and Ronald 1981); the General Linear Models Procedure of SAS was used (SAS Institute 1989). For each treatment, the Weibull distribution was fitted to the data on a daily basis to estimate the lethal times. The SAS procedure LIFEREG was used to fit the distribution. Upper and lower 95% confidence limits were determined.

Results

All isolates caused mortality of *O. senegalensis* (Table 5.1). Mortality occurred within two days and increased to over 98% for all isolates by eight days post-inoculation. On average, 92.7% of cadavers exhibited fungal growth and sporulation within 48 h (Table 5.1). After 72 h, insects were covered with sporulating layers of the fungus (Figure 5.2). These cadavers appeared reddish and hardened shortly after death. By contrast, the remaining cadavers, including all the cadavers from the control group, were soft and discolored; they became black during incubation and no fungal growth or sporulation was observed. Infected grasshoppers were lethargic and ceased feeding at least 24 h before death. Molting did not prevent insects from dying and apparently did not retard infection. Examination of haemolymph of the surviving insects did not reveal the presence of blastospores.

The mean percentage of mortality in the control groups was 9.5%. The analysis of variance using the rank transformations by day and replicate for percent mortality revealed that there were significant differences in percent mortality among isolates; ANOVA, $P < 0.01$, 0.001 and 0.001 on day 3, 4 and 5, respectively (Table 5.2). Isolate BF129 was consistently among the most virulent with the highest early mortality and the shortest time (with isolate BF49) to 100% lethality; by contrast, isolates BF10 and BF251 were the least virulent with low early mortality.

The lethal times to achieve 50% mortality (LT_{50}) (Table 5.3) were in general agreement with the results for percent mortality (Table 5.2). The lethal times separated in three categories. Isolates BF129, BF108, BF49 and GHA had LT_{50}'s between 3.5 and 3.7 days, with BF129 performing best. Isolate BF1 was at an intermediate level at an LT_{50} of 4.2 days. Isolates BF10 and BF251 had the longest LT_{50}, with 4.8 and 4.6 days respectively.

Table 5.1. Daily mortality of *Oedaleus senegalensis* inoculated with seven isolates of *Beauveria bassiana* and oil (control) with number of cadavers exhibiting sporulation in parentheses.

	Mortality[1]							
Day	BF1	BF10	BF49	BF108	BF129	BF251	GHA	Control
1	-	-	1 (0)	1 (0)	1 (0)	-	3 (0)	2 (0)
2	5 (5)	1 (0)	8 (2)	-	12 (8)	7 (4)	4 (2)	4 (0)
3	17 (15)	8 (7)	7 (7)	21 (18)	18 (16)	5 (5)	20 (17)	-
4	28 (28)	15 (15)	33 (32)	32 (32)	17 (17)	14 (14)	26 (25)	-
5	9 (9)	20 (20)	14 (14)	8 (8)	15 (14)	26 (25)	7 (6)	-
6	1 (1)	11 (11)	-	1 (1)	-	8 (8)	3 (3)	-
7	1 (1)	8 (8)	-	-	-	1 (1)	-	-
8	-	-	-	-	-	1 (1)	-	-
9	-	-	-	-	-	-	-	-
% sp.[2]	96.7	96.8	96.8	93.6	87.3	93.6	84.1	0.0

[1] Cumul of three replicates, n = 63 nymphs. Topical application of 10^5 conidia per third-instar nymph.
[2] Percentage of cadavers exhibiting sporulation.

Figure 5.1. *Beauveria bassiana* growth and sporulation on cadavers of *Oedaleus senegalensis* (adults) at 24, 48 and 72 h post inoculation.

