ENVIRONMENTAL FACTORS INFLUENCING THE EFFICACY OF *BEAUVERIA BASSIANA* AGAINST GRASSHOPPERS

by

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B.Sc., University of Alberta 1985

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ABSTRACT

Studies were conducted to elucidate environmental factors that influence the efficacy of the hyphomycete *Beauveria bassiana* as a microbial control agent of grasshoppers. Conidia exposed to sunlight on alfalfa and crested wheatgrass leaves were short-lived and were rapidly killed by exposure to ultraviolet-B radiation. Formulation of conidia in sunscreens prolonged their survival. Simulated rain also decreased conidial persistence. An oil-bait inoculation method was developed to facilitate testing of *B. bassiana*. Two conidial batches of *B. bassiana* that had previously demonstrated differential activity in field experiments, were assessed in laboratory and field studies in 1994. Both batches were highly virulent against *Melanoplus sanguinipes* in the laboratory, but no reductions in field populations or impact on specific taxa were observed despite the deposition of large numbers of conidia onto grasshoppers. However, substantial mycosis (>80%) was observed in grasshoppers sampled immediately after conidial application and kept in greenhouse cages. The prevalence of disease in treated grasshoppers decreased with the sampling date, but the onset of mycosis always occurred 3 to 4 days after collection, suggesting that environmental conditions were responsible for the poor field efficacy of *B. bassiana*. Grasshoppers elevate their body temperature by orientation to solar radiation (basking). The thoracic temperature of basking grasshoppers was 38 to 42°C, and in grasshoppers permitted to bask for 1 h per day, mycosis was decreased by >46%. Grasshoppers also exhibited a "behavioral fever" response to infection. In a field experiment in 1995, conditions were hot and sunny and *B. bassiana* did not reduce grasshopper populations. There was no evidence that *B. bassiana* proliferated in the hemocoels of grasshoppers collected up to 15 days after conidial application. Considerable mycosis was observed in grasshoppers placed in cages in the greenhouse, but not in grasshoppers confined in cages adjacent to the field plots; conditions of daytime temperature were similar but light exposure was reduced by 74% in the greenhouse. A

higher prevalence and more rapid development of disease were observed in grasshoppers placed in shaded cages (83-89%) than in cages exposed to full sunlight (0-15%) or protected from UVB radiation (1-43%) in the field. Conidial survival was equally enhanced in the shaded and UVB-protected relative to the exposed environment. Results of these experiments support the hypothesis that behavioral thermoregulation by grasshoppers can prevent infection and/or arrest mycosis caused by *B. bassiana*. Strategies that overcome the detrimental effects of high temperature on disease development and of UVB deactivation of conidia are required if *B. bassiana* is to be effectively used against acridids.

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This thesis is dedicated to my grandparents,

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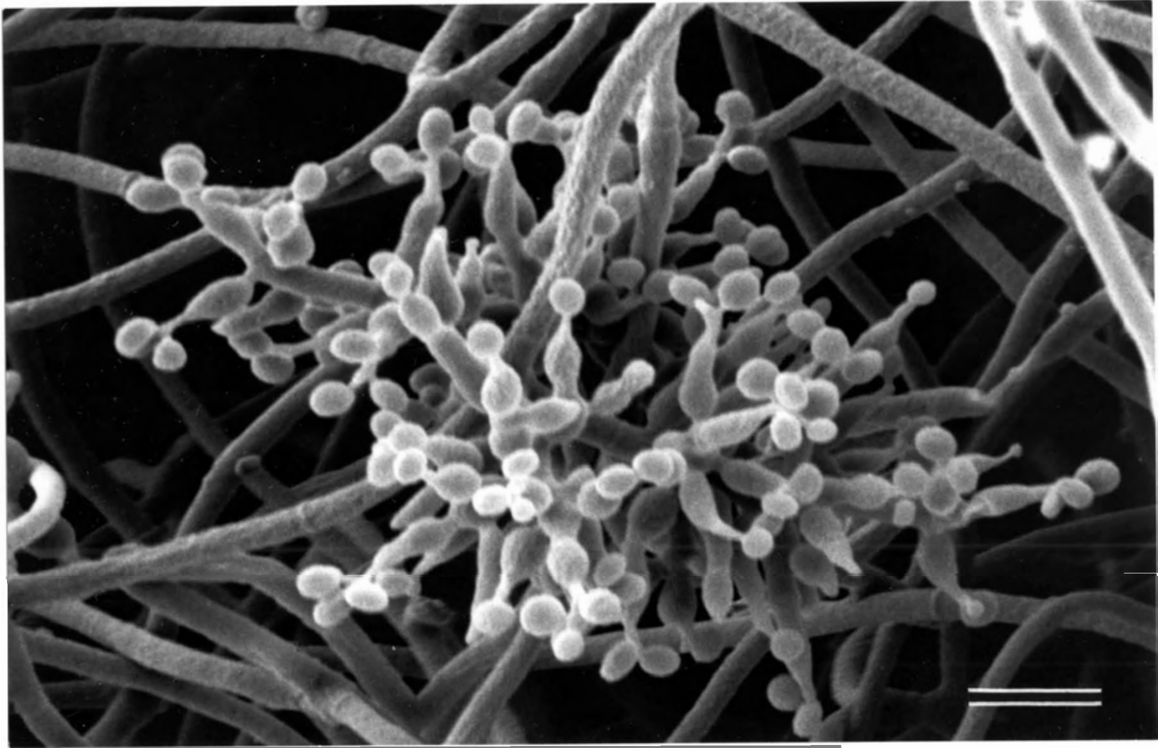
CHAPTER 1

GENERAL INTRODUCTION

Beauveria bassiana (Balsamo) Vuillemin is characterized by the formation of asexual spores (conidia) that are borne sympodially on ampulliform conidiogenous cells (Figure 1.1.1). The teleomorphic (sexual) state for *B. bassiana* is presently unknown although all members of the genus exhibit ascomycetous affinities. Since its sexual state is unknown, *B. bassiana* is placed in the formed class, Hyphomycetes, in the series Sympodulosporae (Barron, 1968). This hyphomycete is a cosmopolitan entomopathogen that is pathogenic to over 700 invertebrate species (Li, 1987; Goettel *et al.*, 1990). However, some isolates have a much more reduced host range, and isozyme analysis indicated considerable variation within the species complex (St. Leger *et al.*, 1992). Indications are that *Beauveria* populations are composed of many distinct genotypes which may be isolated by heterokaryon incompatibility (Couteaudiere *et al.*, 1994).

Grasshoppers and locusts (Orthoptera: Acrididae) are major pests of crops in arid agroecosystems, and in some years, grasshoppers are the chief insect pest on the Canadian Prairies (Johnson *et al.*, 1995). Existing control strategies rely almost exclusively on the use of chemical insecticides. However, recognition of the deleterious s of pesticides have prompted the development of alternative, less obtrusive management strategies. Microbial control agents may offer an alternative that will satisfy the demand for management strategies with low environmental impact. *Beauveria bassiana* has shown considerable potential for the management of insects world-wide (Feng *et al.*, 1994), and it is presently under investigation as a microbial control agent of acridids (Goettel *et al.*, 1995). There are several reports of field infection of acridids by *B. bassiana* (MacLeod, 1954; Humber and Soper, 1986; Li, 1987; Moore and Erlandson, 1988) and pathogenicity of *B. bassiana* towards grasshoppers has been demonstrated under laboratory (e.g., Marcandier and Khachatourians, 1987; Johnson *et al.*, 1988; Moore and Erlandson, 1988;

Figure 1.1.1 Conidiogenesis in *Beauveria bassiana*. Globose to subglobose, hyaline conidia formed sympodially on a rachis originating from ampulliform conidiogenous cells. Bar = 5 μm.



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Bidochka and Khachatourians, 1990) and field (e.g., Johnson *et al.*, 1992; Lobo Lima *et al.*, 1992; Johnson and Goettel, 1993) conditions. Although the precise route of invasion in grasshoppers is unknown, it is generally believed that infection usually occurs through the external integument (e.g., Inglis *et al.*, 1996a). After conidia attach to the host cuticle (Boucias *et al.*, 1988; Boucias and Pendland, 1991), they germinate and hyphae penetrate the host integument by a combination of enzymatic hydrolysis and physical pressure (Charnley, 1992; Goettel, 1992). High humidity was initially thought to be essential for infection, but evidence now suggests that infection occurs independent of ambient humidity (Ferron, 1977; Ramoska, 1984; Marcandier and Khachatourians, 1987) putatively due to a boundary layer of high humidity adjacent to the cuticle. Once in the hemocoel, hyphae may ramify throughout the host and/or the fungus may proliferate by forming yeast-like hyphal bodies (blastospores). The host responds to infection by encapsulation of the fungus (Roberts and Humber, 1981) which may involve the formation of hemocytic aggregations or nodules (Bidochka and Khachatourians, 1987; Hou and Chang, 1985). Although the mechanism by which *B. bassiana* overcomes the immune response of the host is uncertain, the rapid growth rate of *B. bassiana* (Hou and Chang, 1985), production of toxins (Roberts, 1981), and/or proteases (Bidochka and Khachatourians, 1987) have been suggested to play a role. Host death is thought to be caused by a combination of the action of toxins, physical obstruction of blood circulation, nutrient depletion and invasion of organs. After host death and under moist conditions, hyphae emerge and produce a sporulating layer on the cadaver surface from which conidia may be disseminated. Although *B. bassiana* is a facultative pathogen and it is frequently isolated from soil (Domsch *et al.*, 1980), it is unknown whether the fungus can proliferate saprotrophically in soil or if it is present as conidia in the proximity of insect cadavers.

There are three basic approaches in microbial control that are presently being pursued against grasshoppers. The first is the classical approach, in which a pathogen is

introduced to a new geographical area. With this approach, the goal is to establish the pathogen and provide long-term suppression of grasshopper populations. The second approach is the inoculative strategy, which has the goal of augmenting natural inoculum levels to initiate the development of an epizootic. The third strategy is the inundative or microbial pesticide approach, in which the pathogen is released in large quantities with the goal of short-term suppression of the pest population. *Beauveria bassiana* is being developed as an inundative microbial control agent of grasshoppers by Mycotech Corporation Inc. (Butte, MT). In some field trials the inundative application of conidia has resulted in substantial decreases in grasshopper populations (Johnson and Goettel, 1993). In others, results have been less promising (Johnson *et al.*, 1992; Lobo Lima *et al.*, 1992). Disease occurs only when a susceptible insect (suscept) is exposed to a pathogen in a suitable environment, and this interaction is commonly termed the "disease triangle". Microbial control occurs when humans manipulate the interaction (e.g., application of a pathogen) in a manner that favors the occurrence of an epizootic in an insect population; for this reason, humans are included in the disease model, and the interaction is commonly referred to as the "disease tetrad" (Figure 1.1.2). While all of the vertices are essential in the initiation and development of disease under natural or man-manipulated systems, the interactions that occur among each of the components do not always affect one another equally (Carruthers and Soper, 1987). Much of the research conducted on microbial control of insects with *B. bassiana* has emphasized the pathogen and has focused on the selection of virulent genotypes under controlled conditions and on the delivery of propagules to the host (targeting). Considerably less attention has been placed on the environment-pathogen-host interaction, and if entomopathogens such as *B. bassiana* are to be used to effectively manage insects, an understanding of this dynamic interaction is essential. Therefore, the primary objective of my research was to identify environmental factors that influence disease initiation and development in grasshoppers. Once these factors have been

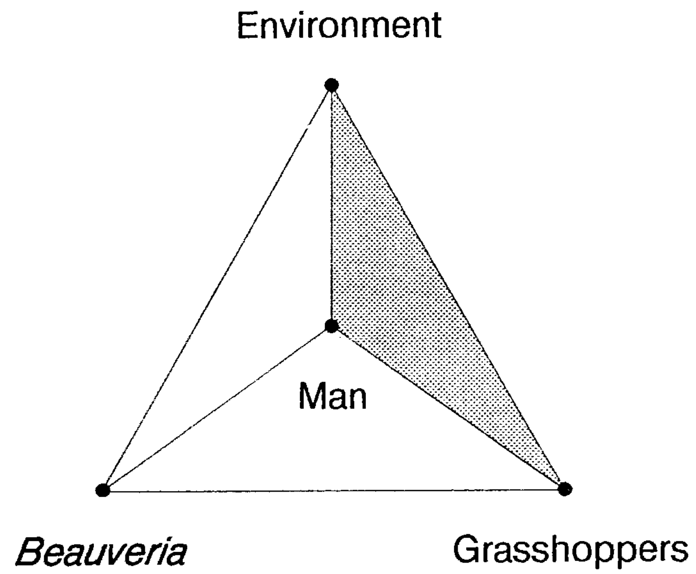

Figure 1.1.2 The disease tetrahedron representing interactions between the primary components responsible for disease development (*Beauveria bassiana*, grasshoppers and the environment) including the effects induced by man.

identified, biorational methods and deployment strategies can be devised.

This thesis contains six chapters. Chapter one is the general introduction. Chapter two describes the general materials and methods concerning the rearing of grasshoppers, preparation of conidial inoculum, assessment of conidial viability, isolation of conidia from leaves and coverslips, colonization of cadavers by *B. bassiana*, and general experimental design and statistical analyses. Chapter three describes the results of field experiments designed to identify factors that affect conidial persistence in epigeal habitats (Figure 1.1.3), and is divided into three sections: A) conidial survival on the leaves of two crops at two canopy positions (top and middle) was measured to determine the relative importance of temperature, relative humidity and solar radiation on conidial survival; B) sunscreen formulations were used to elucidate the importance of ultraviolet B radiation (280-320 nm) on conidial persistence; and C) the degree to which simulated rain caused the removal of conidia from leaves of two crops with different canopy structures (alfalfa and wheat) was measured. Chapter four describes a series of experiments in which the field efficacy of *B. bassiana* against grasshoppers was tested (Figure 1.1.3), and is divided into two parts: A) the development of a bioassay method to test relative virulence of *B. bassiana* strains; and B) the field evaluation of *B. bassiana* clones (conidial batches), which had previously demonstrated differential field efficacy. The objective of this series of experiments was to determine if the poor field efficacy of this fungus could be explained by the pathogen alone (e.g., lost virulence or poor targeting) or by other aspects of the disease interaction. Chapter five presents a series of experiments to determine if the elevation of grasshopper body temperatures (i.e., behavioral thermoregulation) influences disease development (Figure 1.1.3), and is divided into two parts: A) controlled environment studies; and B) field evaluation. Chapter six is devoted to the general discussion where the salient findings of the research are summarized and are discussed relative to methods which may be used to overcome environmental constraints.

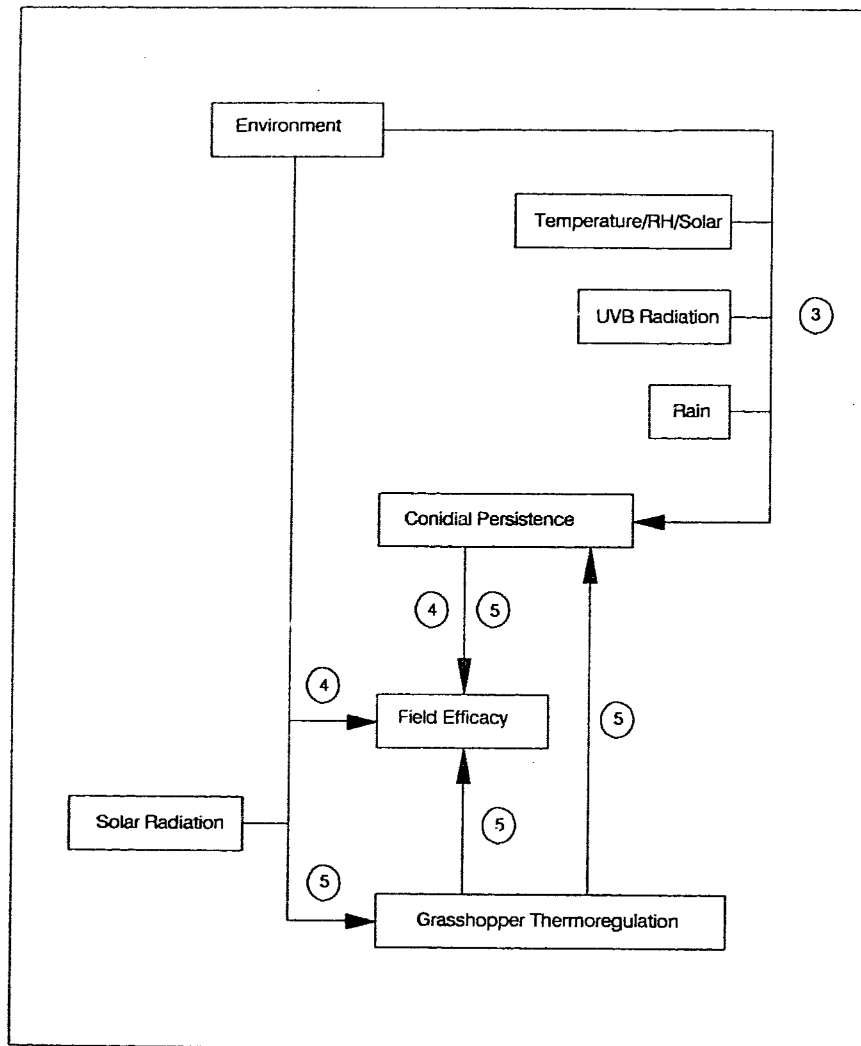

Figure 1.1.3 Studies conducted with *Beauveria bassiana* against grasshoppers. Chapter three experiments investigated the effects of solar radiation (UVB), temperature, relative humidity and rain on conidial persistence. In chapter four, the efficacy of *B. bassiana* against grasshoppers in controlled and field experiments was studied. Chapter five studies investigated the effects of grasshopper thermoregulation and conidial deactivation by UVB radiation on the efficacy of *B. bassiana.* Numbers in circles refer to thesis chapters.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 GRASSHOPPERS

The migratory grasshopper, *Melanoplus sanguinipes* (Fabricius), is an oligophagous grasshopper that is a cosmopolitan pest of cultivated crops and grasses in North America (Vickery and Kevan, 1985). Nymphs of a non-diapause strain (Pickford and Randell, 1969) were used in most experiments. Eggs were hatched in sand-vermiculite substrate and nymphs were reared on a diet of bran and wheat leaves at an ambient temperature of 20 to 25°C; a vertical heat gradient was produced in the rearing cage (40 x 40 x 30 cm) by a 25-W incandescent light bulb. In several experiments, nymphs from field-collected *M. sanguinipes* grasshoppers (F_1 laboratory generation) were used. Adults collected in the fall were placed in cages in the laboratory, permitted to copulate and oviposit into a sand substrate. Egg pods were sifted from the sand, placed in clean moist sand, held at room temperature (20 to 25°C) for 40 days to permit embryogenesis and then transferred to 5°C for a minimum of 90 days to complete diapause. After the cold treatment, eggs were allowed to hatch in the sand-vermiculite substrate and nymphs were reared in the same manner as non-diapause nymphs. Unless indicated otherwise, third-instars were used in all experiments.