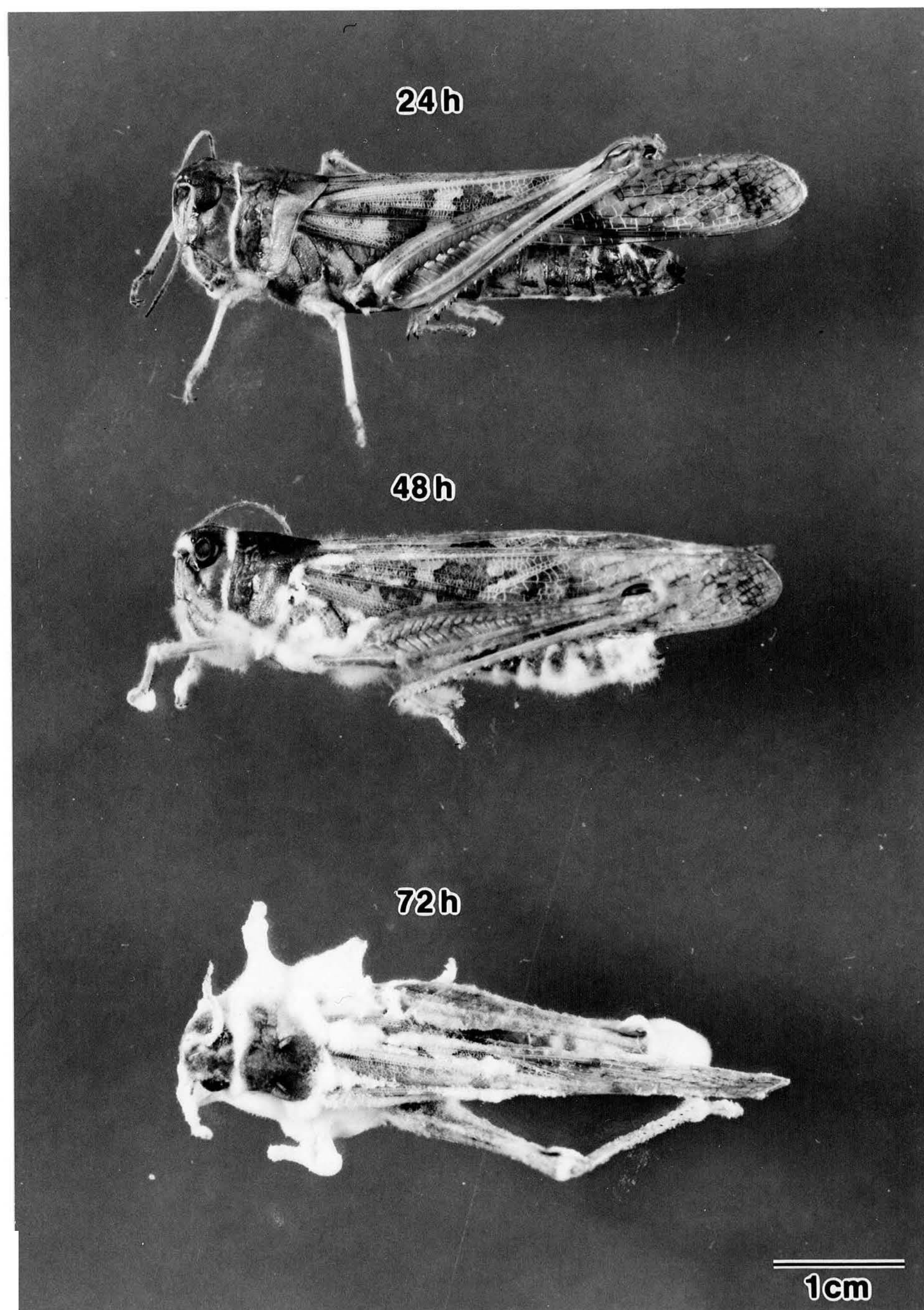

24 h
48 h
72 h
1cm

Table 5.2. Comparisons of mean percent mortalities of *Oedaleus senegalensis* on days 3, 4 and 5 after topical application of 7 isolates of *Beauveria bassiana*.

	Mean Percent Mortality[1]					
	Day 3		Day 4		Day 5	
Isolate	Mean (SE)		Mean (SE)		Mean (SE)	
BF1	28.0 (12.27)	ab	77.1 (1.78)	ab	85.9 (4.64)	bc
BF10	5.2 (0.02)	b	31.5 (0.03)	d	66.6 (3.49)	d
BF49	17.5 (28.03)	ab	75.3 (4.62)	bc	100	a
BF108	28.0 (4.62)	ab	84.1 (3.05)	a	98.2 (1.77)	a
BF129	42.0 (5.25)	a	73.6 (3.04)	c	100	a
BF251	8.7 (4.63)	b	35.0 (6.34)	d	78.9 (6.08)	c
GHA	36.7 (8.04)	ab	80.6 (3.52)	ab	94.6 (0.01)	b

[1] Percent mortality in experimental treatments was adjusted for control mortality. Rank transformation of percent mortality. On ady 3, 4 and 5, ANOVA, $P < 0.01$. Means with the same letter within a column are not significantly different; Tukey's HSD test ($P < .05$).

Table 5.3. Median lethal times (LT_{50}'s) in days in *Oedaleus senegalensis* inoculated with 7 isolates of *Beauveria bassiana* at a dose of 10^5 conidia per third instar.

		Confidence Limits (95%)	
Isolate	LT_{50}	Lower	Upper
BF1	4.2	3.9	4.5
BF10	4.8	4.5	5.2
BF49	3.7	3.5	3.9
BF108	3.6	3.4	3.9
BF129	3.5	3.3	3.8
BF251	4.6	4.4	5.0
GHA	3.7	3.5	4.0

Discussion

The observations of fungal growth and sporulation on cadavers (Figure 5.1) for all isolates under conditions of high relative humidity are consistent with similar observations on other insects (Dunn and Mechalas 1963; Ferron 1977; Moorhouse *et al.* 1993a; Schaefer 1936; Schaerffenberg 1964), including grasshoppers (Johnson and Goettel 1993; Johnson *et al.* 1988a, 1988b; Marcandier and Khachatourians 1987). In nature, horizontal transmission of the disease via sporulating cadavers requires high levels of moisture (Ferron 1977). The lack of visible external fungal growth on some insects that died within the first three days (Table 5.1) may indicate mortality caused by septicemia due to fungal proliferation (Marcandier and Khachatourians 1987) or bacterial infection of wounds caused by fungal penetration (Vey and Fargues 1977). Alternatively, the development of intestinal bacteria after host death may have obscured fungal growth (Ferron 1977).

The treatment of control insects with oil may have contributed to the observed mortality (Table 5.1). Goettel and Johnson (1992) reported that paraffin oil (Sunspray® Sun Refining & Marketing Co., Philadelphia) applied at 2 µl was more toxic to third-instar grasshoppers than sunflower oil (Safflo® Culinar Foods Inc., Toronto). Oil may be adhesive and disruptive to the waxy layer of the insect's epicuticle (Bateman *et al.* 1993) favoring fungal penetration or bacterial invasion. The use of sunflower oil and the relatively small volume of application (1 µl) may explain the low and acceptable mortality observed in this study.