2.2 PREPARATION OF CONIDIAL INOCULUM

Dry conidia of *B. bassiana* (strain GHA supplied by Mycotech Corporation Inc) were maintained at -10°C until used. Conidia contain hydrophobic proteins in their cell walls (hydrophobins) and to uniformly suspend them in water, it was necessary to use mechanical suspension methods. Unless indicated otherwise, two methods were used to suspend conidia in water and oil: for large volumes (> 5 mL), conidia were suspended using Potter-Elvehjem tubes; and for small volumes (≈ 1 mL), conidia were suspended using Kontes micropestles (Appendix 1.1). Once conidia were uniformly suspended, concentrations were

estimated with a hemocytometer and adjusted as required.

Conidial viability was measured by germinating conidia on potato dextrose agar (PDA) amended with Benlate (Appendix 1.1) unless indicated otherwise. Immediately prior (*ca* 1 or 2 days) to the preparation of inoculum, conidia were suspended in water (10^7 conidia/mL) and 100 μl of the suspension was spread onto the surface of PDA in a 8.5 cm-diam petri dish. To prevent overgrowth of hyphae from early germinating propagules, 0.005% Benlate 50 WP (a.i. benomyl; DuPont, Wilmington, DE) was incorporated into the medium; the medium also contained 0.04% Pen G (Sigma Chemical Co., Mississauga, ON), and 0.1% streptomycin sulfate (Sigma) to prevent growth of bacteria. At low concentrations, benomyl permits germ-tube formation but prevents hyphal development. Once the water had absorbed into the medium, cultures were incubated at 25 ± 1°C in the dark for a specified period (usually 24 hr). Conidia were fixed in lactophenol by placing a couple of drops of the fixative on the surface of the medium and then placing a glass coverslip over the fixed area. The fixed area was then examined microscopically. Sample conidia ($n \geq 500$ per culture) were considered viable if a germ-tube was greater than two times the diameter of the conidium.

2.3 RECOVERY OF *BEAUVERIA BASSIANA* FROM LEAVES AND COVERSLIPS

Conidia were isolated from leaves and glass coverslips (13-mm diam) by washing. Following collection, leaves or coverslips were stored at 5°C for a maximum of 48 h. Leaves were cut aseptically into pieces approximately 1 cm long, and leaf pieces (10 to 20) or coverslips from individual treatments were combined in 5 mL of 0.01 M phosphate buffer (pH 7.0) amended with 0.05% Tween 80 in 20 mL vials. The vials were vigorously shaken for 2 h using a rotary shaker (300 rpm), the wash solution was diluted 2 to 3 times in a 10-fold dilution series, and 100 μl aliquots for each dilution was spread onto a semi-selective oatmeal-dodine agar medium (Appendix 1.2) based on the medium of Chase *et al.* (1986). The cultures were incubated at 25°C for 6 to 7 days and the number of colony-forming

units (cfu) of *B. bassiana* were counted at the dilution yielding 30 to 300 cfu per dish (Appendix 1.3). Following washing, the total area of the leaf pieces was determined with a leaf area meter (Model 3100, Li-Cor Inc., Lincoln, NE) and the mean numbers of cfu per cm^2 of leaf or coverslip area were calculated. Representative colonies were isolated, grown in slide culture and examined microscopically to confirm the identity of *B. bassiana* (Figure 1.1.1).

2.4 COLONIZATION OF CADAVERS

Nymphs that died during the course of an experiment were placed on moistened filter paper in a petri dish. Nymphs that subsequently produced hyphal growth of *B. bassiana* after 3 to 7 days were considered to have died from mycosis. Those not producing hyphal growth were classed as "other mortality". Nymphs that survived the duration of the experiment were killed by freezing and similarly placed on moistened filter paper; freezing has no effect on *B. bassiana* conidia (Inglis *et al.*, 1996a).

2.5 EXPERIMENTAL DESIGN AND STATISTICAL ANALYSES

Most analyses were performed using the GLM, REG, LIFEREG and TTEST procedures of SAS (SAS Institute Inc., 1988). Residuals were always plotted against predicted values and where necessary the appropriate transformations were used to normalize variances. Standard errors of the means were calculated from individual treatments and are usually presented in parentheses following the mean. Unless indicated otherwise, experiments were analysed as randomized complete block designs (RCBD). For repeated measure data (e.g., disease progress and conidial persistence data), split-plot models in time were used (Gomez and Gomez, 1984). In most instances, a Box correction was used as a conservative test for the time and time by treatment interactions (Milliken and Johnson, 1984); the Box correction reduces the degrees of freedom for time, the time by treatment interaction, and the residual error(time) by time-1.

CHAPTER 3

PERSISTENCE OF *BEAUVERIA BASSIANA*

3.1 INTRODUCTION

Control of a variety of insects by *B. bassiana* has been obtained by application of conidia in epigeal habitats (Feng *et al.*, 1994). However, the persistence of conidia in these habitats is generally poor. Since a threshold of inoculum is required to cause disease in insects, the poor survival of conidia could seriously decrease the efficacy of conidia applied on foliage by limiting the time that conidial populations remain above inoculum thresholds. Environmental factors that limit the survival of conidia of *B. bassiana* in epigeal habitats have not been extensively studied and if these habitats are to be targeted for the management of insect populations with *B. bassiana*, identification of the factors limiting conidial persistence is necessary.

This chapter is divided into three additional sections (3.2 to 3.4), each examining one aspect of *B. bassiana* conidial persistence. In section 3.2, the influence of canopy and crop type (alfalfa or crested wheatgrass) on penetration, coverage and persistence of conidial populations in a field environment was investigated. Daoust and Pereira (1986a) suggested that exposure to sunlight was the most important factor limiting survival of *B. bassiana* conidia on cowpea foliage, and the field experiment conducted here was designed to identify factors, including sunlight, that have an effect on conidial persistence in a field setting. Recently, several entomopathogens have been applied in oil at ultralow volumes (Pan *et al.*, 1988; Delgado *et al.*, 1991; Bateman *et al.*, 1992; Johnson *et al.*, 1992; Lobo Lima *et al.*, 1992) in an attempt to increase their efficacy. The application of entomopathogens in oil allows decreased spray volumes. However, the possibility of increased adhesion of propagules either to plant or insect cuticles, increased resistance of propagules to suboptimal environmental conditions, and the possible predisposition of insects to infection by conidial applied in oil are unknown. The objectives of this study

were to: 1) measure the persistence of conidia of *B. bassiana* on phylloplanes of crested wheatgrass and alfalfa, crops with substantially different architecture; 2) compare the survival of conidia at the surface of, and within the canopy of the two crops; 3) contrast the application efficacy and persistence of conidia applied in water and an oil emulsion at high volumes, and of conidia applied in oil at ultralow volumes; and 4) determine the infectivity of applied conidia to grasshopper nymphs fed foliage immediately and 2 days after application of conidia.

In section 3.3, the impact of UVB radiation on the persistence of *B. bassiana* conidia was investigated in the field using sunlight protectants. The UVB (280-320 nm) component of sunlight is detrimental to all microorganisms (Tevini, 1993). However, a number of substances have been used to protect and enhance the persistence of entomopathogenic baculoviruses (Ignoffo and Batzer, 1971; Shapiro *et al.*, 1983; Martignoni and Iwai, 1985; Shapiro, 1989; Shapiro, 1992), *Bacillus thuringiensis* (Morris, 1983; Cohen *et al.*, 1991), the entomopathogenic fungus, *Metarhizium flavoviride* (Moore *et al.*, 1993) and the nematode, *Steinernema carpocapsae* (Nickle and Shapiro, 1992), exposed to artificial UVB radiation. The efficacy of UVB protectants for increasing the persistence of fungal propagules in a field environment has not been previously studied. Therefore, the objectives of this study were to: 1) test and compare water and paraffinic oil formulations for UV protection of *B. bassiana* conidia; 2) screen a number of potential UV protectants in the laboratory; and 3) test their efficacy in a field environment.

The occurrence of precipitation after conidial application is presumed to reduce conidial persistence (e.g., Gardner *et al.*, 1977; Daoust and Pereira, 1986b; Johnson *et al.*, 1992). In section 3.4, the objective was to test this possibility in a controlled setting by quantifying the effect of simulated rain on removal of *B. bassiana* conidia from wheat and alfalfa leaves.

3.2 PERSISTENCE OF CONIDIA ON PHYLLOPLANES OF CRESTED WHEATGRASS AND ALFALFA[1]

Materials and Methods

Conidial formulation. The number of *B. bassiana* conidia/g was estimated using a hemocytometer. Immediately prior to use, conidia were suspended in either a paraffinic formulation oil (Mycotech #9209), in a 5% formulation oil - water emulsion (v/v), or in water with 0.05% Tween 80 (Sigma). The target concentrations of conidia were 1.0×10^{10} conidia/mL in oil, and 5.0×10^{8} conidia/mL in water-Tween and the oil emulsion formulations. For both the water-Tween and the oil emulsion formulations, conidial suspensions were homogenized in a Waring blender for 1 min and then passed through a double layer of cheese cloth. Concentrations of conidia in water-Tween were estimated with a hemocytometer. Germination percentages of conidia in water-Tween were determined by placing approximately 20 μl of the conidial suspension in 100 μl of Sabouraud's dextrose broth, incubating the conidia for 12 h and counting the number of conidia forming germ-tubes; a minimum of 200 conidia were used. Germination percentages were consistently greater than 80% prior to use.

Field plots. Adjacent fields of alfalfa (lucerne, *Medicago sativa* L.) cv. Beaver and crested wheatgrass (*Agropyron cristatum* L.) were established at the Agriculture and Agri-Food Canada (AAFC) Research Centre near Lethbridge, AB. Fields of alfalfa used for trials one, two and three, were established in 1991, 1989 and 1991, respectively. Fields used in trial one and two had row spacings of 35 cm, whereas in trial three the row spacing was 17.5 cm. Alfalfa plants were cut 20, 37, and 24 days prior to the application of conidia in

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[1]A version of this section has been published as: "Persistence of the entomopathogenic fungus, *Beauveria bassiana*, on leaves of crested wheatgrass and alfalfa" Inglis, G.D., M.S. Goettel and D.L. Johnson. 1993. Biological Control 3:258-270.

trials one, two and three, respectively. The field of wheatgrass was seeded in 1989 in 17.5 cm wide rows at a rate of 11 kg/ha. The wheatgrass was last cut in June, 1991.

Three replicate plots per formulation, each measuring 3.0 m by 1.5 m were established in each field. At the time of conidial application, crop height was measured and all foliage within a 0.5-m^2 quadrat was harvested and leaf areas measured with a leaf area meter. Leaf area indices (LAI) were determined by dividing the total leaf areas by the soil surface areas.

Application of conidia. Conidia in the water-Tween and the oil emulsion were applied at a rate of 100 L/ha (45 mL/plot), using a compressed CO_2 (40 PSI) bicycle sprayer (R&D Sprayers Inc., Opelousas, LA) equipped with three 015-F80 nozzles (Lurmark Ltd., Longstanton, Cambridge, UK). To obtain the optimal spray pattern, the height of the boom was adjusted according to the height of the wheatgrass or alfalfa canopy. Conidia in oil were applied with a ultralow volume (ULV) spinning disk sprayer (Micron Sprayers Ltd., Bromyard, UK) operated at 7,000 rpm. Conidia in oil were applied at a rate of 5 L/ha (2.25 mL/plot). To reduce drift, plots were surrounded with a 1.8 m high sheet of polyethylene during the spray application. Water and oil sensitive papers were randomly placed at the top of the canopy and on the soil surface in selected plots for each crop to evaluate the spray distribution. Target concentrations of conidia in all formulations were 2.25×10^{10} conidia/plot (5.0×10^{13} conidia/ha). Conidia were applied at times of low wind velocity (< 4 m/s) on the evenings of July 15, August 2 and August 31, 1992, for trials one, two and three, respectively.

Leaf collection and preparation. Ten leaves/leaflets from the top and middle of the canopy (sampling height was determined at the time of application) were arbitrarily collected from the center of each of the three replicate plots (center row). Times of sampling were immediately after (time 0), and 0.5, 1, 2, 4, 8 and 16 days post-application. At the 8 and 16 day sample times, care was taken to choose older leaves present at the

time of conidial application. Within one hour of collection, leaves were returned to the laboratory in plastic bags and stored at 5°C for a maximum of 12 h. Each of the 10 leaves per sample were aseptically cut across the laminae into pieces of approximately 1 cm and 0.5 cm long for wheatgrass and alfalfa, respectively.

Microbiological analysis. Conidia were isolated from the leaf pieces using the wash method and cfu per cm^2 were determined on oatmeal-dodine agar. The efficacy of the wash procedure was investigated by recovering known quantities of conidia from the surfaces of alfalfa leaflet segments. Equal weights of conidia were suspended in either water-Tween, the oil emulsion or oil, and 5 μl aliquots for the water-Tween and oil emulsion and 2 μl of the oil formulation were placed on each of 30 leaflet segments (10 in each of three replicates). The leaflet segments were then washed, and cfu enumerated on oatmeal-dodine agar. Simultaneously, conidial concentrations in the original formulations were enumerated by diluting the suspension three to five times in a 10-fold dilution series replicated three times, spreading 100 μl aliquots from each onto oatmeal-dodine agar and enumerating cfu at 6 to 7 days. The recovery efficacy was calculated as a percent cfu of the original concentration.

Grasshopper bioassay. Nymphs of a non-diapause strain of *M. sanguinipes* were used. Third- to fourth- instars were placed in plastic containers (20 nymphs per replicate) and maintained in a controlled environment chamber (CEC) at a 16/8 h light/dark photoperiod and 25/20°C day/night temperature. The protocol required that nymphs be fed either alfalfa leaflets or wheatgrass leaves collected 0 and 2 days post-application of *B. bassiana*. However, due to inclement weather, application of conidia was delayed 24 h in trial two. This necessitated that nymphs fed leaves immediately after application of conidia, be starved for 12 h, fed *ad libitum* for 12 h, and then starved for an additional 12 h. Nymphs fed leaves at 2 days post-application of conidia were starved for 12 h. Following the starvation period, nymphs were fed 100 g (at 12 h intervals) of arbitrarily

collected leaves from each crop at each sample time. Nymphs were then maintained on a diet of wheat and lettuce leaves for 11 days. During this period dead nymphs were removed daily, placed on moistened filter paper and those colonized by *B. bassiana* recorded.

Scanning electron microscopy (SEM). Leaf pieces from the top of the wheatgrass and alfalfa canopies in trial three were arbitrarily selected at 0, 1, 2 and 4 days post-application. Leaves were immediately fixed in 2% glutaraldehyde in 0.05 M phosphate buffer. Replicate leaf samples also obtained immediately after application, were air-dried. Glutaraldehyde-fixed leaf segments were dehydrated in ethanol and critical-point dried in liquid CO_2. Both air-dried and critical-point dried specimens were sputter coated with gold and examined with a Hitachi S-570 scanning electron microscope at an accelerating voltage of 10 kV.

Weather data. Mean hourly temperatures and relative humidities at a height of 1 m above the soil surface were measured at a weather station located adjacent to the field plots (Figure 3.2.1). Incoming solar radiation (300-2800 nm), precipitation, wind direction and velocities also were recorded. In addition, mean hourly temperatures and relative humidities at the mid-canopy position of both crops were recorded with CR21 microloggers (Campbell Scientific Co., Edmonton, AB).

Statistical analyses. All experiments were arranged as RCBD's. To normalize the conidial population data, $\log_{10}$ transformations were used. For each trial and crop, experiments were analyzed separately as a split plot in time with three levels of formulation, two levels of canopy position and seven sample times. When significant differences ($P \leq 0.05$) were observed for a formulation, the data were analyzed at each sample time as a factorial experiment, and Duncan's multiple range test ($\alpha = 0.05$) was used to separate means. Where indicated, formulation treatments were combined ($n = 9$). Individual formulations or combined formulation data were fitted to linear models within each canopy

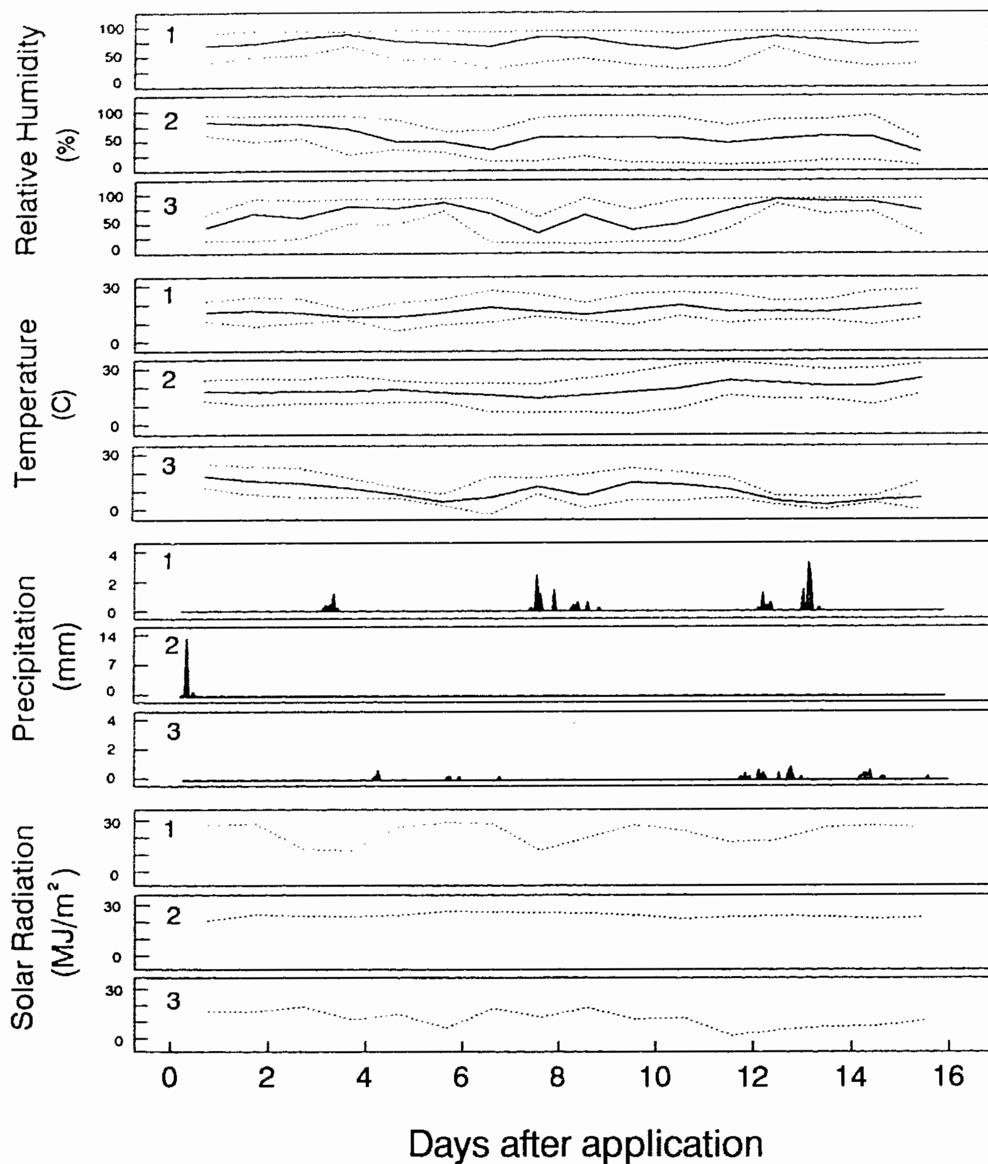


Figure 3.2.1 Daily maximum, minimum and mean hourly relative humilities; daily maximum, minimum and mean hourly temperatures; hourly precipitation; and daily cumulative hourly incoming light (300-2800 nm) for trials one, two and three.

position. At the top of the wheatgrass canopy, the best fit of the regression lines required $\log_{10}$-transformation of the x-axis (time); for all other regressions the x-axis was not transformed. In addition to time, best fit regressions were conducted against cumulative degree days (>10°C) and cumulative solar radiation. Comparisons of slopes and initial populations (y-intercepts) between trials for each crop were conducted using analysis of covariance with time used as the covariate (SAS Institute Inc., 1991). In most instances, no *B. bassiana* conidia were isolated from leaf segments collected from untreated plots and when conidia were isolated , it was at very low levels (< 10 cfu/cm^2). Therefore, the control treatment was excluded from the analyses of conidial persistence. Grasshopper mortality data were analyzed as a factorial experiment with three formulations and two crops. Separate analyses were conducted for each sample time. There was no mortality attributed to *B. bassiana* in grasshoppers fed non-inoculated leaf segments, and therefore this treatment was excluded from the analysis of *B. bassiana* mortality; for other mortality it was included.