Although all the isolates were shown to be pathogenic to *O. senegalensis*, they differed in virulence. Isolates BF10 and BF251 with low early mortality (Table 5.2) and the longest LT_{50}s (Table 5.3) are the least virulent; by contrast isolate BF129 was consistently among the most virulent with the highest early mortality (Table 5.2) and the lowest LT_{50} (Table 5.3). The dose of 10^5 conidia per grasshopper may have been too

high to achieve adequate discrimination between isolates. Such discrimination may be facilitated by retesting the five best isolates at low doses (McCoy *et al.* 1985).

Selection of preferred isolates might also be improved by testing them under suboptimal conditions. For example, Soares *et al.* (1983) found that three strains of *M. anisopliae* were equally virulent at 20°C, but were significantly different in virulence at 15°C. The rapid and synchronized germination of BF1, BF49, BF108, BF129 and GHA achieved in this study may have played an important role in increasing their virulence (Al-Aidroos and Roberts 1978; Daoust and Roberts 1982; Heale *et al.* 1989; Samuels *et al.* 1989). Isolate BF251 had slow germination and weak virulence, but rapid germination of isolate BF10 failed to import high virulence, suggesting that low virulence may have a different genetic basis in the two isolates. Jackson *et al.* (1985) found that the virulence of *Verticilium lecanii* was not necessarily correlated with *in vitro* germination. This suggests that, while tests of *in vitro* germination are useful, care should be taken when extrapolating from them with regard to virulence *in vivo*. From these observations, it can be concluded that: 1) the temperatures used were favorable to most isolates and probably did not permit differentiation on the basis of absolute mortality; and 2) high germination appeared to correspond with high virulence with the exception of isolate BF10.

Differences in virulence occur among isolates of *B. bassiana* from different geographic locations and hosts (McCoy *et al.* 1985). In this study, it was possible that genetic variation among isolates from fairly widespread locations (Table 3.1) may have been the cause of differences in virulence among isolates. There are also reports that isolates from the test species or closely related species are more virulent than others (Goettel *et al.* 1990; Latch 1976; Poprawski *et al.* 1985; Soares *et al.* 1983). These observations are consistent with the superior virulence of isolate BF129 which was recovered from *O. senegalensis*. However, Feng and Johnson (1990) suggested that neither the phylogenetic relationship nor the host origin is a reliable indicator of probable virulence. Certain strains possess high pathogenicity to previously unencountered hosts

(Moorhouse *et al.* 1993a; Prior 1990), as exemplified in this study by isolate GHA, which came from different geographical location than the test insect.

My results demonstrate that the tested isolates were similar in pathogenicity to *O. senegalensis* as other isolates of *B. bassiana* tested on grasshoppers (Appendix 4, 5; Goettel and Johnson 1992; Moore and Erlandson 1988). Isolates from the Sahel showed equal or greater virulence than the isolate GHA, known as a promising candidate for grasshopper control in North America (Johnson and Goettel 1993). However, in comparison to chemical pesticides, *B. bassiana* was slow to kill grasshoppers, requiring four to six days post-inoculation for the majority of insects to die. In field conditions, if applications are targeted on second or third instars, this level of virulence would significantly reduce damage on crops. Therefore, further studies to differentiate between isolates and to bring the best isolates to field trials are essential.

CHAPTER 6

EVALUATION OF ORAL AND TOPICAL APPLICATIONS OF *BEAUVERIA BASSIANA* AGAINST *OEDALEUS SENEGALENSIS*

Introduction

For migratory pests such as *Oedaleus senegalensis,* both oral and contact exposure to pesticides are needed. For instance, it is desirable that grasshoppers migrating into sprayed plots become infected with pathogens by feeding on foliage or baits. Alternatively, when resident grasshoppers became contaminated after pesticide sprays, migration will become too late to prevent infection. Both oral and contact routes are used by *Beauveria bassiana* to infect insects (Bao and Yendol 1971; Broome *et al.* 1976; Ferron 1978; Gabriel 1959; Miranpuri and Khachatourians 1991; Yanagita 1987; Siebeneicher *et al.* 1992), although infection through the integument is most common (Allee *et al.* 1990; Ferron 1978; Payne 1988; Pekrul and Grula 1979). There is evidence that grasshoppers become infected after feeding on *Beauveria*-contaminated food (Goettel and Johnson 1992; Johnson *et al.* 1988a; Lobo Lima *et al.* 1992; Moore and Erlandson 1988), but no detailed study has been done on the comparative effectiveness of oral and topical applications of conidia.

The objective of this experiment was to compare mortality caused by *B. bassiana* on *O. senegalensis* after ingestion of inoculum or after direct application of conidia to the integument.

Materials and Methods

Two isolates of *B. bassiana*, BF49 (Table 3.1) and GHA (Chapter 4), were tested. Dry conidia of the two isolates were supplied by Mycotech Corporation (Butte, Montana)

as a powder containing 8.7 x 10^{10} and 8.0 x 10^{10} conidia per gram for GHA and BF49, respectively. Conidia were stored at 4°C. Conidia suspensions were prepared using sunflower oil (Safflo® - Culinar foods Inc., Toronto) at concentrations of 2 x 10^8 conidia per ml (Chapter 2). Conidial viability of each isolate was predetermined to be over 85% after incubation at 25°C for 24 h on Sabouraud's dextrose broth medium supplemented with 2% yeast.