Results

Efficacy of the wash method for recovery of conidia. The efficacy of the wash method was investigated by comparing numbers of conidia recovered from washed leaf segments inoculated with known quantities of conidia suspended in water-Tween, an oil emulsion, or oil. From leaf segments inoculated with conidia in water-Tween, 102 % (SE=0.6) was recovered. When conidia were applied in an oil emulsion, 139 % (SE=4.9) was recovered, and from leaf segments inoculated with conidia in formulation oil, 91.6 % (SE=3.3) was recovered. Although only 2-μl aliquots of conidia in oil were used per leaflet, substantial penetration of the oil through the leaf cuticle into the mesophyll cells was observed.

Field Application of conidia. Only concentrations of conidia suspended in water-Tween could be accurately verified with a hemocytometer. In both the oil and 5% oil

emulsion formulations, the oil visually obscured the conidia, therefore, conidial concentrations in these formulations were verified using the dilution-spread plate technique. In general, numbers of conidia isolated on the oatmeal-dodine medium for the oil emulsion formulation were slightly less (< 10%) than those for the other two formulations. This was attributed to aggregation of conidia which resulted in the development of a singie colony from multiple conidia.

Immediately following application of conidia, there were no interactions (F=0.01-2.9; df=2,10; P≥0.10) between formulation and canopy position, and with the exception of crested wheatgrass in trial one (F=7.3; df=2,10; P=0.011), there were no significant differences (F=0.19-3.7; df=2,10; P≥0.063) between the formulation treatments in either crop (Figures 3.2.2-3.2.4). Populations estimates of *B. bassiana* isolated from leaves at the top of the canopy immediately following application ranged from 3.71 (SE=0.48) to 4.54 (SE=0.12) log cfu/cm^2 and from 3.92 (SE=0.13) to 5.09 (SE=0.15) log cfu/cm^2 for wheatgrass and alfalfa, respectively. Conidia applied in the oil emulsion in trial one were less numerous (α=0.05) than conidia applied in either water-Tween or oil on wheatgrass phylloplanes (Figure 3.2.2A). Although winds were light (1.5 to 3.8 m/s), ULV droplets were observed to drift during application in all three trials.

In general, fewer droplets per cm^2 were observed on oil and water-sensitive papers at the bottom (soil level) of the canopy relative to the top of the canopy in alfalfa but not wheatgrass. There were no differences (F=0.02-1.5; df=1,10; P≥0.25) in numbers of cfu isolated from leaves at either the top or middle of the crested wheatgrass canopy; LAI were 0.55, 0.24 and 0.35 in trials one, two and three, respectively (Figures 3.2.2A, 3.2.3A, 3.2.4A). Fewer cfu (F=9.1-44.6; df=1,10; P<0.001) were isolated from leaves at the middle than the top of the alfalfa canopy in trials one and three; populations ranged from log 3.52 (SE=0.25) to 4.34 (SE=0.07) log cfu/cm^2 (Figures 3.2.2B, 3.2.4B). For these trials, LAI were 1.8 and 1.9, respectively. However, in trial three, when the LAI of alfalfa

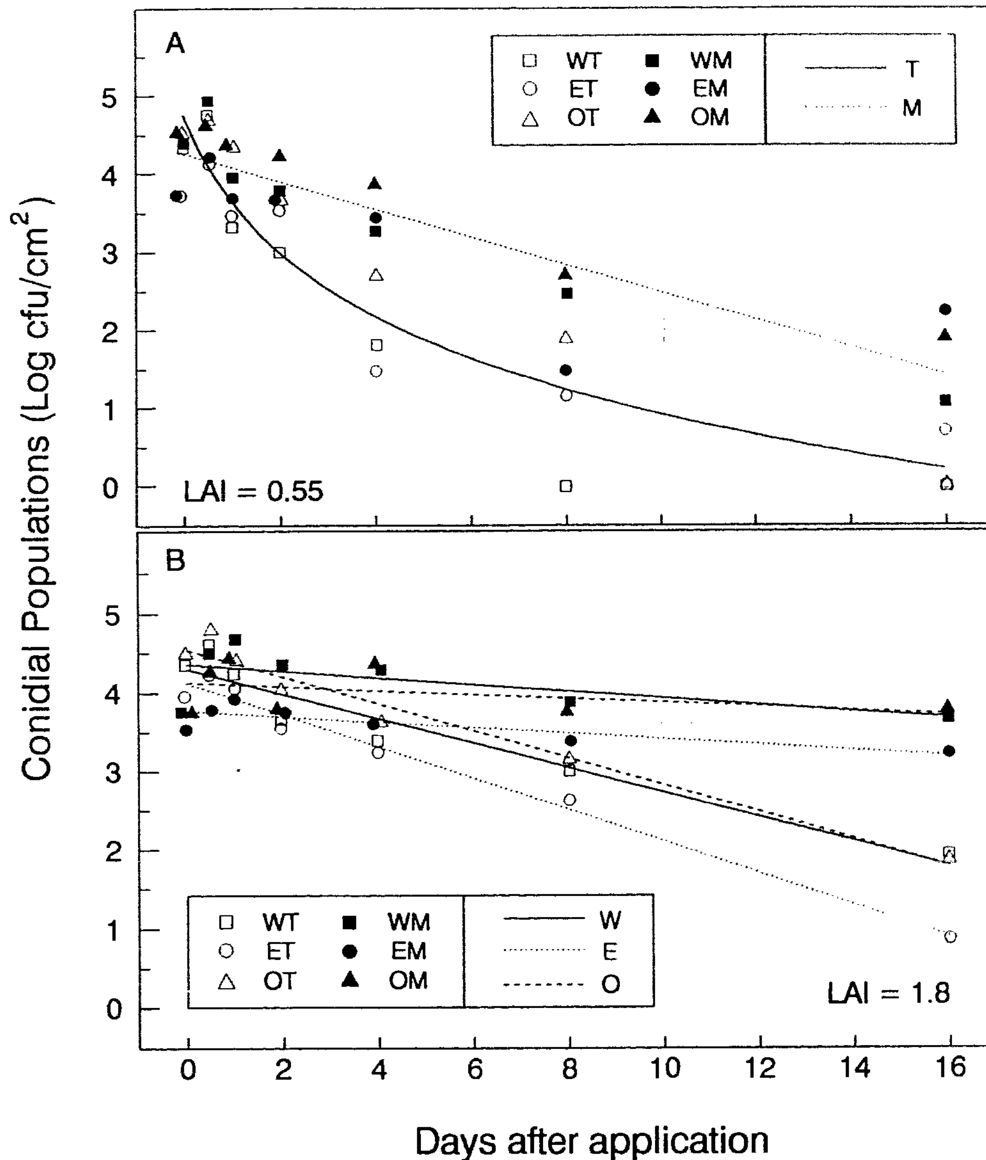


Figure 3.2.2 Persistence of *Beauveria bassiana* conidia on the phylloplanes of crested wheatgrass and alfalfa from July 15 to 31. A) Conidial populations (log_{10} colony-forming units (cfu)/cm^2 of leaf area) for conidia applied on phylloplanes of crested wheatgrass in water amended with 0.05% Tween 80 (W), in a 5% oil emulsion (E) and in formulation oil (O) at the top (T) and middle (M) of the canopy; and B) populations of conidia (log cfu/cm^2) applied on phylloplanes of alfalfa. Leaf area indexes (LAI) for both crops were measured at the time of application.

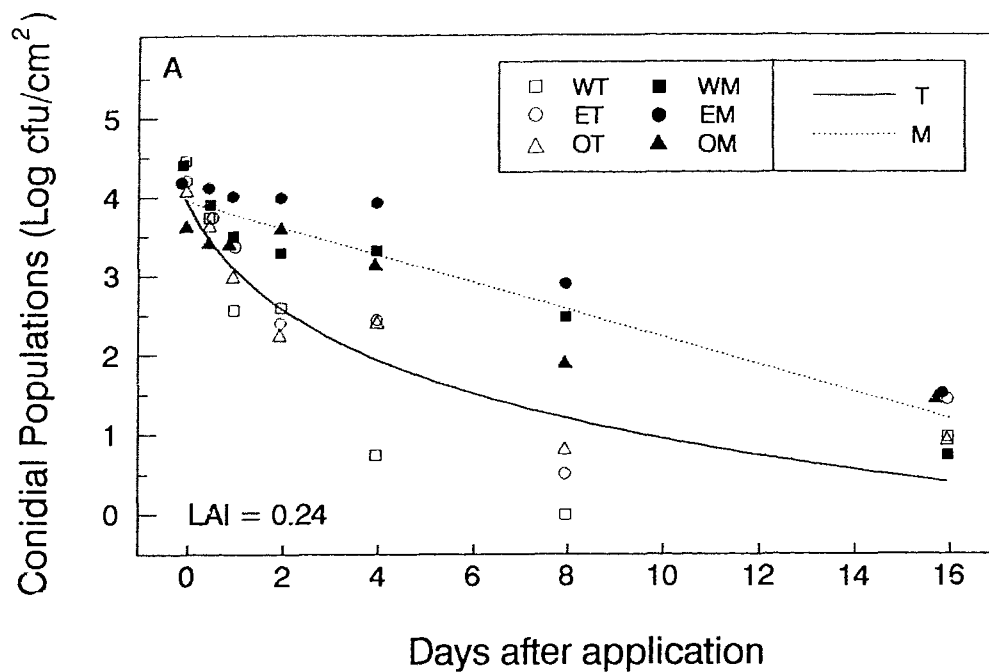


Figure 3.2.3 Persistence of *Beauveria bassiana* conidia on phylloplanes of crested wheatgrass from August 2 to 18. A) conidial populations ($\log_{10}$ cfu/cm^2 of leaf area) for conidia applied in water amended with 0.05% Tween 80 (W), in a 5% oil emulsion (E) and in formulation oil (O) at the top (T) and middle (M) of the canopy. LAI was estimated at the time of application.

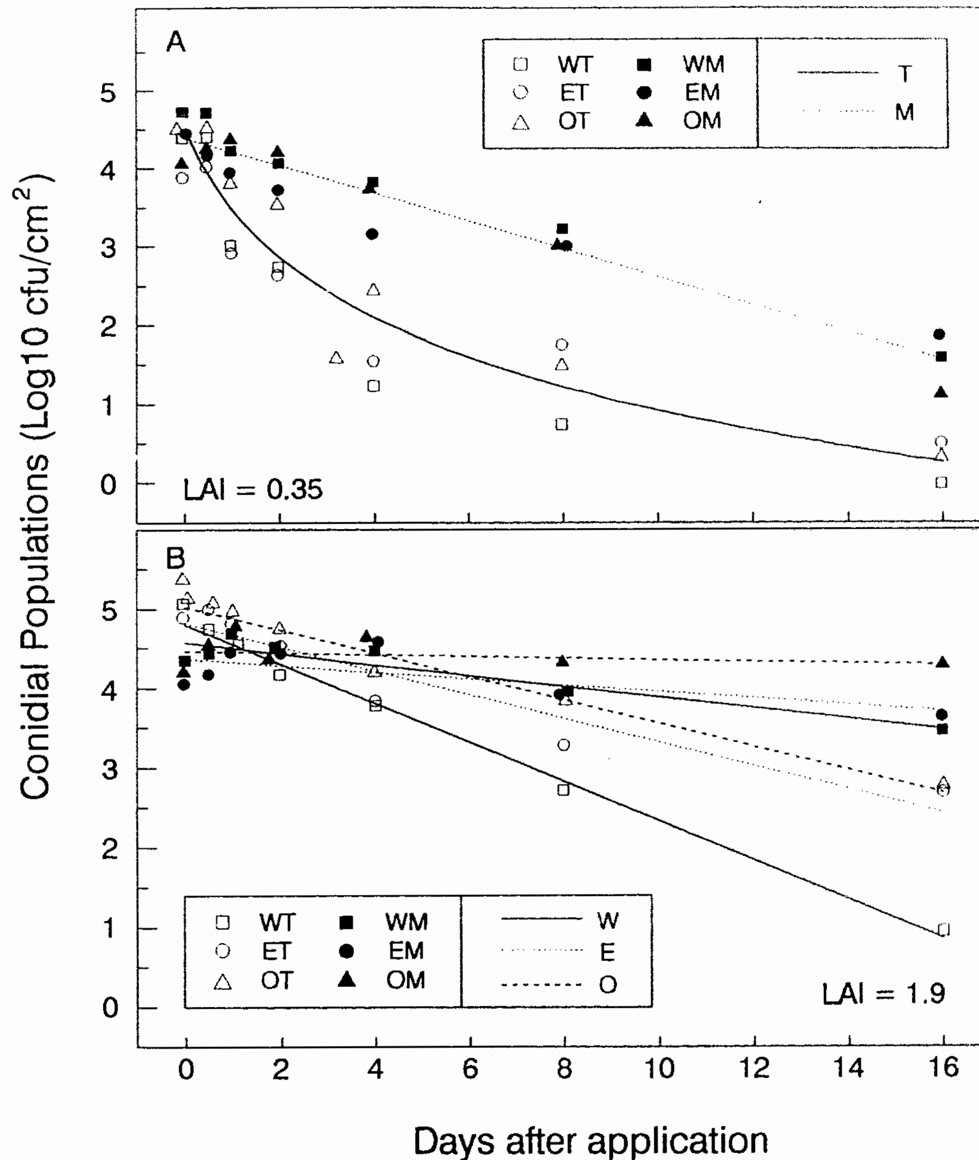


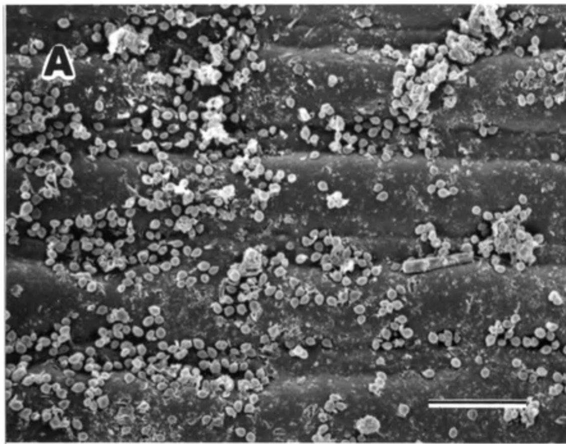
Figure 3.2.4 Persistence of *Beauveria bassiana* conidia on the phylloplanes of crested wheatgrass and alfalfa from August 31 to September 16. A) conidial populations (log_{10} cfu/cm^2 of leaf area) for conidia applied on phylloplanes of crested wheatgrass in water amended with 0.05% Tween 80 (W), in a 5% oil emulsion (E) and in formulation oil (O) at the top (T) and middle (M) of the canopy; and B) populations of conidia (log cfu/cm^2) applied on phylloplanes of alfalfa. LAI for both crops were measured at the time of application.

was greater than 2, there was no effect (F=2.2; df=1,10; P=0.17) of canopy position on the recovery of *B. bassiana* conidia. Microscopic observations of leaf surfaces showed a degree of conidial aggregation for all formulations (Figure 3.2.5 A-D). In several instances, aggregation of conidia in circles around the point of droplet impact on wheatgrass leaves were observed for conidia applied in water and the oil emulsion (Figure 3.2.5 E-F).

Weather conditions. Conditions of precipitation, temperature and incoming solar radiation differed substantially between the three trials (Figure 3.2.1). Cumulative light was similar between trials one (3.7×10^5 kJ/m^2) and two (3.9×10^5 kJ/m^2) but substantially less in trial three (2.2×10^5 kJ/m^2). In trial one, two and three, mean hourly temperatures at 1 m were 16.6, 19.2, and 9.7°C, respectively. Total precipitation was 22, 15 and 8.4 mm for trials one, two, and three respectively. Immediately following application of conidia in trial two, an intense storm lasting less than 1 h occurred; greater than 14 mm of rain fell accompanied by hail, and winds gusted in excess of 80 kph. Relative humidities at 1 m averaged 75, 58, and 69% for the three trials, respectively. Both temperature and relative humidity at canopy mid-height for crested wheatgrass were similar to ambient conditions over a 24 h period. In the alfalfa canopy, temperatures were similar but relative humidities were slightly higher by 3 to 5% on average. During daylight hours over a 12 h period (800 am to 800 pm), there were minimal differences in temperature due to canopy position in both wheatgrass and alfalfa. However, relative humidities were higher by 6 to 9% in alfalfa; there were no conspicuous differences in relative humidities in the wheatgrass canopy.

Persistence of conidia on crested wheatgrass. The only interaction that consistently influenced persistence of *B. bassiana* conidia was between canopy position and time (F=5.8-10.9; df=6,72; P<0.001). There were no interactions (F=0.36-1.2; 12,72; P≥0.30) between canopy, formulation and sampling time. Interactions between formulation

Figure 3.2.5 Scanning electron micrographs of *Beauveria bassiana* conidia on phyllopianes of crested wheatgrass and alfalfa immediately following application. A-C) Bars = 20 μm. A) conidia applied onto a wheatgrass leaf in water amended with 0.05% Tween 80; B) conidia applied onto a wheatgrass leaf in a 5% oil emulsion; C) conidia applied onto a alfalfa leaflet in oil; D) conidia adhering to an alfalfa cuticular hair. Bar = 5 μm. E-F) aggregation of conidia in circles around the site of droplet impact. E) conidia applied in a 5% oil emulsion. Bar = 20 μm; F) conidia applied in water-Tween. Bar = 10 μm.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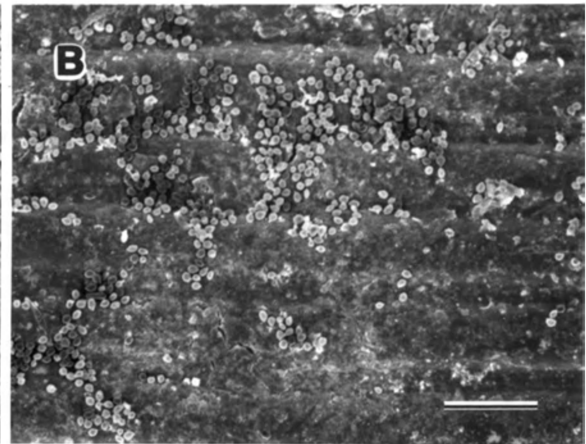
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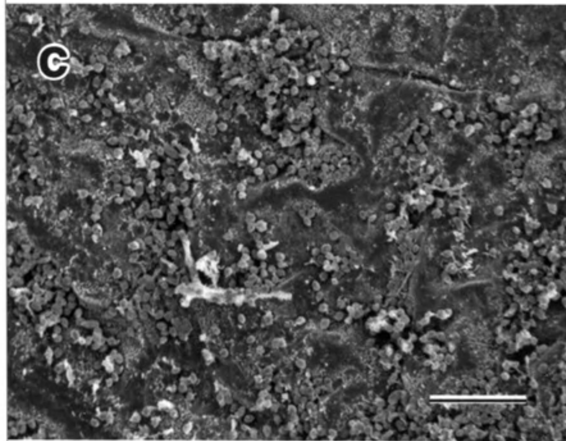
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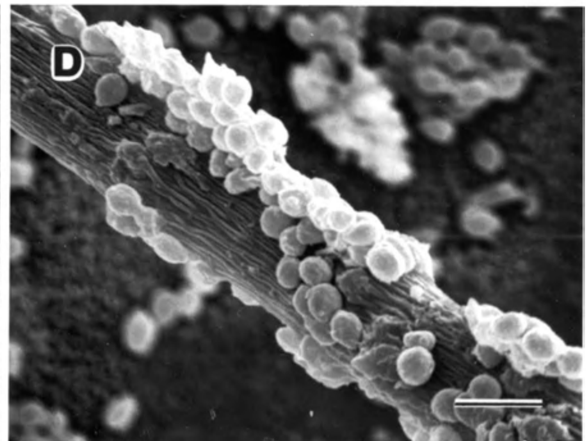
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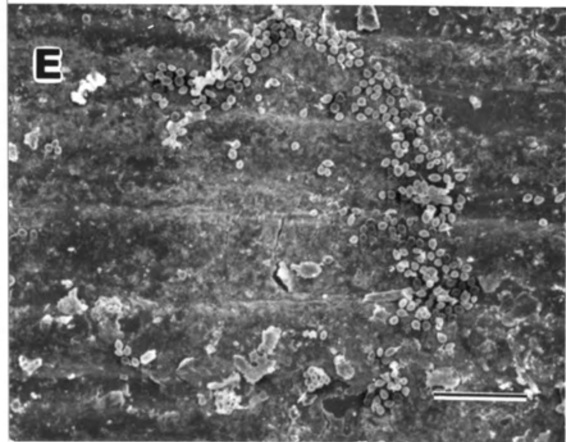
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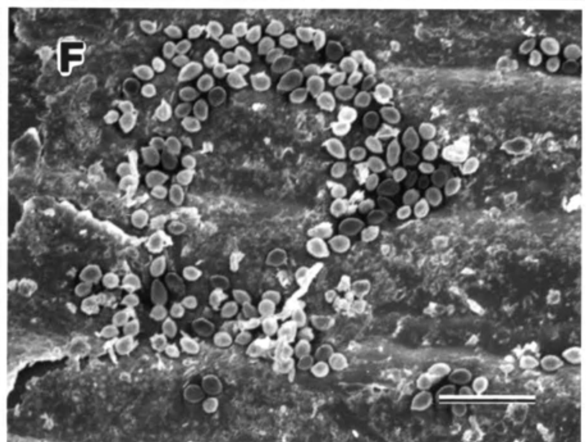
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and canopy also were non-significant ($F=0.31$-1.3; $df=2,10$; $P\geq0.31$). Time was highly significant ($F=80.5$-97.4; $df=6,72$; $P<0.001$) and with the exception of trial one ($F=2.2$; $df=12,72$; $P=0.022$), there were no interactions ($F=1.3$; $df=12,72$; $P=0.24$) between formulation and time; the positive interaction in trial one was attributed to the oil emulsion formulation at the mid-canopy position (Figure 3.2.2A). The influence of canopy position alone was significant ($F=8.9$-22.5; $df=1,10$; $P\leq0.014$); a canopy effect was first observed ($\alpha=0.05$) at 1 day post-application of conidia and was subsequently maintained throughout the 16-day duration of the experiment. Formulation had no effect ($F=0.58$-3.5; $df=2,10$; $P\geq0.070$) on persistence of conidia; therefore, data for formulations were combined at each canopy position and a single regression equation was used to model the response of conidial survival to time. Substantial aggregation of conidia was observed by SEM on leaves 1 to 4 days post-application for all formulations. However, in contrast to conidia on leaves collected immediately after application, conidia were found to be congregated in the intercellular depressions of wheatgrass leaves (Figure 3.2.6A). At the middle of the wheatgrass canopy, conidial persistence declined logarithmically over time (Figures 3.2.2A, 3.2.3A, 3.2.4A). In contrast, at the top of the canopy, data were fitted to linear regressions following log-transformation of both cfu and time data. Formulations were combined within each canopy position to calculate the coefficient of determination (r^2); these ranged over the three trials from 0.66 to 0.78 at the top and from 0.65 to 0.72 from the middle of the canopy (Table 3.2.1). Slopes ranged from -2.9 to -3.7 for the top and from -0.17 to -0.18 for the middle canopy position. There were no differences ($F=0.03$-2.3; $df=2,183$; $P\geq0.10$) between slopes of conidial persistence between trials for either the top or middle canopy positions. Slopes for each canopy position differed ($F=128$-167; $df=3,183$; $P<0.001$) from zero. However, the y-intercepts (average initial populations) differed between trials for both the top ($F=4.8$; $df=2,183$; $P=0.009$) and middle ($F=4.5$;

Table 3.2.1 Linear regression data of field persistence of *Beauveria bassiana* conidia on alfalfa leaflets[a]

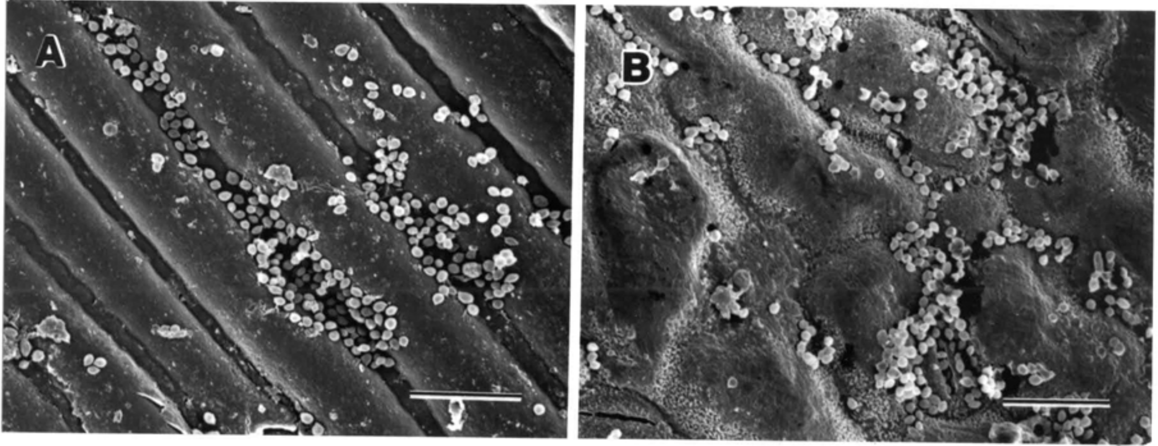
	Trial one					Trial three				
Canopy/Formulation[b]	a	SE(a)	b	SE(b)	r^2	a	SE(a)	b	SE(b)	r^2
Top of Canopy										
Water	4.29	0.12	-0.16	0.016	0.83	4.80	0.10	-0.25	0.015	0.93
Oil Emulsion	4.11	0.09	-0.20	0.013	0.93	4.81	0.10	-0.15	0.014	0.86
Oil	4.53	0.12	-0.17	0.018	0.83	5.02	0.09	-0.15	0.012	0.88
Middle of Canopy										
Water	4.34	0.12	-0.042	0.017	0.23	4.57	0.07	-0.068	0.010	0.71
Oil Emulsion	3.75	0.07	-0.035	0.010	0.38	4.37	0.10	-0.041	0.014	0.30
Oil	4.12	0.10	-0.025[c]	0.014	0.14	4.46	0.09	-0.010[c]	0.013	0.03

[a]The regression equation is: $\log_{10}$ cfu/cm^2 = a + b (days).

[b]Formulations consisted of water amended with 0.05% Tween 80 (W), a 5% oil-emulsion (E); formulation oil (O) and leaves were sampled at the top (T) or middle (M) of the canopy.

[c]Slopes that are not significantly different from zero ($P>0.05$).

Figure 3.2.6 Scanning electron micrographs of *Beauveria bassiana* conidia on phylloplanes of crested wheatgrass and alfalfa 4 days post-application. A) aggregation of conidia in intercellular depressions of a wheatgrass leaf; B) conidia aggregated in a depression of an alfalfa leaflet. Note the conidia in a stoma. Bars = 20 μm.


A
B

df=2,183; P=0.012) canopy positions. Accumulated degree days and cumulative light were less effective as predictors of conidial persistence at the top of the crop canopies than was time.

Persistence of conidia on alfalfa. The severe storm encountered immediately after application of conidia in trial two disrupted the canopy structure of the alfalfa. Therefore, only data for time 0 at both canopy positions, and for the times 0 to 4 days for the top of the canopy were used in this trial. Substantial numbers of cfu were isolated from the top of the canopy following the rain storm. Populations isolated from the top of the canopy 12 h after the application of conidia were 3.22 (SE=0.33), 3.91 (SE=0.19) and 3.79 (SE=0.39) log cfu/cm^2 for the water-Tween, the oil emulsion and oil formulations, respectively.

In trials one and three, there were no interactions (F=0.81-1.5; df=12,72; P≥0.14) between canopy, formulation and sampling time. The interaction between canopy position and time was highly significant (F=27.6-40.7; df=6,72; P<0.001) in both these trials. There was no interaction (F=2.2; df=2,10; P=0.16) between canopy position and formulation in trial one, whereas, a weak interaction (F=5.8; df=2,10; P=0.020) was observed in trial three. In trial three (F=4.6; df=12,72; P<0.001) but not one (F=0.43; df=12,72; P=0.95), formulation influenced the rate of decline over time. Similar to the case of wheatgrass, both time (F=59.7-89.8; df=6,72; P<0.001) and canopy (F=39.3-9.2; df=1,10; P≤0.013) were highly significant. In both trials, the effect of formulation on the persistence of conidia was highly significant (F=25.1-10.8; df=2,10; P≤0.003). Therefore, data for the three formulations were not combined as in the case of wheatgrass, and individual regressions were calculated for each formulation (Table 3.2.1). A comparison of slopes between trials for each formulation and canopy position, indicated that for conidia applied in both water-Tween and the oil emulsion at the top of the alfalfa canopy, slopes differed (F=7.4-16.6; df=1,38; P<0.010) between trials one and three (Table 3.2.1). For all other formulation-canopy combinations there were no significant differences (F=0.13-

1.6; df=1,38; P≥0.21) between slopes. With the exception of conidia applied in water and sampled at the middle of the canopy (F=2.6; df=1,38; P=0.12), initial populations (y-intercepts) differed (F=6.6-29.2; df=1,38; P≤0.014) between trials for all formulation-canopy combinations. Analysis of covariance, indicated that slopes (combined across trials) for all three formulations differed from zero at the top (F=7.8-16.8; df=1,40; P≤0.008) but not the middle (F=0.06-0.99; df=1,40; P≥0.33) of the canopy. Although the surface topography of alfalfa leaflets differed substantially from wheatgrass leaves, similarly to wheatgrass, conidial aggregations in intercellular depressions were observed on leaves 1 to 4 days post-application (Figure 3.2.6B).

Grasshopper bioassay. Average total mortality of grasshopper nymphs fed crested wheatgrass and alfalfa leaves immediately after the application of *B. bassiana* conidia ranged from 51 to 82% for the three formulations (Table 3.2.2). Considerable variation between replicates was observed and there was no difference between either crops (F=0.02; df=1,10; P=0.89) or formulations (F=0.8; df=2,10; P=0.49) in the prevalence of mortality attributed to infection by *B. bassiana*; such mortality ranged from 31 to 47%. In nymphs fed untreated leaves, there was no mortality that could be attributed to *B. bassiana*. Other mortality ranged from 17 to 34%, and there was no difference between crops (F=2,9; df=1,14; P=0.11) or formulation (F=0.19; df=3,14; P=0.90).

Both *B. bassiana*-incited and other mortality in nymphs fed crested wheatgrass and alfalfa leaves 2 days post-application of *B. bassiana* were considerably less than those fed leaves immediately after application (Table 3.2.2). Mortality attributed to *B. bassiana* ranged from 0 to 5.5%, and the prevalence of other mortality ranged from 0 to 17%. A difference (F=8.1; 1,14; P=0.014) in other mortality due to crop was observed in nymphs fed leaves 2 days post-application of conidia. For both sample times, the number of

Table 3.2.2 Mortality of grasshopper (*Melanoplus sanguinipes*) nymphs fed leaf segments of alfalfa and crested wheatgrass collected from the top of the canopy at 0 and 2 days post-application of conidia of *Beauveria bassiana*.

	Mortality (%)			
	0 days[a]		2 days[b]	
Crop/Formulation	*Beauveria*[c]	Other[d]	*Beauveria*[c]	Other[d]
Crested Wheatgrass				
Water-Tween[e]	46.6 (17.8)[g]	23.5 (2.7)	0.0	17.4 (4.6)
Oil Emulsion (5%)[e]	40.8 (2.3)	34.3 (1.0)	5.1 (5.1)	2.4 (2.4)
Oil-ULV[f]	44.8 (12.1)	28.4 (7.1)	0.0	7.9 (0.8)
Untreated	0.0	27.7 (5.6)	0.0	8.4 (5.3)
Alfalfa				
Water-Tween	31.2 (12.1)	19.8 (5.5)	0.0	0.0
Oil Emulsion (5%)	46.4 (12.3)	16.5 (8.4)	3.9 (3.9)	0.0
Oil-ULV	58.2 (18.8)	23.3 (5.5)	5.5 (2.8)	11.4 (2.7)
Untreated	0.0	23.3 (12.0)	0.0	0.0

[a]n = 30-35 nymphs per formulation per crop.

[b]n = 35-41 nymphs per formulation per crop.

[c]Nymph cadavers (%) exhibiting growth of *B. bassiana.*

[d]Nymph cadavers (%) not exhibiting growth of *B. bassiana.*

[e]Conidia in distilled water amended with 0.05% Tween 80 and in a 5% oil emulsion were applied using a bicycle sprayer at a rate of 100 L/ha (5×10^{13} conidia/ha).

[f]Conidia in oil applied using an ultralow volume applicator at a rate of 5 L/ha (5×10^{13} conidia/ha).

[g]Values in parentheses represent standard errors of the mean.

surviving nymphs killed at the end of the experiment (12 days) and subsequently exhibiting growth of *B. bassiana* was low (2 to 3%).

Discussion

Conidia of *B. bassiana* on phylloplanes of crested wheatgrass and alfalfa in a field environment were found to be relatively short-lived at the top of the canopy for both crops. Persistence of conidia appeared to be somewhat enhanced at the top of the alfalfa canopy relative to wheatgrass; by 4 days, populations were reduced by over 99% on wheatgrass and by 75-90% on alfalfa leaves. Daoust and Pereira (1986a) observed reduced germination of *B. bassiana* conidia isolated from cowpea foliage in a field setting; conidial half-lives were 1-2 days and no conidia were viable after 1 week. Conidia at the mid-canopy level of both wheatgrass and alfalfa survived substantially longer than those at the top. Conidia on leaves within the canopy of alfalfa survived much longer than those in the wheatgrass canopy. At the middle of the wheatgrass canopy, conidial populations were reduced by 79 to 81% after 4 days. On alfalfa leaves at the mid-canopy position, populations were only reduced by 8 to 47% after 4 days, and by 16 days, substantial numbers of viable conidia were still isolated (populations reduced by 29 to 85%). Feeding by grasshoppers is not limited to the top of the canopy. Therefore the enhanced persistence of conidia may have positive consequences for implementation of a biological control program with *B. bassiana* due to the lengthened time that conidial populations remain above inoculum thresholds within canopies.

In the present study, conditions of temperature, relative humidity and precipitation fluctuated considerably within and between trials. In trial two, substantial rainfall and hail accompanied by driving winds occurred immediately following application of conidia, yet only slight population decreases were observed in both crops. When the slopes of conidial persistence between trials were compared, no significant differences at either the top or middle of the canopy of the wheatgrass were found. Similarly, slopes of conidial

persistence between trials at the middle of the alfalfa canopy generally did not differ. These observations suggest that temperature, relative humidity and rainfall do not have an overriding impact on conidial survival. However, the possibility that critical weather events affect *B. bassiana* at the microclimate level requires further study.

Solar radiation is detrimental to fungi on phylloplanes, and is known to be particularly important in inactivating conidia of *B. bassiana* (Roberts and Campbell, 1977; Daoust and Pereira, 1986a; 1986b). Mortality of grasshoppers ingesting vegetation sprayed with *B. bassiana* conidia was less in Africa than in Montana (Delgado *et al.*, 1991; Johnson *et al.*, 1992; Lobo Lima *et al.*, 1992), and the increased light intensity encountered in Africa may have negatively affected conidial survival (Johnson *et al.*, 1992). In the present study, conidia of *B. bassiana* were found to persist longer within the canopy of alfalfa relative to that of wheatgrass. Since leaf area indices (LAI) were much greater for alfalfa (LAI = 1.8 to 1.9) than for wheatgrass (LAI = 0.24 to 0.55), and temperatures and relative humidities were only slightly more favorable for conidial survival at the middle than at the top of the alfalfa canopy, this implicates solar radiation as the most important parameter affecting survival of conidia on phylloplanes. However, I found no apparent correlation between cumulative radiation and conidial persistence; in trial three, exposure to light (300-2800 nm) at the top of the canopy was substantially reduced, yet slopes of conidial persistence in other trials were generally no different. The germicidal portion of the spectrum was not measured. Ultraviolet radiation affects the survival of entomopathogenic Hyphomycetes (e.g., Fargues *et al.*, 1988) but the influence of wavelengths less than 300 nm on conidia of *B. bassiana* on phylloplanes is unknown.

An endophytic existence would buffer *B. bassiana* from the sub-optimal environmental conditions encountered on phylloplanes and it has been reported to exist as an endophyte in corn (Bing and Lewis, 1991; 1992). Although there are numerous reports of direct penetration of insect cuticles by *B. bassiana* (Ferron, 1978), direct penetration of

plant tissues has not been documented. Germination of *B. bassiana* can occur in the presence of free water or at relative humidities greater than 90% (Walstad *et al*, 1970; Kuberappa and Jayaramaiah, 1987), both of which occurred in the present study. However, conidial germination was not observed on leaves at the top of the canopy for either crop, and whether germination occurred at the more favorable microclimate within the canopy is unknown. In several instances conidia were observed within alfalfa leaf stomata (Figure 3.2.6B), and whether stomata serve as possible sites of penetration requires investigation.