Third instars of *O. senegalensis* were placed individually in foam-stoppered glass vials (7 x 2 cm) and starved overnight. They were inoculated by allowing them to feed individually on a lettuce disk (5 mm diam.) treated with 0.5 µl of conidial suspension (10^5 conidia per grasshopper), or by topical application with an identical dose (Chapter 2). Grasshoppers that did not completely consume the lettuce within 12 h were discarded. The experiment comprised six treatments involving the two isolates (GHA and BF49), the two application methods (topical and oral) and two controls (oil alone). Nine to twelve nymphs were randomly selected from the colony and assigned to one of the six treatments. The experiment was repeated three times on different occasions. Food was supplied and the frass was removed from the containers (Chapter 2). Mortality was recorded daily for 18 days, and cadavers were checked for mycosis (Chapter 2). Examination of the haemolymph of survivors for the presence of blastospores was performed 22 days after inoculation.

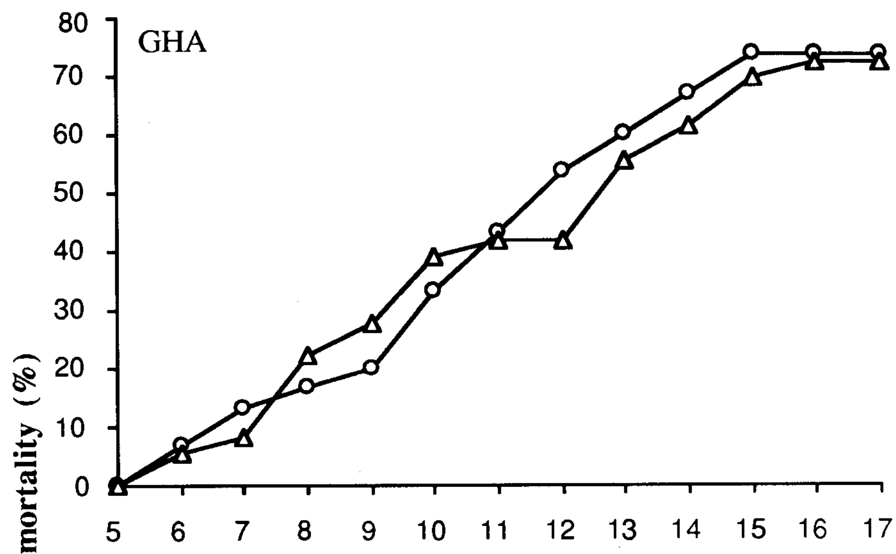
The analysis of variance was performed on final mortalities subjected to rank transformations (Conover and Ronald 1981). The General Linear Models Procedure of SAS was used. The Weibull distribution was fitted to the daily mortality data to estimate the median lethal times (LT_{50}) using the LIFEREG procedure of SAS (SAS Institute 1989). Upper and lower 95% confidence limits were determined.

Results

Mortality occurred within seven days of inoculation in all experimental treatments (Figure 6.1). There was no mortality in the two control groups. The final percent mortalities (day 18) were as follows: 1) isolate GHA, 73.3 and 72.2% for oral and topical applications, respectively; 2) isolate BF49, 76.0 and 71.3% for oral and topical applications, respectively. Over 98% of the grasshoppers that died showed fungal sporulation. The fungus emerged from the intersegmental areas within 24 h and sporulation occurred within 48 h. Examination of haemolymph 22 days post-inoculation revealed the presence of blastospores in three (two from topical treatment with isolate GHA and one from oral application of BF49) of the surviving grasshoppers .

There was no significant difference in final percentage of mortality (ANOVA, $P >$ 0.05). The LT_{50}s were: 1) isolate GHA, 13.4 and 13.7 days for oral and topical applications, respectively; and 2) isolate BF49, 14.1 and 14.9 days for oral and topical applications, respectively (Table 6.1). The intervals of LTs were overlapping.

Fig 6.1. Cumulative mortality of *Oedaleus senegalensis* after oral (—o—) and topical (Δ—) applications of *Beauveria bassiana,* isolates GHA and BF49 at a dose of 10^5 conidia per third instar.



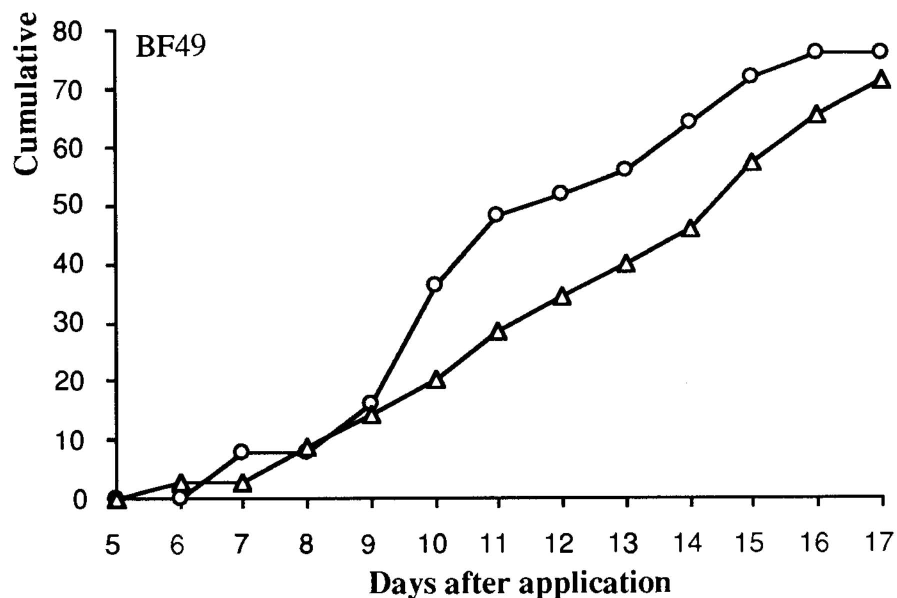


Table 6.1. Median lethal times (LT_{50}) in days in *Oedaleus senegalensis*, after oral and topical applications of 2 isolates (GHA and BF49) of *Beauveria bassiana* at a dose of 10^5 conidia per third instar.