Conidia of *B. bassiana* are hydrophobic and were found to disperse better in oil than in water-Tween. Substantial clumping of conidia was observed in the oil emulsion even after homogenization but aggregation of conidia was observed for all three formulations on phylloplanes (Figures 3.2.5 A-D, 3.2.6 A-B). The attachment of conidia to wheatgrass and alfalfa cuticle was considerable regardless of formulation, and in several instances conidia were observed attached to cuticular hairs of alfalfa (Figure 3.2.5D). Passive and non-specific adherence of *B. bassiana* conidia to insect cuticles mediated by their hydrophobicity has been reported (Boucias *et al.*, 1988; Boucias and Pendland, 1991). It seems likely that the similar adherence occurs on plant cuticles, and reductions in numbers of conidia adherent to leaves was not observed for up to 4 days following their application. However, conidia appeared to congregate in intercellular depressions of both wheatgrass and alfalfa leaves (Figures 3.2.6 A-B). Although conidia appeared to be strongly attached to leaves, I found that conidia suspended in all three formulations were effectively removed from their surfaces by vigorous washing in buffer amended with Tween 80. For the oil emulsion formulation, recovery of cfu was greater than 100% and it seems likely that the high rate of recovery of conidia applied in this formulation resulted from the release of conidia from aggregations.

Differences between the three formulations with respect to application efficacy and subsequent persistence of conidia were minimal. Although the alfalfa canopy influenced foliar coverage of conidia, there was no difference between formulations. Conversely, the wheatgrass canopy had no effect on penetration of conidia but LAI were much lower than in alfalfa. The application of propagules in oil onto phylloplanes has been reported for a number of fungi (Bateman *et al.*, 1992; Johnson *et al.*, 1992; Lobo Lima *et al.*, 1992). Some putative advantages to formulation of fungal propagules in oil include decreased spray volumes, infection of locusts by *Metarhizium flavoviride* at low humidities (Bateman *et al.*, 1993), stimulated germination of *Bipolaris* species (Winder and Van Dyke, 1990), prolonged viability of *B. bassiana* conidia (Prior *et al.*, 1988), and decreased sensitivity of *M. flavoviride* conidia to light (Moore *et al.*, 1993). The application of conidia in oil allowed decreased spray volumes, but the small plots had to be enclosed in polyethylene to prevent drift. Persistence of conidia applied onto phylloplanes in oil was not significantly different from that of conidia formulated in either water-Tween or a 5% oil emulsion. Oil deposited on both wheatgrass and alfalfa leaves was rapidly absorbed into the mesophyll cells leaving conidia exposed on their surfaces. The observed absorption of oil through the leaf cuticle may explain the reduced protection from UV light provided by the formulation oil.

Most investigations into persistence of conidia on phylloplanes have focused on infectivity of applied propagules over time. Gardner *et al.* (1977) showed that infection of fall armyworm larvae by conidia of *B. bassiana* applied onto soybean foliage was reduced by 5 days and non-existent by 10 days. Similarly, infection of cabbage looper larvae by conidia applied onto leaves of collard and soybeans was significantly reduced 1 day after application (Ignoffo *et al.*, 1979). Repeated application of conidia on potato phylloplanes provided inconsistent control of Colorado potato beetle (Hajek *et al.*, 1987). To test if reductions in populations over time corresponded with infectivity of conidia, grasshopper nymphs were fed either wheatgrass or alfalfa leaves from the top of the canopy

immediately, and 2 days after, application of conidia in trial two. Mortality ranging from 31-58% was observed in nymphs fed leaves immediately after application. Significant mortality of grasshoppers also was reported in North America and Africa following application of *B. bassiana* conidia onto foliage (Johnson *et. al.*, 1988; Delgado *et al.*, 1991; Lobo Lima *et al.*, 1992). The results suggest that the infectivity of conidia on wheatgrass and alfalfa leaves corresponds with reductions in conidial numbers. Although nymphs were fed *ad libitum* in the present study, by 2 days post-application of conidia populations on phylloplanes of wheatgrass and alfalfa were reduced by 99 and 75%, respectively from the top of the canopy and the corresponding mortality in nymphs was reduced to 0-6%.

Conidia of *B. bassiana* applied on phylloplanes of crested wheatgrass and alfalfa were found to be relatively short-lived at the top of the canopy for both crops. Slopes of conidial persistence were similar between trials although conditions of temperature, relative humidity, precipitation and solar radiation (> 300 nm) varied substantially. However, the degree of canopy coverage in alfalfa was greater than that of wheatgrass and mortality of conidia within the alfalfa canopy was reduced. This was attributed to protection from solar radiation and not to more favorable conditions of relative humidity and temperature. Oil had little or no effect on persistence of *B. bassiana* conidia relative to conidia applied in water or an oil emulsion. Mortality of grasshopper nymphs appeared to correspond with the conidial persistence data; by 2 days post-application of conidia on phylloplanes populations were reduced by more than 75%. Since UV radiation affects the survival of hyaline propagules, it may be possible to use UV protectants to increase persistence and this possibility is investigated in section 3.3. If phylloplanes or aerial plant parts are targeted in a strategy to control grasshoppers with *B. bassiana*, in addition to virulence, isolates should also be selected on their ability to persist in these habitats.

3.3 INFLUENCE OF UVB RADIATION ON CONIDIAL PERSISTENCE[2]

Materials and Methods

Conidial inoculum. Dry conidia were suspended in sterile deionized water or in paraffinic formulation oil using a Kontes mechanical pestle or a Potter-Elvehjem homogenizer. In the laboratory experiments, a paraffinic emulsifiable oil was used, whereas a paraffinic oil flowable (no emulsifier added) was used in the field experiment. Conidial concentrations were estimated with a hemocytometer and adjusted as required.

Influence of substrate. The survival of conidia exposed to UVB radiation on leaves and on glass was compared. Conidia (2×10^5 conidia/μl) suspended in water or oil were pipetted (1 μl) onto sterile round coverslips (13 mm diam) and onto the surface of leaf pieces (approximately 2 x 0.5 cm) of field-collected crested wheatgrass (*Agropyron cristatum* L.) attached to a white plastic tray using double-sided tape. The coverslips and leaf pieces were then placed 10 cm below a UVB fluorescent bulb (Ultra-Lum, Carson, CA) for 0, 15, 30, 45 or 60 min at 25 $\pm$ 1°C. Radiation from this bulb ranges in wavelength from 260 to 400 nm, with a peak near 300 to 320 nm (Figure 3.3.1). Intensity of UVB radiation was measured using a UVX radiometer (UVP Inc., San Gabriel, CA, USA) equipped with a UVX-31 sensor (310 nm peak); UV flux at a distance of 10 cm ranged from 601 to 675 μW/cm^2 along the length of the bulb. Following exposure, conidia were isolated from leaf pieces by washing and cfu were enumerated on oatmeal-dodine agar as detailed previously. Conidial survival relative to the control (time$_0$) was calculated as ((cfu T_0 - cfu T_x) / cfu T_0) x 100. An initial experiment indicated that incubation of conidia in water and oil at 25°C in the dark for up to 6 h had no effect on conidial viability.

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[2]A version of this section has been published as: "Influence of ultraviolet light protectants on persistence of the entomopathogenic fungus, *Beauveria bassiana*. Inglis, G.D., M.S. Goettel, and D.L. Johnson. 1995. Biological Control 3:258-270.

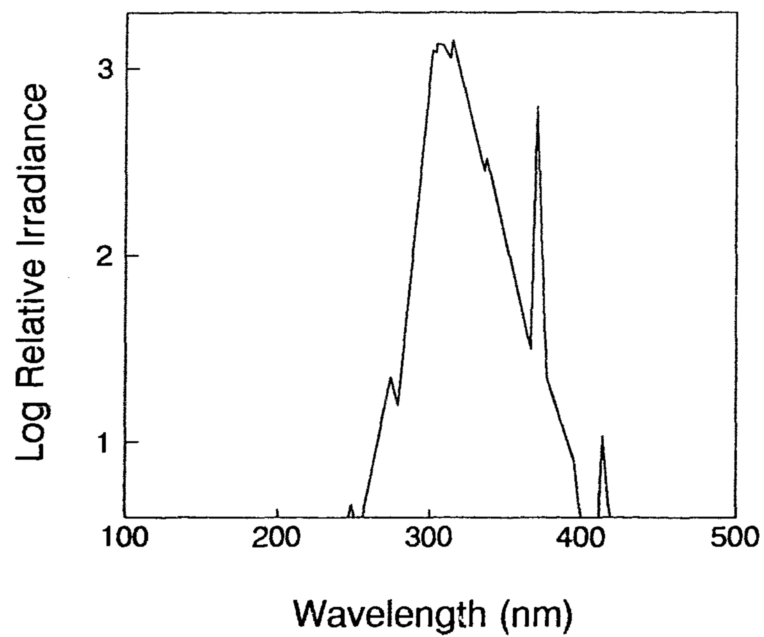

Figure 3.3.1 Spectral distribution of irradiative energy from the Ultra-Lum UV-B fluorescent bulb used for irradiating *Beauveria bassiana* conidia.

Sunscreens. The adjuvants tested were divided into water- or oil-compatible compounds. The water-compatible adjuvants tested were: Congo Red (Sigma); diethanolamine-4-methoxycinnamate (Nipasorb D; Graessorb D; Nipa Laboratories, Clwyd, UK); and eight stilbene brighteners provided by Dr. M. Shapiro, USDA-ARS, Beltsville, Maryland, USA. In addition, an attapulgite clay formulation (consisting of 15% oil (v/v), 12% clay (w/v) and 73% deionized water), and a 5% oil-water emulsion formulation (v/v) were tested. The oil-compatible adjuvants used were: butyl-methoxy-dibenzoylmethane (Parsol 1789; Givaudan & Co. Ltd., Surrey, UK); ethyl trans-cinnamate (Sigma); 4-isopropyl-dibenzoylmethane (Eusolex 8020; Merck, Darmstadt, Germany); magnesium silicate (Florisil 60-100 mesh; Fisher Scientific, Edmonton, AB); octyl-p-methoxycinnamate (Parsol MCX; Givaudan); octyl-salicylate (Graessorb S; Nipa Laboratories); 2-hydroxy-4-methoxybenzophenone (oxybenzone; Sigma), and; 2,2-dihydroxy-4-methoxybenzophenone and 2,2-hydroxy-4-octoxybenzophenone supplied by Mycotech Corporation Inc.

Solubility and toxicity of sunscreens. The solubilities of the sunscreens were determined in water and oil at room temperature (20 to 22°C). The maximum sunscreen concentration tested was 5%; saturated solutions at room temperature were used if sunscreens were insoluble at 5%. To measure possible toxicity, non-germinated conidia (2×10^5 conidia/μl) were suspended in each of the sunscreens. After 12 h at 25°C, 1 μl aliquots were pipetted onto sterile coverslips, and the coverslips were washed and cfu enumerated on oatmeal-dodine agar as previously described.

Sunscreen selection. Conidia were suspended in each of the formulations so that the final concentration was 2×10^5 conidia/μl; sunscreen concentrations were the same as for the toxicity experiment. Within 15 min of preparation, conidial suspensions (1 μl) were pipetted onto the surfaces of leaf pieces taped to a plastic tray, and the leaf pieces were exposed to UVB radiation for 1.5 and 3.0 h. The leaf pieces were washed and viable conidia were enumerated on oatmeal-dodine agar. Control treatments consisted of conidia

applied in oil or water onto leaf pieces that were either exposed to UVB radiation or maintained in the dark for the same period.

Field evaluations. Conidia in various UV-protectants were sprayed on crested wheatgrass at AAFC (Lethbridge) as described in section 3.2. The field used was seeded in 1989 and mowed in April, 1992, 3 months prior to commencement of the experiment. Treatments were arranged as a RCBD with three replicate plots measuring 3.0 m by 1.5 m. The target concentration was 3.0×10^{13} conidia/ha or 1.4×10^{10} conidia/plot. The water treatments tested were Tinopal LPW (Calcofluor white; M_2R; 5% w/v), Blankophor BSU (5% w/v), Congo Red (5% w/v), clay (12% w/v), and water alone. Tinopal LPW was obtained by adjusting the pH of Blankophor, BBH to 9.5 with 1 N potassium hydroxide. The oil treatments consisted of oxybenzone (5% w/v), ethyl-cinnamate (5% v/v), octyl-salicylate (5% v/v) and oil alone. An uninoculated control treatment was also included.

Water-compatible formulations were applied at a rate of 100 L/ha (45 mL/plot), using the bicycle sprayer (see section 3.2). Oil-compatible formulations were applied at a rate of 5 L/ha using the ULV spinning disk sprayer. To eliminate drift, plots were enclosed in a polyethylene tent (3.0 x 1.5 x 1.5 m) during the ULV spray application. Water and oil sensitive papers were arbitrarily placed on the soil surface in selected plots to evaluate the spray distribution. Conidia were applied at times of low wind velocity (< 4 m/s) on the morning of July 28, 1993 for trial one and August 12 for trial two.

Ten leaves from the top of the canopy were arbitrarily collected from the centre of each of the three replicate plots. Times of sampling were immediately after (time 0), and 1, 2, 4, 6, 8, 12 and 16 days post-application. Leaves were returned to the laboratory in plastic bags, and each of the 10 leaves per replicate were aseptically cut across the laminae into pieces of about 1 cm long. Propagules were isolated on oatmeal-dodine agar using the wash method. Sample leaf areas per plot averaged 6.07 and 6.72 cm^2 in trials one and two, respectively.

Weather data. Solar radiation (300 to 2800 nm), temperature, relative humidity, precipitation, wind direction and velocity were recorded at a weather station adjacent to the field plots. The pyranometer malfunctioned 4 and 5 days after application of conidia in trial one. Hours of bright sunshine during these two days were 13.4 and 8.7 h, respectively (Environment Canada, Lethbridge). Using hours of bright sunshine, theoretical total incoming solar radiation at ground level (Q_s theoretical) on these days was calculated using the equations of Baier and Robertson (1965) and Robertson (1968) as 3.17×10^4 kJ/m^2 for day 4 and 2.40×10^4 kJ/m^2 for day 5. These values were higher than those recorded by the pyranometer (Q_s actual) for days with comparable hours of bright sunshine. Therefore, Q_s theoretical was plotted against Q_s actual for the period of July 28 to August 28, 1993. The coefficient of determination (r^2) observed was 0.96 and the equation used to describe this relationship was Q_s actual = 3967.9 + 1.08(Q_s theoretical); standard errors of the mean (SE) were 903.4 and 0.044 kJ/m^2 for a and b respectively. From the equation, Q_s was estimated as 2.58×10^4 kJ/m^2 for day 4 and 1.87×10^4 kJ/m^2 for day 5.

Statistical analyses. All experiments in controlled environments were arranged as completely randomized designs (CRD). The conidial population data was $\log_{10}$ transformed, and in two cases in the substrate selection experiment, the percent reduction data were arcsine-transformed but untransformed means and SE are presented. The substrate selection experiment was analyzed as a split plot in time with two levels of formulation and substrate, and five levels of time. This experiment was conducted three times for the water formulation and twice for the oil formulation; data from the different trials were treated as replicates and combined for analysis. The toxicity and selection experiments were repeated once, and with the exception of the clay formulation treatment, they were analyzed using analysis of variance. In conjunction with a significant F test, Tukey's studentized range test ($\alpha = 0.05$) was used to separate means. For the clay formulation, comparisons were made using the TTEST procedure of SAS.

In the field experiment, the water- and oil-compatible formulations were analyzed separately as a split plot in time with a Box correction. When a significant ($P \leq 0.05$) interaction was observed for formulation and time, pairwise comparisons of the slopes of conidial persistence between the control and test formulation were conducted using analysis of covariance. For the SAS REG procedure, the mean persistence data for each formulation were used to fit linear models; log_{10}-transformation of the x-axis was used for water-compatible formulations in both trials, and for the oil-compatible formulations in trial one but not two. In addition to time, the predictability of cumulative solar radiation on conidial survival was examined by regression analysis. In most instances, no *B. bassiana* conidia were isolated from leaf segments collected from untreated plots, and when conidia were isolated , it was at very low levels (< 10 cfu/cm^2). Therefore, the uninoculated control treatment was excluded from the analyses of conidial persistence.

Results

Influence of substrate. On both leaves and coverslips, droplets of water were localized and evaporated within 15 min of placement. Oil droplets (4 to 5 mm diam) covered a larger area than did water droplets (1.5 to 2.0 mm diam) on coverslips. On wheatgrass leaves, oil spread rapidly across the lamina and an oil sheen was usually observed.

When exposed to UVB radiation in the laboratory, significant interactions were observed between formulation (oil and water) and duration of exposure ($F=8.2$; $df=4,112$; $P<0.001$), and formulation and substrate ($F=13.0$; $df=1,19$; $P=0.002$). Comparisons between the oil and water formulations for individual substrates indicated that conidial survival was enhanced in oil on glass (Table 3.3.1); formulation ($F=65.4$; $df=1,5$; $P<0.001$), time ($F=23.4$; $df=4,56$; $P<0.001$) and the interaction between formulation and time ($F=8.5$; $df=4,56$; $P<0.001$) were significant. Although there was no interaction ($F=1.95$; $df=4,56$; $P=0.11$) between formulation and time, more conidia ($F=8.3$; $df=1,5$;

Table 3.3.1 Influence of formulation and substrate on survival of *Beauveria bassiana* conidia exposed to UVB radiation.

Duration (Min)	Glass		Leaves	
	$\log_{10}$ cfu[a]	% reduction[b]	$\log_{10}$ cfu[a]	% reduction[b]
Water Formulation				
0	3.96 (0.06) a[c]	--	4.11 (0.06) a	--
15	2.46 (0.11) b	96.0 (0.78) a[d]	2.36 (0.28) b	96.1 (1.0) a[d]
30	1.59 (0.27) bc	99.0 (0.27) b	1.70 (0.30) bc	98.8 (0.42) ab
45	0.66 (0.34) c	99.3 (0.41) b	1.66 (0.31) bc	98.6 (0.66) b
60	1.12 (0.31) c	99.4 (0.26) b	0.74 (0.30) c	99.7 (0.10) b
Oil Formulation				
0	3.99 (0.08) a	--	3.75 (0.15) a	--
15	3.92 (0.07) ab	22.4 (6.0) a	3.24 (0.22) a	49.2 (18.0) a
30	3.58 (0.20) ab	49.9 (16.4) ab	2.64 (0.54) ab	75.7 (7.1) ab
45	3.32 (0.27) ab	62.2 (14.5) ab	2.80 (0.24) ab	82.2 (5.2) ab
60	3.13 (0.26) b	74.4 (9.3) b	1.79 (0.38) b	97.4 (0.89) b

[a]Conidia ($\log_{10}$ colony-forming units (cfu)) recovered from glass coverslips or crested wheatgrass leaves. Values in parentheses following means represent standard errors of the means.

[b]Percent reduction was calculated as $((cfu_{T0} - cfu_{Tx}) / cfu_{T0})100$.

[c]Means not followed by the same letter within each formulation-substrate group are significantly different ($\alpha = 0.05$) according to Tukey's studentized range test. The experiment was conducted three times for the water formulation ($n = 10$) and two times for the oil formulation ($n = 6$).

[d]Data was arcsine transformed

P=0.035) were isolated from leaves treated with conidia in oil (averaged over time) than with conidia in water.

Comparisons between substrates for individual formulations indicated that the survival of conidia applied in oil on coverslips was greater ($F=21.5$; $df=1,5$; $P=0.006$) than the survival of conidia applied in oil to leaves (Table 3.3.1). Time alone was highly significant ($F=8.6$; $df=4,40$; $P<0.001$), and there was no interaction ($F=1.3$; $df=4,40$; $P=0.29$) between time and substrate. In water, there was no difference ($F=0.74$; $df=1,9$; $P=0.41$) in survival of conidia applied to either substrate (Table 3.3.1). As with oil, there was no interaction ($F=2.0$; $df=4,72$; $P=0.10$) between time and substrate and time alone was highly significant ($F=46.6$; $df=4,72$; $P<0.001$).

Solubility and toxicity of sunscreens. Of the adjuvants tested, 5 of 10, and 6 of 9 were highly soluble in water and oil (>5% w/v), respectively. The Blankophor brighteners, BBH, DML, HRS, LPG and RKH were marginally soluble in water, and Florisil, HOB and HMB exhibited low solubility in oil. Saturated formulations (room temperature) of the marginally soluble adjuvants were subsequently tested for UV protection. None of the water- ($F=0.85$; $df=10,11$; $P=0.60$) or oil-compatible ($F=1.19$; $df=7,8$; $P=0.40$) adjuvants tested were toxic to non-germinated conidia of *B. bassiana* after 12 h incubation at 25°C. The increased pH of Blankophor, BBH (9.5) required to enhance its solubility in water, had no effect ($\alpha=0.05$) on conidial viability.

Sunscreen selection. After 1.5 h exposure to UVB radiation ($F=27.3$; $df=12,65$; $P<0.001$), conidial survival in 9 of 11 water-compatible formulations was greater ($\alpha=0.05$) than that of conidia exposed to UVB radiation in water alone (Table 3.3.2). Conidial survival was equal ($\alpha=0.05$) to that of non-exposed conidia applied in water in all but three of the formulations. Five of the adjuvants protected conidia ($\alpha=0.05$) exposed to UVB radiation for 3 h ($F=30.4$; $df=12,64$; $P<0.001$). These included Congo Red and the

Table 3.3.2 Influence of water-compatible formulation adjuvants on survival of *Beauveria bassiana* conidia applied to leaves and exposed to UVB radiation for 1.5 and 3.0 h.

Formulation	Concentration	$\log_{10}$ cfu/leaf[a] (SE of the mean)	
	(%)	1.5 h	3.0 h
BSU-Optical brightener (OB)[b]	5	4.68 (0.04) a[c]	4.41 (0.07) a[c]
Congo Red[b]	5	4.58 (0.03) a	4.45 (0.10) a
Tinopal LPW[b]	5	4.46 (0.06) a	4.12 (0.12) ab
BBH-OB	0.25	4.35 (0.15) a	4.22 (0.12) ab
P167-OB	5	4.27 (0.31) a	4.43 (0.04) a
HRS-OB	2	4.13 (0.10) ab	3.49 (0.15) abc
LPG-OB	0.25	4.01 (0.14) ab	3.55 (0.23) abc
DML-OB	0.25	4.10 (0.05) ab	3.18 (0.32) bc
RKH-OB	0.25	2.86 (0.34) b	3.27 (0.32) abc
Diethanolamine-4-methoxycinnamate	5	1.16 (0.52) c	0.41 (0.41) d
Oil emulsion	5	0.74 (0.49) c	0.53 (0.53) d
Water	--	1.10 (0.52) c	2.77 (0.16) c
Water (No UV Exposure)	--	4.68 (0.06) a	4.44 (0.06) a

[a]Conidia ($\log_{10}$ cfu/leaf) recovered from crested wheatgrass leaves.

[b]Adjuvants selected for subsequent field evaluations.

[c]Means not followed by the same letter are significantly different ($\alpha = 0.05$) according to Tukey's studentized range test. The experiment was conducted two times ($n = 6$).

optical brighteners, BSU, BBH, P167, and Tinopal LPW; BBH was tested at a concentration of only 0.25% (w/v). The clay formulation treatment was analyzed separately. There was no difference in survival ($t=1.51$; $df=10$; $P=0.16$) between conidia exposed to UVB for 1.5 h (3.74, SE=0.20 log cfu/leaf) and conidia maintained in the dark (4.14, SE=0.17 log cfu/leaf). In contrast, conidial survival in clay was reduced ($t=2.57$; $df=10$; $P=0.028$) after 3.0 h exposure to UVB. From leaves treated with conidia in clay and exposed to UVB for 3.0 h, 4.19 (SE=0.11) log cfu/leaf were isolated compared to 4.55 (SE=0.089) log cfu/leaf from non-exposed leaves. Congo red, clay, Tinopal LPW, and Blankophor, BSU were selected for evaluation in the field experiment.

None of the nine oil-compatible formulations ($F=3.5$; $df=10,55$; $P<0.001$) tested enhanced ($\alpha=0.05$) survival relative to oil alone after 1.5 h exposure to UVB radiation (Table 3.3.3). After 3.0 h exposure to UVB radiation ($F=6.9$; $df=10,55$; $P<0.001$) conidial survival in Parsol MCX and 2,2-hydroxy-4-octoxybenzophenone was superior ($\alpha=0.05$) to the survival of conidia applied in oil alone. On the basis of availability and a previous report of their efficacy in protecting *M. flavoviride* conidia from artificial UVB radiation (Moore *et al.*, 1993), oxybenzone, ethyl-cinnamate and octyl-salicylate were selected for evaluation in the field experiment.

Field evaluations. Conditions of incoming solar radiation, temperature, precipitation and relative humidity fluctuated within and between trials (Figure 3.3.2). Total incoming solar radiation was 3.72×10^5 kJ/m^2 in trial one, and 25.1% less (2.79×10^5 kJ/m^2) in trial two. Hourly incoming solar radiation, averaged over the 16 days of the trials (daylight hours), was 1430 (SE=74.9) and 1327 (SE=62.6) kJ/m^2, respectively. Mean hourly temperatures were 16.2 (SE=0.29) and 15.2 (SE=0.23) °C, and relative humidities averaged 67.8 (SE=1.1) and 75.8 (SE=0.94) % in trials one and two, respectively. Five periods of light precipitation (< 3.0 mm per event) were recorded in trial one. In trial

Table 3.3.3 Influence of oil-compatible formulation adjuvants on survival of *Beauveria bassiana* conidia applied to leaves and exposed to UVB radiation for 1.5 and 3.0 h.

Formulation	Concentration	$\log_{10}$ cfu/leaf (SE of the mean)	
	(%)	1.5 h	3.0 h
Oxybenzone[b]	5	3.71 (0.17) ab[c]	1.40 (0.47) bcd[c]
Ethyl-cinnamate[b]	5	3.44 (0.17) ab	1.19 (0.38) bcd
2,2-dihydroxy-4-methoxybenzophenone	2	3.36 (0.22) ab	1.75 (0.35) abc
Parsol MCX	5	3.21 (0.23) ab	2.49 (0.50) ab
Octyl-salicylate[b]	5	3.16 (0.41) ab	0.85 (0.35) bcd
Eusolex	5	2.96 (0.35) ab	1.64 (0.37) abcd
Parsol 1789	5	2.82 (0.04) ab	0.00 (0.00) d
2,2-hydroxy-4-octoxybenzophenone	4	2.31 (0.77) b	2.37 (0.13) ab
Florisil	2	2.17 (0.48) b	1.19 (0.38) bcd
Oil	-	2.51 (0.17) b	0.50 (0.50) cd
Oil (No UV exposure)	-	4.41 (0.18) a	3.36 (0.14) a

[a]Conidia ($\log_{10}$ cfu/leaf) recovered from crested wheatgrass leaves.

[b]Adjuvants selected for subsequent field evaluations.

[c]Means not followed by the same letter are significantly different ($\alpha = 0.05$) according to Tukey's studentized range test. The experiment was conducted two times ($n = 6$).

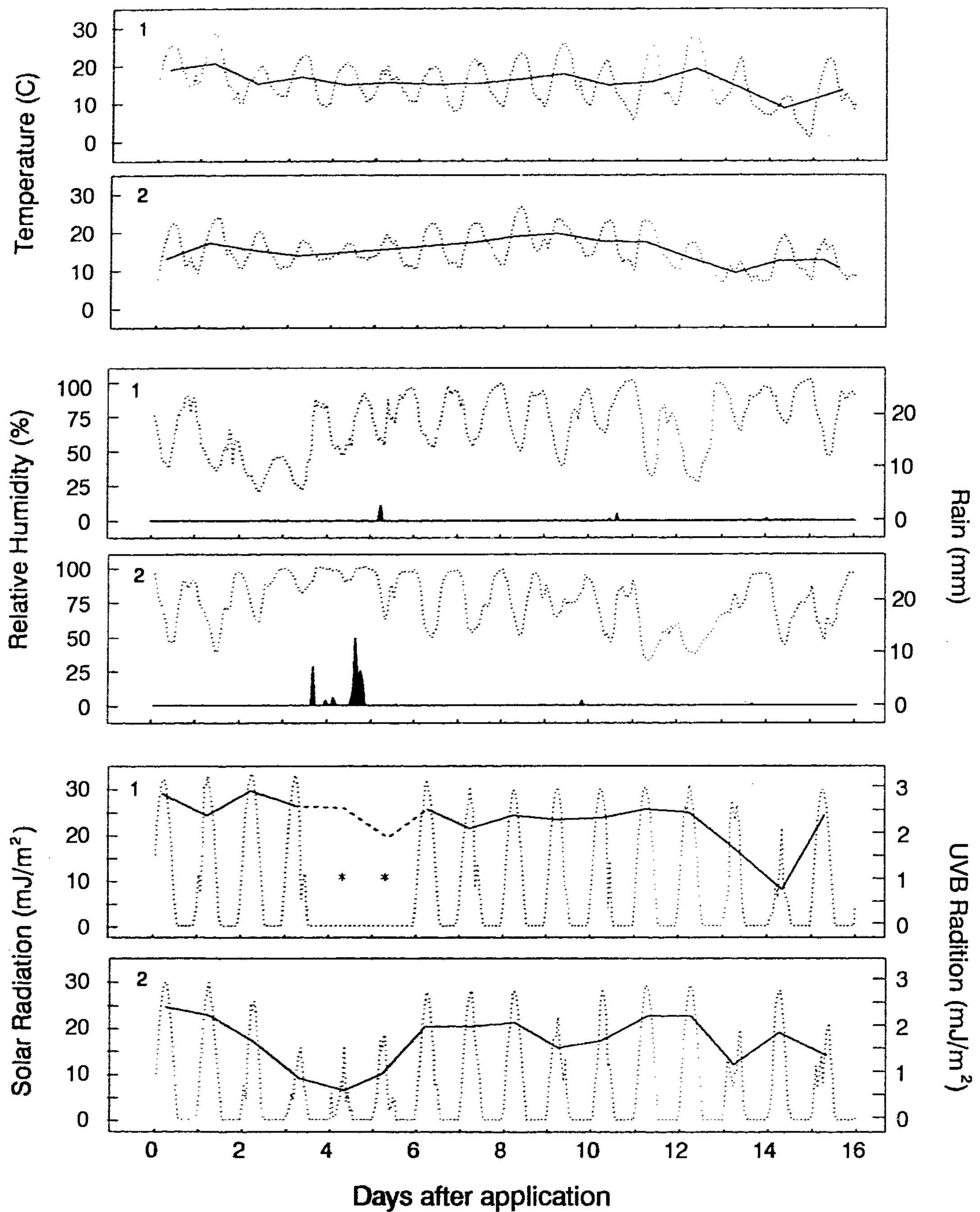

Figure 3.3.2 Hourly (dotted lines) and mean (solid lines) temperature, relative humidity, precipitation (peaks), hourly (dotted lines) and total daily (solid lines) solar radiation (300-2800 nm) during trials one and two. Asterisks represent missing solar radiation data; daily solar radiation during this period (dashed line) was estimated using hours of bright sunshine.

two, 51 mm of rain fell 3 to 5 days after application, followed by two additional periods of light rain (< 1 mm).

Of the five water-compatible formulations, there was no difference ($F=1.37$ and 1.60; df=4,8; $P=0.33$ and $P=0.26$) in conidial populations between the formulations immediately after application (T_0) in either trial one or two; populations ranged from 4.10 (SE=0.12) to 5.05 (SE=0.21) log cfu/cm^3. For all the water-compatible formulations, conidial survival declined logarithmically over time (Figures 3.3.3, 3.3.4), and the persistence data were fitted to linear regressions following log-transformation of both the cfu and time data. Coefficients of determination (r^2) ranged from 0.84 to 0.95, and from 0.85 to 0.98 for trials one and two, respectively (Table 3.3.4). Slopes of conidial persistence for the formulations ranged from -1.7 to -4.2, and both slopes ($t=-5.6$ to -16.9; df=6; $P<0.001$) and y-intercepts ($t=12.0$ to 36.5; df=6; $P<0.001$) were significantly different from zero. For individual treatments, neither slopes nor y-intercepts differed ($F=0.24$ to 2.96; df=1,44; $P\geq0.09$) between trials. Although a strong relationship was observed between cumulative solar radiation and conidial persistence, light was generally a less effective predictor of conidial survival ($r^2 = 0.67$ to 0.98) than was time (log-transformed). However, in trial two for the clay formulation, a stronger relationship was observed between conidial persistence and light ($r^2 = 0.98$) than with time ($r^2 = 0.85$).

In both trials, their was no interaction ($F=1.84$ and $F=1.91$; df=4,10; $P\geq0.19$) between time and formulation. However, time ($F=67.3$ and $F=75.8$; df=1,10; $P<0.001$) and formulation ($F=9.97$ and $F=20.6$; df=4,8; $P=0.0034$ and $P=0.001$) were highly significant. Pairwise comparisons of the formulations with the control treatment using analysis of covariance, indicated that Tinopal LPW ($F=6.19$ and $F=8.42$; df=1,44; $P=0.0167$ and $P=0.0058$) and clay ($F=4.30$ and $F=3.97$; df=1,44; $P=0.0439$ and $P=0.0527$) enhanced survival of conidia in both trials (Figures 3.3.3, 3.3.4) and that

Table 3.3.4 Linear regression data for *Beauveria bassiana* conidial persistence on crested wheatgrass leaves in the field experiment[a].

	Trial one					Trial two				
	a	SE(a)	b	SE(b)	r^2	a	SE(a)	b	SE(b)	r^2
Water-compatible										
Water	4.50[b]	0.37	-3.33[b]	0.46	0.90	4.31	0.14	-2.71	0.17	0.98
BSU	5.08	0.32	-4.19	0.40	0.95	4.53	0.18	-3.68	0.22	0.98
Tinopal LPW	4.59	0.19	-2.11	0.24	0.93	4.18	0.12	-1.73	0.14	0.96
Congo Red	4.85	0.30	-3.08	0.37	0.92	4.62	0.16	-2.78	0.20	0.97
Clay	4.92	0.36	-2.49	0.44	0.84	4.66	0.27	-1.96	0.33	0.86
Oil-compatible										
Oil	5.03	0.31	-3.83	0.39	0.94	4.18	0.16	-0.20	0.02	0.94
Oxybenzone	5.08	0.21	-3.07	0.26	0.96	4.49	0.12	-0.23	0.02	0.97
Ethyl-cinnamate	5.06	0.18	-3.43	0.22	0.98	4.54	0.07	-0.23	0.01	0.99
Octyl-salicylate	4.89	0.33	-3.06	0.41	0.90	4.41	0.09	-0.22	0.01	0.98

[a]For the water-compatible formulations in both trials and the oil-compatible formulations in trial one, the x-axis was $\log_{10}$-transformed and the regression equation is: $\log_{10}$ cfu/g of soil = a + b ($\log_{10}$ days + 1) where n = 8. For the oil-compatible formulations in trial two, the x-axis was untransformed and the regression equation used is: $\log_{10}$ cfu/g of soil = a + b (days).

[b]All y-intercepts and slopes are significantly different from zero ($P \leq 0.01$).

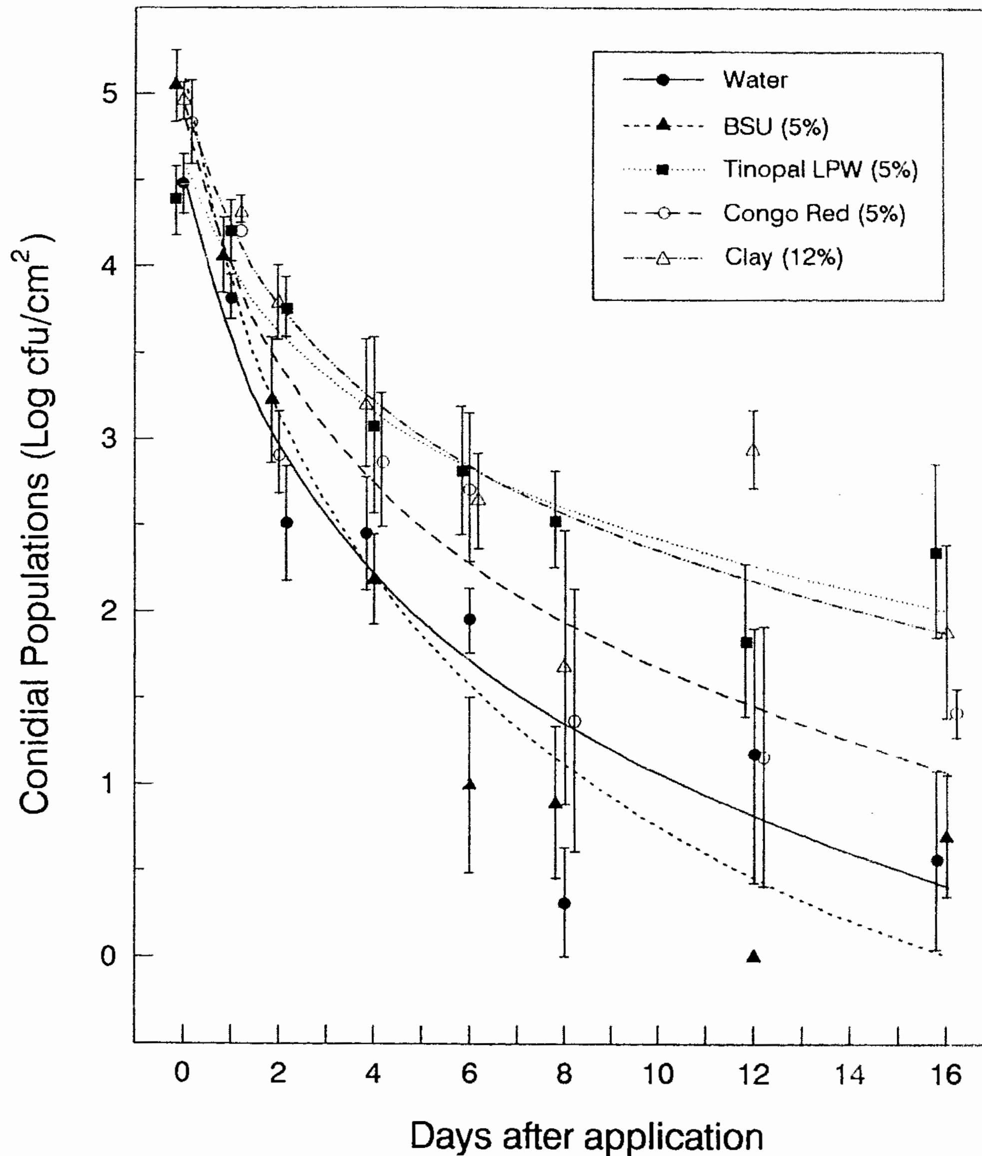

Figure 3.3.3 Persistence of *Beauveria bassiana* conidia in water-compatible sunscreens on crested wheatgrass in trial one (July 28 to August 13, 1993). Populations were quantified as $\log_{10}$ colony-forming units (cfu)/cm^2 of leaf area and vertical lines represent standard errors of means (n=3). To avoid superimposition of standard error bars, means are offset along the x-axis.