Isolate		LT50	Confidence Limits (95%)	
			Lower	Upper
GHA	Oral	13.4	11.5	15.6
	Topical	13.7	11.9	15.7
BF49	Oral	14.1	12.0	16.5
	Topical	14.9	13.0	17.1

Discussion

Minimizing mortality in the control group, apparently by using a low (0.5 μl) dose of oil, allows a good assessment of virulence. The mortality with both isolates confirms their pathogenicity to *O. senegalensis*. The delay in the onset and the extended occurrence of mortality (Figure 6.1) in comparison to earlier experiment (Chapter 5) may have been caused by poor germination at 30°C. The day:night regime of 30:25°C may have been less favorable compared to the regime of 25:20°C used in the germination test (Chapter 4). Reduced germination would likely result in a low infection rate and the few germ tubes that penetrate the host would be exposed to hemocytic granulomas, structures which envelop short hyphae or blastospores (Vey and Fargues 1977). In the Sahel, temperatures may rise well above 30°C representing a limiting factor to the use of both isolates. However, because temperatures during the night are cooler, application at the end of the day may provide sufficient time and favorable temperature to allow germination.

It is surprising that insects containing blastospores in the haemolymph at 22 days post-inoculation were able to survive that long. No apparent symptoms of disease were observed in these grasshoppers. It is not known how grasshopper physiology is affected by sub-lethal doses of *B. bassiana*. Fargues *et al.* (1991) reported that fecundity was reduced in Colorado potato beetle *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) that survived infection of *B. bassiana*; They observed similar effects with sub-lethal doses. Because a low reproductive rate would reduce the rate of population growth and could limit outbreaks, these effects should be examined in subsequent studies.

The mortality achieved with both application methods confirms earlier findings that the fungus on baits can infect grasshoppers (Goettel and Johnson 1992, Johnson and Goettel 1993; Johnson *et al* 1988a; 1992; Moore and Erlandson 1988). However, the

mechanism of infection is not known. *Beauveria bassiana* is capable of producing pathogenesis by the digestive system (Bao and Yendol 1971; Broome *et al.* 1976) when conidia are ingested, but Ferron (1981) reported that fungal spores do not germinate in the digestive system. Siebeneicher *et al.* (1992) observed conidia germinating in the crop of the red imported fire ant, *Solenopsis invicta* (Hymenoptera: Formicidae). Infection through the mouth parts has also been reported (Siebeneicher *et al.* 1992; Veen 1966). Such infection could not be ruled out in the present study, as a portion of conidia may have attached to the mouth parts during feeding on lettuce. Another portion may have germinated in the gut (Moore and Erlandson 1988). However, Dillon and Charnley (1986a, 1986b, 1988) showed that bacteria in the gut of the desert locust *Schistocerca gregaria* (Acrididae: Cyrtacanthacridinae) produced toxins capable of inhibiting the infectivity of *Metarhizium anisopliae.* An abundant bacterial flora has been isolated from grasshoppers (Mead *et al.* 1988), but their interaction with *B. bassiana* is not known. It is also believed that rapid passage of food through the gut may reduce the incidence of infection (Allee *et al.* 1990). In the migratory grasshopper, *Melanoplus sanguinipes*, the egestion time is relatively short, 3.0 to 5.7 h (Mead *et al.* 1988), and may support such a conclusion. It is unlikely that within such a short period the fungus is able to germinate and penetrate the gut wall. Moore and Erlandson (1988) presented data indicating that oral infection was retarded in comparison to topical application or injection of conidia into the gut. By contrast, the results with the two-striped grasshopper, *Melanoplus bivittatus,* indicate that the delayed mortality was only pronounced at low doses (Appendix 4, 5).

My results confirm that *B. bassiana* is infective regardless of the mode of application, and suggest that conidia could be applied operationally against *O. senegalensis* either in baits or as contact sprays.

CHAPTER 7

GENERAL DISCUSSION

The potential use of *Beauveria bassiana* against the Senegalese grasshopper, *Oedaleus senegalensis,* which causes extensive damage on crops (Bernadi 1986), was investigated. The pest is controlled at present in the Sahel by ground and aerial applications of chemical insecticides. This type of control is expensive (Appendix 1; Brader 1988, cited by Prior *et al.* 1992; Kawasaki 1990) and has raised environmental concerns (Berger 1991; Everts 1990; Matteson 1992). The search for alternative methods of control that are efficient, economic and that minimize adverse environmental impact, recently focussed on the use of pathogens. However, studies on this subject have been limited to a few reports on the susceptibility of *O. senegalensis* to *Nosema locustae* , viruses (Henry *et al.* 1985a, 1985b) and *B. bassiana* (Johnson *et al* 1992; Lobo Lima *et al.* 1992). I was interested in *B. bassiana* because it may represent a cheap and efficient control agent for grasshoppers (Prior and Greathead 1989; Johnson *et al.* 1988a), which does not possess the deleterious attributes of chemical pesticides (Goettel *et al.* 1990; Vandenberg 1990).