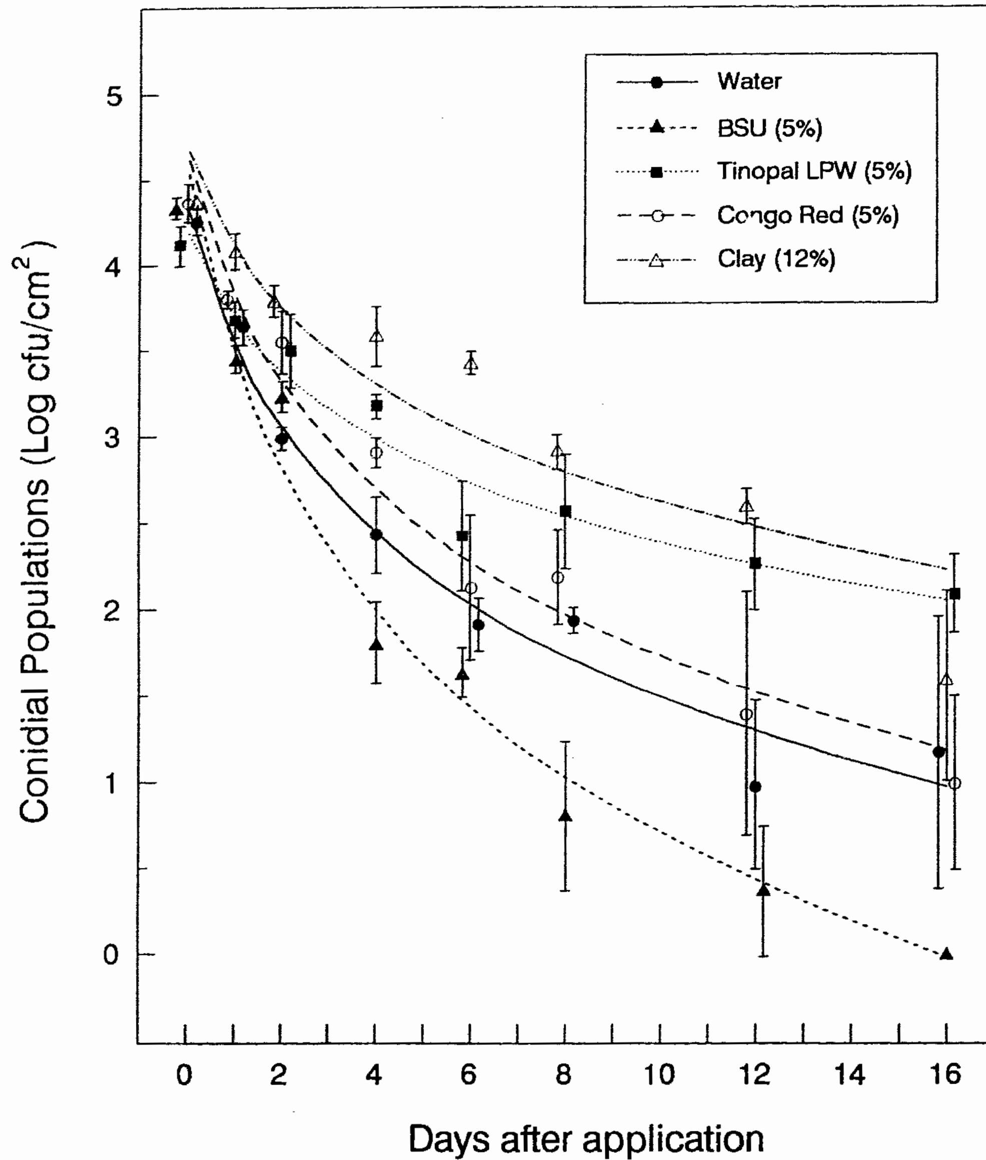

Figure 3.3.4 Persistence of *Beauveria bassiana* conidia in water-compatible sunscreens on crested wheatgrass in trial two (August 12 to 28). Populations were quantified as log_{10} colony-forming units (cfu)/cm^2 of leaf area and vertical lines represent standard errors of means (n=3). To avoid superimposition of standard error bars, means are offset along the x-axis.

neither formulation was superior ($F=0.60$ and $F=0.62$; df = 1,44; $P=0.44$ and $P=0.43$). Congo Red did not affect ($F=0.21$ and $F=0.04$; df = 1,44; $P=0.65$ and $P=0.85$) the persistence of conidia in either trial. Although similar ($F=2.88$; df = 1,44; $P=0.097$) to the control treatment in trial one, the survival of conidia in BSU was less ($F=7.74$; df = 1,44; $P=0.0079$) than in water in trial two.

Immediately after application, conidial populations were similar ($F=0.10$ and $F=0.29$; df = 3,6; $P=0.96$ and $P=0.82$) in the four oil-compatible formulations in both trials. In contrast to the water-compatible formulations, slopes of conidial persistence differed between the trials. In trial one, best fit regression analysis required a $\log_{10}$-transformation of both cfu and time data, and slopes of conidial persistence ranged from -3.1 to -3.8 (Table 3.3.4). In contrast, only the cfu and not the time data required log-transformation in trial two; slopes ranged from -0.20 to -0.23. In both trials, all slopes ($t=$ -6.4 to -24.8; df = 6; $P<0.001$) and y-intercepts ($t=14.7$ to 62.1; df = 6; $P<0.001$) were significantly different from zero. Coefficients of determination for the oil-compatible formulations ranged from 0.90 to 0.99 (Table 3.3.4). As with the water-formulations, a strong relationship was observed between cumulative light and conidial persistence for oil (r^2 = 0.85 and 0.94), oxybenzone (r^2 = 0.95 and 0.97), ethyl-cinnamate (r^2 = 0.95 and 0.99) and octyl-salicylate (r^2 = 0.74 and 0.98). For the oil and octyl-salicylate formulations in trial one, and all formulations in trial two, light was almost as good a predictor of conidial survival as was time.

For the oil-compatible formulations, time ($F=51.0$ and $F=87.8$; df = 1,8; $P<0.001$) but not formulation ($F=3.65$ and $F=0.80$; df = 3,6; $P=0.083$ and $P=0.53$) influenced conidial persistence, and there was no interaction ($F=0.59$ and $F=0.44$; df = 3,8; $P \geq 0.64$) between formulation and time (Figures 3.3.5, 3.3.6). Comparison of water and oil controls over time indicated no difference in conidial persistence in either trial ($F=0.48$-1.1; df = 1,4; $P \geq 0.35$). Observed slopes of conidial persistence for oil and water control treatments were

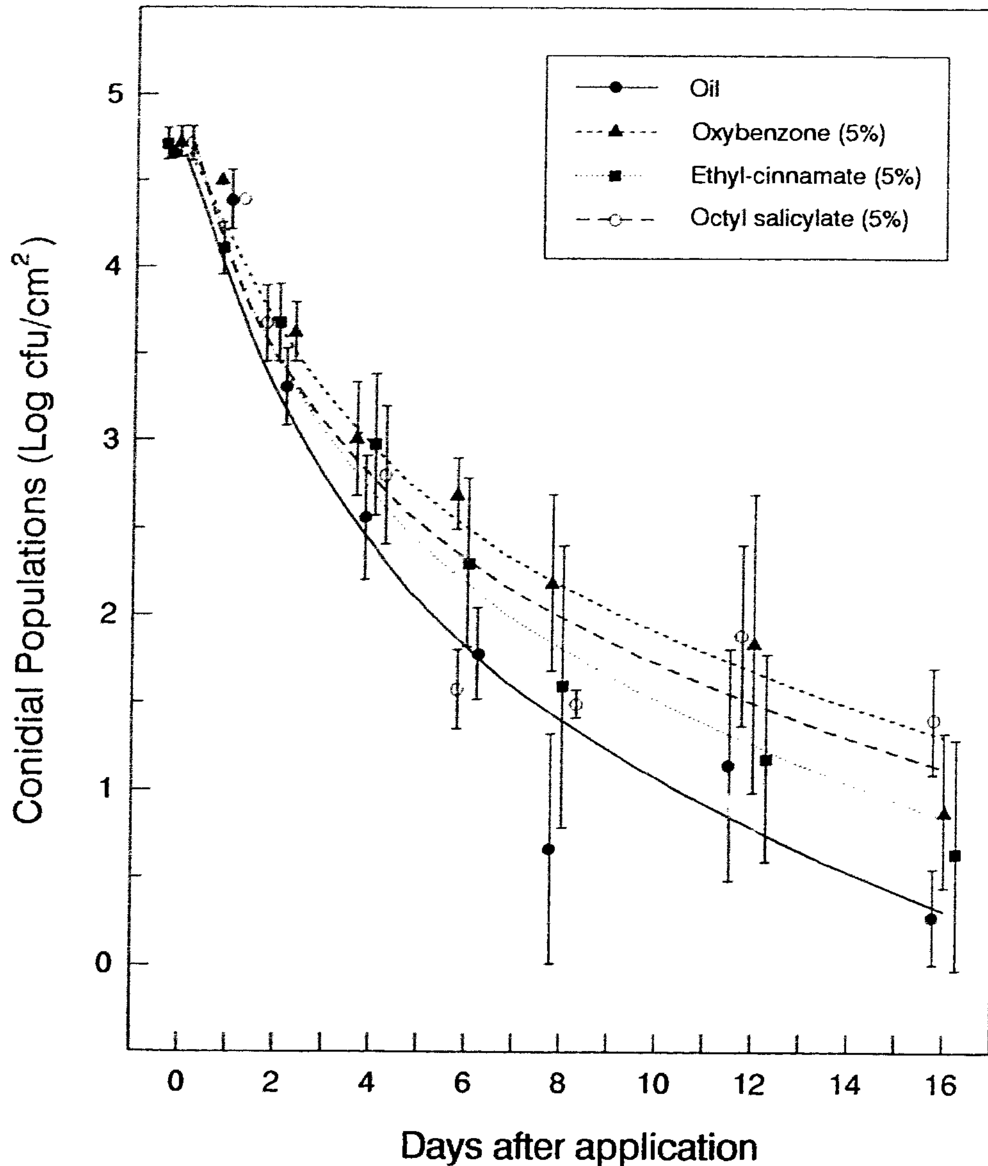

Figure 3.3.5 Persistence of *Beauveria bassiana* conidia in oil-compatible sunscreens on crested wheatgrass in trial one (July 28 to August 13, 1993). Populations were quantified as $\log_{10}$ colony-forming units (cfu)/cm^2 of leaf area and vertical lines represent standard errors of means (n=3). To avoid superimposition of standard error bars, means are offset along the x-axis.

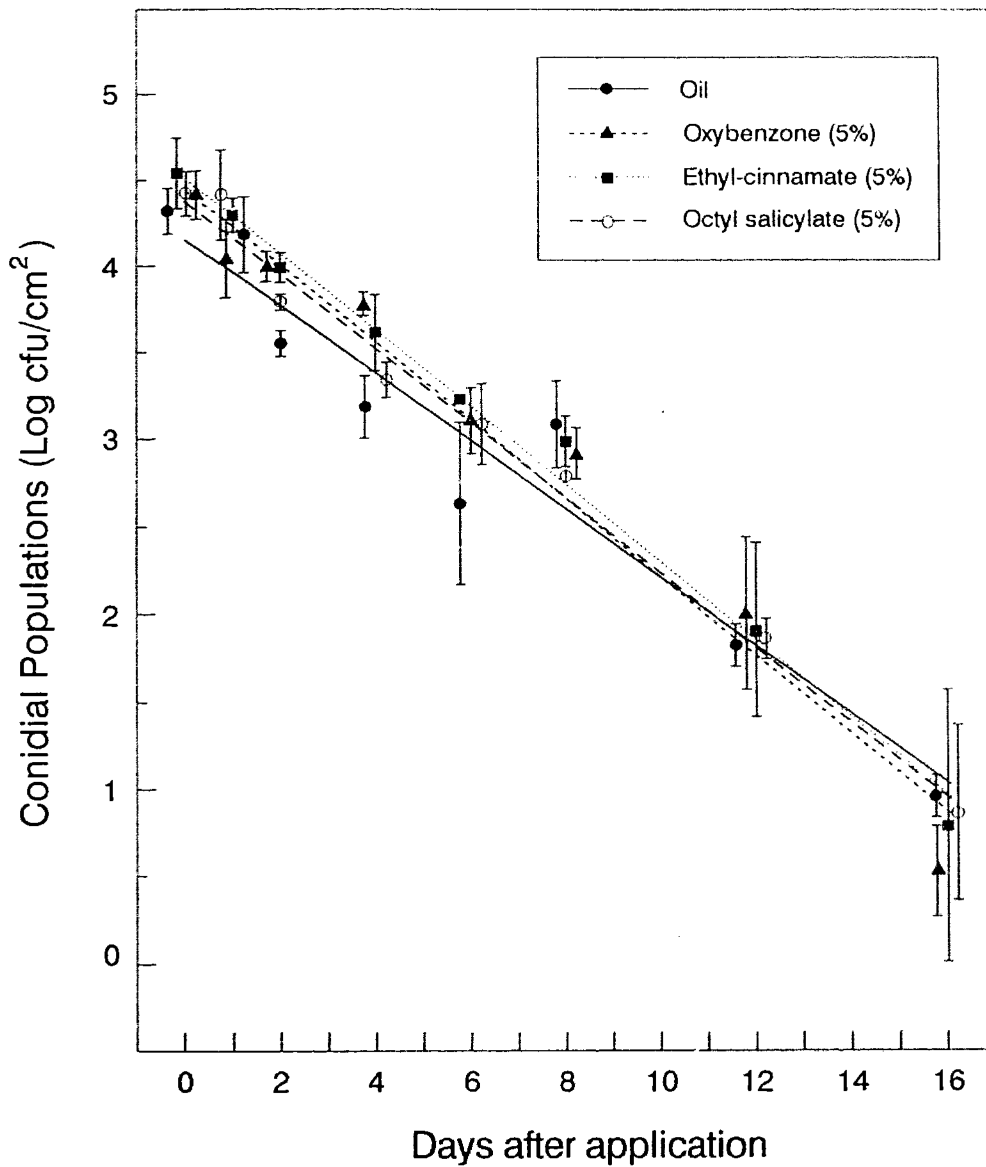

Figure 3.3.6 Persistence of *Beauveria bassiana* conidia in oil-compatible sunscreens on crested wheatgrass phylloplanes in trial two (August 12 to 28). Populations were quantified as $\log_{10}$ colony-forming units (cfu)/cm^2 of leaf area and vertical lines represent standard errors of means (n = 3). To avoid superimposition of standard error bars, means are offset along the x-axis.

similar to those observed in the previous study at the top of the wheatgrass canopy (section 3.2).

Discussion

The impact of UVB radiation on fungal populations in natural habitats has not been extensively studied. *Beauveria bassiana*, a hyaline fungus, is soil-borne and is rarely isolated from plant foliage. Furthermore, the survival of *B. bassiana* conidia applied to foliage is very poor (section 3.2), and the most important parameter limiting survival of conidia in epigeal habitats appears to be sunlight (Daoust and Pereira, 1986a; section 3.2). Ultraviolet radiation causes primary (e.g., nucleic acid mutations) and/or secondary (e.g., photoreactions) damage to exposed microorganisms, either of which may lead to cellular death (Tevini, 1993). Conidia of *B. bassiana* were highly sensitive to artificial UVB radiation, but the mechanism causing death is unknown. It seems likely that the extreme sensitivity of *B. bassiana* to the UVB portion of the solar spectrum limits its persistence in epigeal habitats. Formulation of *B. bassiana* to provide protection from ultraviolet light will be necessary to increase its survival and efficacy in epigeal habitats.

Congo Red, clay and all of the stilbene brighteners tested provided a degree of protection from artificial UVB radiation (Table 3.3.2). Four of the adjuvants were tested in a field environment but only clay and the stilbene brightener, Tinopal LPW, consistently increased the persistence of *B. bassiana* conidia on wheatgrass leaves. Congo Red and the stilbene brightener, BSU were ineffective. Stilbene brighteners readily absorb UVB radiation and have previously been shown to protect nuclear polyhedrosis virus (NPV) occlusion bodies (Martignoni and Iwai, 1985; Shapiro, 1992), *Steinernema carpocapsae* (Nickle and Shapiro, 1992), and *B. thuringiensis* (Morris, 1983) from UVB inactivation. Tinopal LPW has also been shown to enhance the virulence of NPV (Shapiro and Dougherty, 1993). In contrast to the UVB absorbing adjuvants, clay acts as a sunlight blocker. Sunlight blockers have provided effective protection of viruses from UVB radiation (Ignoffo and Batzer, 1971;

Jaques, 1971; Shapiro *et al.*, 1983), and the use of blockers such as clay, starch or carbon may be preferable because they are either environmentally innocuous or easily decomposed.

Several entomopathogens, including *B. bassiana*, have been applied in oil at ultralow volumes (ULV) in attempts to increase their efficacy (Feng *et al.*, 1994). Similarly to a previous field study (section 3.2), no marked increase in the survival of *B. bassiana* conidia applied in oil at ULV was observed relative to conidia sprayed in water at conventional volumes. However, in the laboratory experiment, *B. bassiana* conidia exposed to UVB radiation survived better in oil than in water on glass. Conidia of *M. flavoviride* in oil on agar were found to be less sensitive to UVB radiation (below 305 nm) than were conidia in water; this was attributed to absorption of UVB radiation by the oil (Moore *et al.*, 1993). Although the survival of conidia was also greater on leaves in oil than in water, conidial persistence in oil was substantially reduced on leaves relative to that on glass. Despite the presence of an oil sheen, the decreased efficacy of oil on leaves, at least in part, was attributed to the absorption of oil into mesophyll cells. Under field conditions, the volume of oil deposited on leaves at ULV is considerably less than the volume of oil (1 μl) that was pipetted onto the leaf segments in the laboratory. Therefore, it would be expected that the rate and degree of absorption of oil into leaf tissues applied at ULV would be greater, and the rapid absorption of oil into leaf tissues may explain the poor protection of *B. bassiana* conidia from UVB radiation observed on leaves in the field experiment.