My study had two main objectives . The first objective was to identify new isolates of *B. bassiana* in the native range of the pest. The reason for this approach is that isolates from the pest breeding areas are assumed to be virulent and well-adapted to local conditions. The second objective was to evaluate the new isolates with regard to the effects of temperature on germination and pathogenicity and the efficacy of oral and topical applications of conidia.

Ten new isolates of *B. bassiana* were recovered from soil and grasshoppers in Burkina Faso, in zones of intensive locust and grasshopper activity. This is the first record of the natural occurrence of *B. bassiana* in Burkina Faso. Although the recovery level may appear low, these findings represent a clear demonstration that *B. bassiana* is

present in the breeding grounds of the pest. Six isolates were tested and were found to be highly pathogenic to *O senegalensis*, causing over 98% mortality eight days after inoculation, with the majority of mortality occurring after three to five days post-inoculation. Because the insects ceased feeding at least 24 h before death, such mortality would result in a considerable reduction in damage. Future assessments of the fungus should include both host mortality and reduction of damage to vegetation (Johnson and Pavlikova 1986). Treatments against early instars would be most effective, because damage by *O. senegalensis* is caused mainly by fourth and fifth instars and young adults (Cheke 1990).

The most virulent isolate had an LT_{50} of 3.5 days. Differences in LT_{50} and germination success at different temperatures confirm that it is necessary to select the most virulent isolates, even if they come from the same region. To evaluate isolates further, the use of either small doses (McCoy *et al.* 1985), or of many levels of temperatures would be necessary, as fungal growth and development are temperature-dependent (Carruthers *et al.* 1985; Hall and Bell 1960, 1961; Walstad *et al.* 1970).

The lack of any significant difference in virulence between oral and topical applications of 10^5 conidia per third instar indicates that conidia could be applied as contact sprays in a carrier oil, or could be deployed via edible baits in a similar way as chemical pesticides. In field conditions, use of both contact and oral routes of infection would likely improve efficacy because the two methods would act in a complementary manner.

This thesis has demonstrated that selection of virulent isolates can be conducted on pathotypes collected in the Sahel, within the breeding zone of locusts and grasshoppers. My results show that there is excellent potential for the use of *B. bassiana* in the Sahel for the management of *O. senegalensis*.

Appendix 1. Amount and value of insecticides used for control of locusts and grasshoppers in the 6 Sahelian countries, 1986. (Rachadi 1986, cited by Kawasaki 1990).

Product	Country	Quantity (liters or tons)	Value (1,000FFr)	Area treated (ha)
Chlorpyrlphos-ethyl	Senegal	4,000 l	300	12,000
Deltamethrin	Chad	4,000 l	280	5,400
	Niger	2,000 l	140	
Diazinon 960	Burkina Faso	17,000 l	1,267	297,360
	Mali	56,400 l	4,130	
	Mauritania	10,500 l	754	
	Senegal	40,000 l	2,950	
Fenitrothion ULV500, 960, 1000, 500EC	Burkina Faso	59,780 l	4,349	2,099,000
	Chad	169,000 l	9,954	
	Mali	235,025 l	14,016	
	Mauritania	37,500 l	2,842	
	Niger	15,000 l	1,150	
	Senegal	287,320 l	17,111	
Fenitrothion 2%, 2.5%, 3%	Senegal	1,945 t	13,331	282,500
Fenitrothion + Fenvalerate	Senegal	20,000 t	1,583	20,000
Fenitrothion + Fenvalerate	Senegal	100 t	1,148	7500
Fenvalerate	Mauritania	4,000 t	400	8,000
HCH 25	Mali	514 t	5,140	42,834
Lindane 5%	Niger	300 t	2,100	50,000
Malathion ULV 960	Mali	10,000 l	367	474,666
	Mauritania	10,000 l	367	
	Senegal	212,500 l	8,535	
Propoxur 1%, 2%	Burkina Faso	187 t	1,429	328,000
	Chad	350 t	3,482	
	Mauritania	640 t	5,760	
	Niger	15 t	105	
	Senegal	700 t	4,620	
Total			114,676	3,627,260

$1 US. = 4 FF

APPENDIX 2. Haemocytometer Protocol

Use 22x30 cm No. 1 1/2 (M66041-24) canlab microscope cover glass with the improved Neubauer haemocytometer.

1. Place just enough suspension under the coverslip to flood the chamber (Do not float the coverslip off the platform).

2. Count the cells in the four corners and center chambers (use the center square of the haemocytometer) using the hand counter to keep track of cell numbers.

3. Divide the total count from all five chambers by five to get the average number.

4. Multiply this number by 25×10^4. This gives the cell number per ml.

It is often necessary to dilute suspensions in order to accurately count conidia in cells. In this case the cell number must be corrected using the dilution factor.

APPENDIX 3. Oatmeal Agar Medium

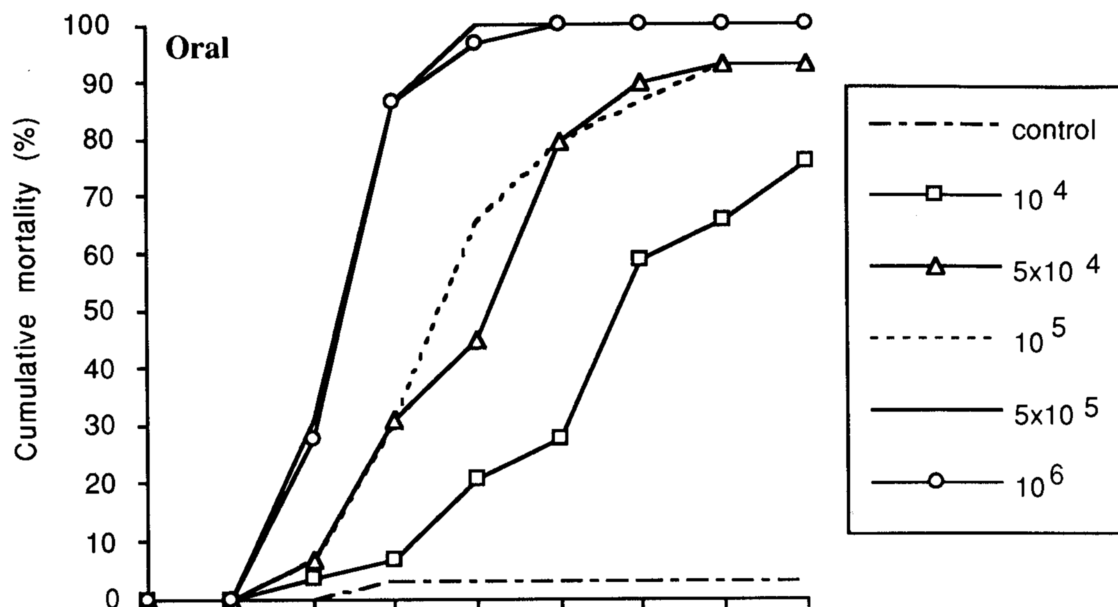
Selective medium for isolation of *Beauveria bassiana* and *Metarhizium anisopliae* (Chase *et al.* 1986)

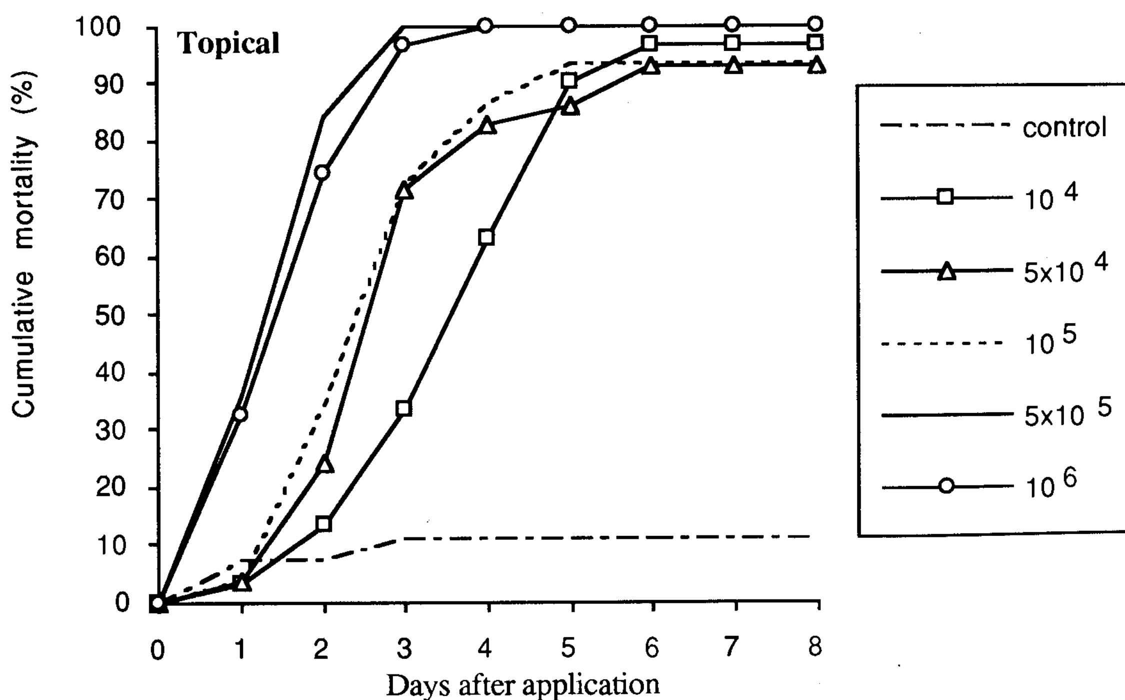
Oatmeal Agar (Difco)	17.5 g
Agar (BBL®)	2.5 g
Cyprex 65WP (dodine)	0.45 g
Crystal violet (Sigma)	2.5 mg
Penicillin G	0.2 g
Streptomycin sulfate	0.5 g
Distilled water	500 ml

Procedure

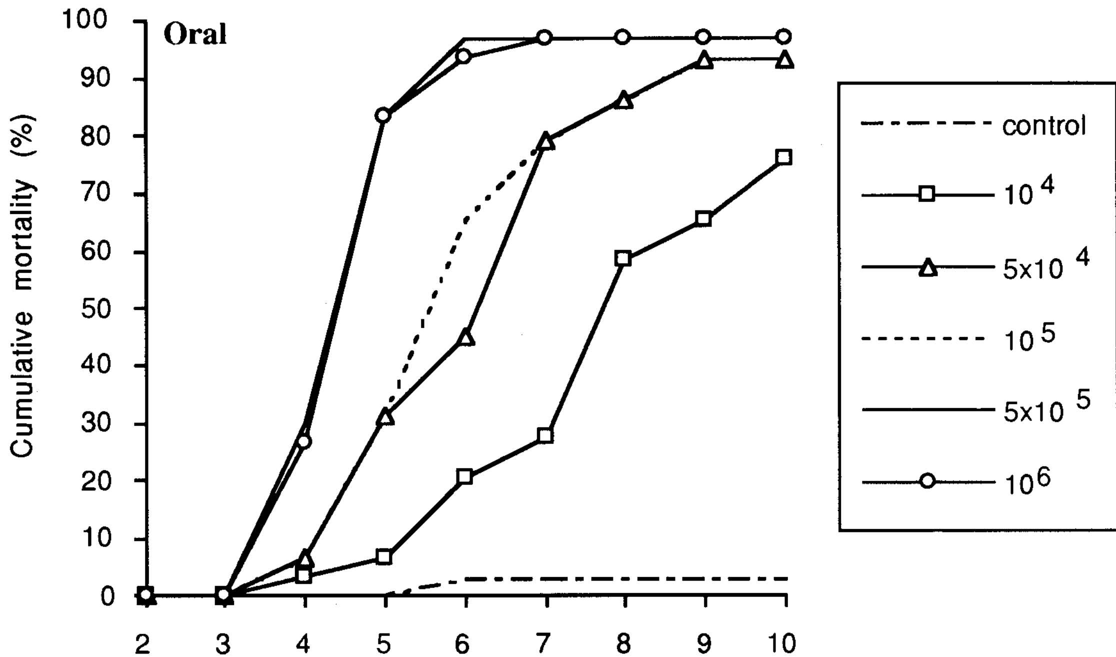
- Weigh Oatmeal and Agar
- Make a smooth slurry with *ca. 50* ml water in separate bowl
- Transfer to an Erlenmeyer flask and bring volume to 500 ml
- Stir vigorously while heating until the mixture boils
- Add Cyprex and 5 ml of the Crystal violet solution while heating
- Autoclave for 20 minutes at 15 psi
- Cool to *ca.* 50-55^{0}C and adjust the pH 5.6 with NaOH if necessary
- Add 2 ml of the antibiotic solution
- Mix the medium well to evenly distribute antibiotics
- Swirl flask while pouring plates to maintain the proper consistency
- Pour 20, 15x100 mm Petri plates per 500 ml batch.

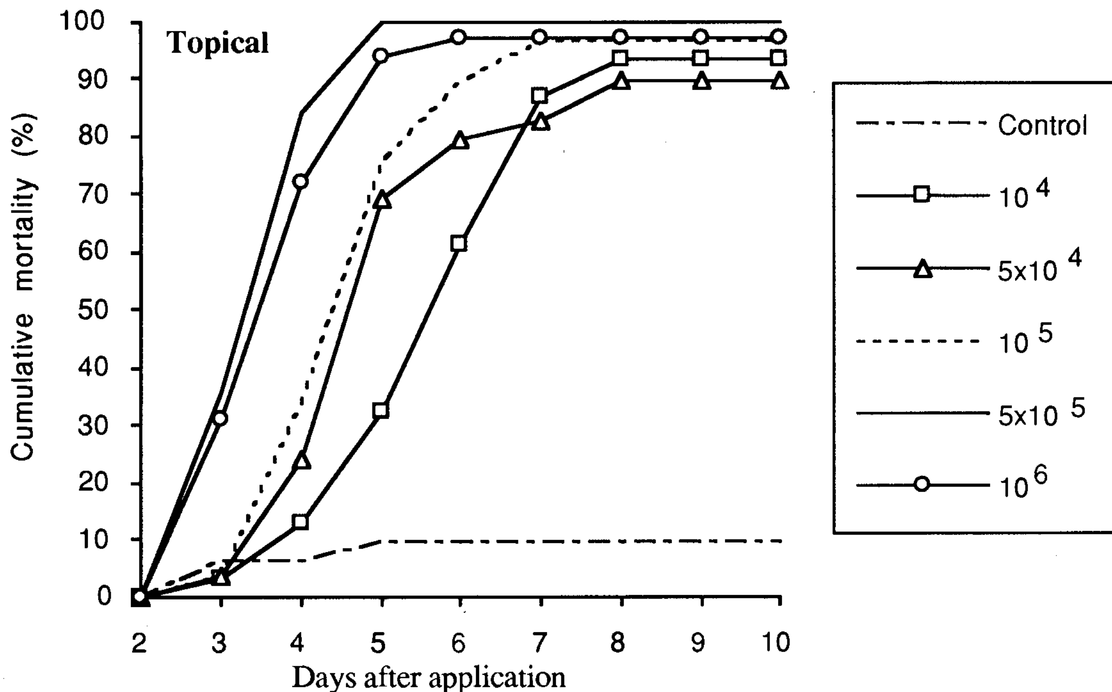
APPENDIX 4. Cumulative mortality in *Melanoplus bivittatus* nymphs after oral and topical applications of *Beauveria bassiana* (Isolate GHA) at doses of 10^4, 5 x 10^4, 10^5, 5 x 10^5 and 10^6 conidia per third-instar nymph. Conidia were suspended in sunflower oil (Safflo® -Culinar foods Inc., Toronto). Control consisted of oil alone. 30 to 32 grasshoppers per treatment.





APPENDIX 5. Cumulative mortality in *Melanoplus bivittatus* adults after oral and topical applications of *Beauveria bassiana* (Isolate GHA) at doses of 10^4, 5 x 10^4, 10^5, 5×10^5 and 10^6 conidia per grasshopper. Conidia were suspended in sunflower oil (Safflo® -Culinar foods Inc., Toronto). Control consisted of oil alone. 22 to 24 grasshoppers per treatment.





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