Oil-soluble sunscreens, which absorb UVB radiation, have been developed for use in the cosmetic industry (Shaath, 1990). Several of these (oxybenzone, ethyl-cinnamate and octyl-salicylate) were found to protect *M. flavoviride* conidia on agar (Moore *et al.*, 1993). Although several of the oil-compatible adjuvants tested protected *B. bassiana* conidia from artificial UVB radiation, they did not protect conidia under field conditions. Reasons for the differential efficacy of these adjuvants between the field and laboratory are unknown. However, absorption of the oil carrier into the leaf tissues may have contributed to the

decreased protection provided by these adjuvants in the field. In trial one but not two, the conidial persistence data for conidia applied in oil were fit to linear models after log-transformation of the x-axis. Reasons for the discrepancy between trials are unknown, but may be due to variability in the data, or the result of differing environmental conditions. For example, less solar radiation (25%) was encountered in trial two which may have affected conidial survival.

This study demonstrated that *B. bassiana* conidia are extremely sensitive to UVB radiation and that the survival of conidia can be prolonged in field environments by UVB protectants. Whether the prolonged survival of conidia provided by UVB protectants is sufficient to enhance the efficacy of insect control warrants investigation.

3.4 INFLUENCE OF SIMULATED RAIN ON CONIDIAL PERSISTENCE[3]

Materials and Methods

Inoculum Preparation and Application. Dry conidia were suspended in sterile deionized water at a concentration of 1.7 x 10^9 viable conidia per mL. Conidia were then applied to leaves of wheat plants (*Triticum aestivum*; cv. AC Reed) and alfalfa (cv. AC Blue J) grown individually in 8.75-cm diameter pots containing Cornell mix. Immediately before treatment, plants were trimmed to a height of 8 to 10 cm. The conidial suspension (100 μl) was applied to individual plants in a 10.8 cm diameter by 35.5 cm high plexiglass cylinder using an airbrush (Artek, Rockford, IL) at 103 kPa. Droplet size, density and distribution pattern were monitored using water-sensitive paper (Teejet Spraying Systems Co., Wheaton, IL). Following inoculation, plants were maintained in the dark at ambient temperature for 15 to 20 min to allow the water carrier to evaporate before exposing dry conidia on leaf surfaces to simulated rain.

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[3]A version of this section has been published as: "Effects of simulated rain on the persistence of *Beauveria bassiana* conidia on leaves of alfalfa and wheat. Inglis, G.D., D.L. Johnson and M.S. Goettel. 1995. Biocontrol Science and Technology 5:365-370.

Rain Simulation. A continuous spray, Guelph rainfall simulator (Tossell *et al.*, 1987) was used in a glasshouse with diffuse light and a temperature of 23° to 25°C. The boom of the simulator was situated 1 m above the bench and plants were randomly arranged within the 0.5 x 0.5 m spray area; the rain coverage within this area had previously been determined to be uniform. Rain was applied (100 kPa) at medium (27 mm h^{-1}) and high (113 mm h^{-1}) intensities using the 1/8 4.3 W and 1/4 14 W nozzles, respectively. Plants were exposed to rain for 30 or 60 min at each rain level.

Conidial Enumeration. Prior to rain exposure, 10 alfalfa leaflets (*ca* 1 x 0.5 cm) and 10, 1 cm wheat leaf segments were arbitrarily collected from plants in each pot. The wheat leaf segments were removed 1 to 2 cm from the tip of the lamina. After exposure to rain, 10 leaflets or leaf segments were removed from the same leaves sampled prior to rain exposure. Control treatments were alfalfa and wheat plants inoculated with conidia but not exposed, although positioned adjacent to the rainfall simulator in the glasshouse for 60 min. No *B. bassiana* conidia were isolated from leaves of the alfalfa and wheat plants sampled prior to inoculation.

Conidia were isolated on oatmeal-dodine agar using the wash method. Leaf areas ranged from 4.0 to 11.9 and 4.2 to 10.3 cm^2 for alfalfa and wheat respectively. Reductions in conidial number due to rain exposure at $time_x$ were calculated as: ((cfu cm^{-2} at time $(T)_0$ - cfu cm^{-2} at T_x) / cfu cm^{-2} at T_0) x 100. If the number of conidia isolated at T_x were larger than at T_0, percent increase in number was calculated as: ((cfu cm^{-2} at time $(T)_0$ - cfu cm^{-2} at T_x) / cfu cm^{-2} at T_x) x 100.

To assess the effectiveness of the wash procedure, conidia sprayed on alfalfa and wheat leaves were isolated and populations per cm^2 of leaf calculated as above. After washing, the leaf segments were rinsed in sterile buffer-Tween, 4.5 mm diameter disks were cut from each, homogenized, diluted in buffer-Tween and the homogenate spread on

oatmeal-dodine agar. Colony-forming units cm^{-2} from the washes and homogenates were compared.

Statistical Analyses. Each of the three trials conducted were arranged as a CRD with two levels of crop, rain intensity and duration, each replicated three times. Log_{10} transformations were required to normalize the conidial population data but untransformed means and standard errors of the means (SE) are presented throughout the text. Prior to combining the data, homogeneity of variance between trials was examined using Bartlett's test. The combined data was analyzed with weighted analysis of covariance (SAS Institute Inc., 1988). Conidial populations on leaves before rain exposure were used as the covariate, and in all instances a trial effect was included in the models. In the absence of interactions between crop, rain intensity, rain duration and the covariate, individual treatments within each crop were compared to the control treatment using weighted analysis of covariance and the least-square means (lsmeans) function of SAS.

Results and Discussion

The wash technique employed recovered 95.6 to 99.7 % of the conidia from alfalfa and wheat leaves. Within 10 min of application, the water carrier had evaporated and initial populations of dry conidia on leaves of the plants ranged from 1.6×10^5 (SE=0.39×10^5) to 3.5×10^5 (SE=0.64×10^5) cfu cm^{-2} (Table 3.4.1). There was no difference (F=1.1; df=1,88; P=0.28) in the deposition of conidia on the two crops. Conidia of *B. bassiana* are rapidly killed by solar radiation under field conditions (section 3.2). However, under the diffuse light conditions of the greenhouse the conidia were not affected by the 60 min exposure (Table 3.4.1; control treatment).

The role of precipitation on the persistence of fungal taxa possessing hydrophobic propagules (e.g., *Beauveria*, *Metarhizium* and *Nomuraea*) in epigeal habitats has not previously been studied. Simulated rain caused the removal of *B. bassiana* conidia from leaves of alfalfa (F=3.5; df=4,39; P=0.017) and wheat (F=3.2; df=4,39; P=0.022),

Table 3.4.1 Effect of simulated rain on retention of *Beauveria bassiana* conidia on leaves of alfalfa and wheat.

Rain Treatment	Alfalfa			Wheat		
	Before	After	Reduction	Before	After	Reduction
	cfu cm^{-2} (x 10^5)		(%)	cfu cm^{-2} (x 10^5)		(%)
Medium intensity[a]						
30 Min	1.96 ± 0.35	1.13 ± 0.12 ab[d]	28.1 ± 12.5	1.90 ± 0.28	1.10 ± 0.20 bc[d]	35.4 ± 11.8
60 Min	2.50 ± 0.43	2.09 ± 0.52 bc	24.6 ± 14.0	1.83 ± 0.20	0.85 ± 0.21 ab	56.0 ± 6.7
High intensity[b]						
30 Min	2.19 ± 0.45	0.74 ± 0.24 a	60.6 ± 9.0	1.60 ± 0.39	1.07 ± 0.50 ab	49.7 ± 14.0
60 Min	2.58 ± 0.81	1.21 ± 0.41 ab	46.8 ± 12.9	1.72 ± 0.48	1.16 ± 0.55 ab	50.7 ± 9.7
Control[c]	3.51 ± 0.64	4.24 ± 0.96 c	-2.2 ± 16.8	3.07 ± 0.46	3.28 ± 0.52 c	-4.0 ± 7.8

[a]Medium intensity rain = 26.7 mm h^{-1}.

[b]High intensity rain = 112.7 mm h^{-1}.

[c]No rain treatment and exposure to the same light conditions for 60 min.

[d]Post-rain exposure treatment means (± standard error) within each crop not followed by the same letter are significantly different ($P \leq 0.05$) according to least-square means. Conidial populations on leaves after rain exposure were adjusted for the covariate (conidial populations before rain exposure) but unadjusted means are presented.

with the decrease in population density ranging from 28.1 (SE=12.5) to 60.6 (SE=9.0) % (Table 3.4.1). The duration of rain exposure had no effect (F=0.7; df=1,61; P=0.42), and conidia were removed equally from leaves of both crops (F=0.2; df=1,61; P=0.65). There was a weak effect (F=4.9; df=1,61; P=0.031) due to the difference in rain intensity. Although the interaction between crop and rain intensity was non-significant (F=2.9; df=1,61; P=0.094), the rain intensity effect occurred only for alfalfa (Table 3.4.1). Rain has been shown to decrease the foliar persistence of *Bacillus thuringiensis* (Frankenhuyzen and Nystrom, 1989), and facilitate the dispersal of some fungal propagules (Fitt *et al.*, 1989). Droplet velocity and size affect dispersal of fungal propagules (Fitt *et al.*, 1989), and although the nozzles and pressure used produced a relatively wide range of drop sizes, the 1 m boom height selected produced rain with relatively low droplet velocities.

Rain, particularly immediately after the application of inoculum, has been implicated with decreased efficacy of *B. bassiana* by many researchers (e.g., Gardner *et al.*, 1977; Johnson *et al.*, 1992). Results from the present study indicate that rain could reduce the efficacy of this entomopathogen in epigeal habitats. However, rain accompanied by wind in excess of 80 km h^{-1} within 30 min of application was observed to have no apparent effect on persistence of *B. bassiana* conidia on leaves (section 3.2). It now appears that the rapid deactivation of conidia by solar radiation that occurs under field conditions obscures the effect of conidial removal by rain, as determined by changes in populations over time.

Although simulated rain removed *B. bassiana* conidia from leaves, a substantial proportion of the conidia applied in water remain after exposure to relatively high intensity rain. Furthermore, the effects of rain are not all detrimental because infection can be enhanced under conditions of high ambient humidity (Schaerffenberg, 1964) provided by precipitation or irrigation (Campbell *et al.*, 1985). Whether precipitation will enhance or jeopardize the development of an epizootic will depend on whether conidial populations are decreased below the inoculum threshold required to incite mycosis in the target insect.

Formulation adjuvants such as sticking agents might also reduce the impact of rain on conidial removal, and prolong the time that inoculum densities remain above the threshold level.

3.5 CONCLUSIONS

Conidia within the alfalfa and wheatgrass canopy survived longer than those at the top of the canopy. Differences in conidial survival between canopy positions did not appear to be due to temperature or relative humidity. The alfalfa canopy was considerably more dense than that of wheatgrass and the persistence of conidia was conspicuously greater with the alfalfa canopy suggesting that solar radiation was the primary factor affecting conidial survival. There was no consistent effect of formulation on survival of conidia. To test the effect of UVB radiation on conidial survival a number of sunscreens were tested in laboratory and field settings. Numerous sunscreens protected conidia from UVB radiation in the laboratory but in a field environment, only two of seven sunscreens consistently prolonged conidial survival. This study demonstrated that exposure to UVB radiation is an important factor limiting conidial persistence, and that the use of UV protectants can prolong their survival. Despite the occurrence of a violent rain storm after the application of conidia in one field trial (section 3.2), no differences were observed in slopes of conidial persistence in other trials where no or little rain occurred. While simulated rain removed significant numbers of conidia from leaves, substantial populations of conidia remained after exposure to high intensity rain. The rapid deactivation of conidia that results from exposure to solar radiation in field environments likely obscures conidial dislodgement by rain as measured by changes in populations over time.

CHAPTER 4

EFFICACY OF *BEAUVERIA BASSIANA* AGAINST GRASSHOPPERS

4.1 INTRODUCTION

Factors that limit the efficacy of *B. bassiana* against grasshoppers in field environments are unknown. Conidia of *B. bassiana* (GHA 92) produced in 1992 and aerially applied in oil were effective against grasshoppers, whereas conidia of the same genotype produced in 1994 (GHA 94) and similarly applied were ineffective (N. Foster, pers. comm.; C. Bradley, pers. comm.). The two most obvious possibilities for the decreased efficacy of GHA 94 were either loss of virulence and/or inadequate targeting of grasshoppers with conidia. By comparing the efficacy of these two conidial batches in laboratory and field experiments, information on the factors of the pathogen-grasshopper-environment interaction that limit the efficacy of *B. bassiana* may be obtained.

This chapter is divided into two sections. In section 4.1, a bioassay method that facilitates evaluations of *B. bassiana* conidia in oil against grasshoppers is described. Grasshopper nymphs are usually targeted in inundative applications with *B. bassiana* in North America, but the small size of early-instar nymphs, the hydrophobicity of their cuticle, their mobility, and their susceptibility to mechanical damage during handling make it difficult to deliver an accurate dose of conidia. Furthermore, some evidence now suggests that oil is a more efficacious carrier than water for propagules of entomopathogenic fungi (Bateman *et al.*, 1993), but topically-applied oil can be toxic to nymphs (Goettel and Johnson, 1992). In section 4.2, GHA 92 and GHA 94 are tested in laboratory and field tests to determine if previous differences between field trials were due to changes in the pathogen or to other aspects of the disease tetrad.

4.2 DEVELOPMENT OF AN OIL-BAIT BIOASSAY METHOD[4]

Materials and Methods

Four isolates of *B. bassiana* from diverse geographical locations and substrates were evaluated. The isolates tested were: Lethbridge Research Centre (LRC) 14, isolated from soil near Magrath, AB in July, 1991; LRC 30, isolated from soil from Burkina Faso, Africa in September, 1992 and shown to be pathogenic to Senegalese grasshoppers (*Oedaleus senegalensis* (Krauss)); LRC 82 (ARSEF 2455; University of Alberta Microfungus Collection and Herbarium 4132) isolated from an alligator at City Zoo, Oklahoma City, OK in May, 1978; and Mycotech Corporation Inc. "GHA", an isolate known to be pathogenic to the migratory grasshopper. All cultures were lyophilized in skim milk. Cultures were established on PDA and grown in the dark at 25°C for 7 to 10 days. The germinability of conidia was measured on PDA with Benlate. Within 24 h of the germination assessments, conidia were collected from the surface of the PDA cultures and suspended in sunflower oil (Safflo, Unico Inc., Concord, ON). Concentrations of viable conidia in oil were then estimated using a hemocytometer and conidial densities were adjusted so that 0.5 μl contained 1×10^5, 3.2×10^4, 1×10^4, 3.2×10^3 or 1×10^3 viable conidia (5.0, 4.5, 4.0, 3.5 or 3.0 $\log_{10}$ conidia).

Nymphs (F_1 laboratory generation) were individually collected in sterile 20-mL vials stoppered with a sterile polyurethane foam plug and starved for 12 h before inoculation. Conidial suspensions from each dose were pipetted (0.5 μl) onto 5 mm-diam lettuce disks; the control treatment consisted of oil alone. Lettuce disks were maintained in the dark at ambient temperature for 15 to 20 min and the bait was presented to individual nymphs in the glass vials. To prevent loss of the oil carrier and/or sticking of the lettuce disk to the

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[4]A version of this section has been published as: "An oil-bait bioassay method used to test the efficacy of *Beauveria bassiana* against grasshoppers" Inglis, G.D, D.L. Johnson and M.S. Goettel. 1996. Journal of Invertebrate Pathology 67:312-315.

wall of the vial, the inoculated disk was pierced with a pin and suspended approximately 2 cm into the vial from the foam plug. Nymphs in vials were placed at 25°C under incandescent and fluorescent light. Although the majority of the nymphs ingested the lettuce disk within 30 min, nymphs were maintained in the vial for 12 h and nymphs that underwent ecdysis or that did not consume the entire disk during this period were removed from the experiment. Groups of 12 to 15 nymphs per treatment were transferred to 21 x 28 x 15 cm plexiglass containers equipped with a perforated metal floor to reduce contact with frass. Nymphs were maintained on a diet of wheat leaves in a CEC under a 16/8 h light/day photoperiod and a 25/20°C day/night temperature regime. Conditions of temperature and relative humidity in the CEC (35 to 40 cm above the bench surface) and within the cages were monitored with a CR21X micrologger. Relative humidities within the CEC varied from 20 to 55%. Although humidities within the cages were similar to those in the CEC for most of the day, they rose 4 to 8% higher for a 2 to 3 h period after placement of the wheat leaves in cages. Temperatures in the cages rose 2.5 to 3.1°C higher during the light period but remained near that of the CEC temperature during the dark period.

The experiment was arranged as a RCBD with four blocks conducted in time. The total number of nymphs per isolate-dose combination ranged from 46 to 61 (1176 nymphs total with 257 to 289 total nymphs per isolate). Nymphs that produced hyphal growth of *B. bassiana* on moistened filter paper were recorded. No ($P=0.33$) interaction was observed between isolate and dose in the prevalence of "other mortality" by 14 days, and when individual treatments were compared to the control treatment with Dunnett's test, no differences ($\alpha=0.05$) were observed. Therefore, all subsequent analyses were conducted on nymphs considered to have died from mycosis. Comparisons of disease progress between doses by isolate and between isolates by dose were conducted as split-plots in time with a Box correction. Subsequent to a significant F-test for the time-treatment interaction, means were compared using lsmeans. The prevalence of final mycosis (14

days) was analyzed with four levels of isolate and five levels of dose; means were also compared using lsmeans. In the absence of a block effect, the mycosis data were combined across blocks, and probit-transformed regressions of final mycosis by dose were fitted by maximum likelihood using a S108 Multiline Quantal Bioassay program (Agriculture and Agri-Food Canada, Sir John Carling Building, 930 Carling Ave, Ottawa, ON, K1A 0C5). The program also tested for goodness of fit, estimated lethal $dose_{50}$ with 95% fiducial limits, and tested for parallelism and for common y-intercepts. For isolate-dose combinations where the prevalence of final mycosis exceeded 50%, data were fitted to a Weibull distribution, and median lethal times with upper and lower 95% confidence limits (CL) were estimated.

Results and Discussion

The oil-bait method facilitated the rapid inoculation of grasshopper nymphs; within 1 h, 350 nymphs were easily inoculated. In a subsequent study using the same method, ≈1650 nymphs were inoculated within a 4 h period (see section 5.3). The oil-bait method unquestionably expedites the inoculation of nymphs relative to direct topical application of conidia. Very low levels of nymphal mortality were observed within 48 h of inoculation (0 to 6.3%), and at the end of the 14-day experimental period, mortality not attributed to *B. bassiana* was 7.3 (SE = 1.7) % in control nymphs. The oil-bait method reduced the handling of nymphs thereby decreasing the potential for mechanical damage. Furthermore, the toxic effects of oil which can occur with direct topical application (Goettel and Johnson, 1992), were minimized.

For each of the four *B. bassiana* isolates tested, a significant ($F = 5.0$-6.0; $df = 4,15$; $P \leq 0.009$) dose response of mycosis was observed (Figure 4.2.1 and 4.2.2). Among isolates, substantial differences ($F = 27.0$; $df = 3,57$; $P < 0.001$) were observed in the prevalence of final mycosis averaged over dose (Figure 4.2.1). With the exception of the 3.0 $\log_{10}$ dose, a higher ($P \leq 0.01$) prevalence of final mycosis was observed for the GHA

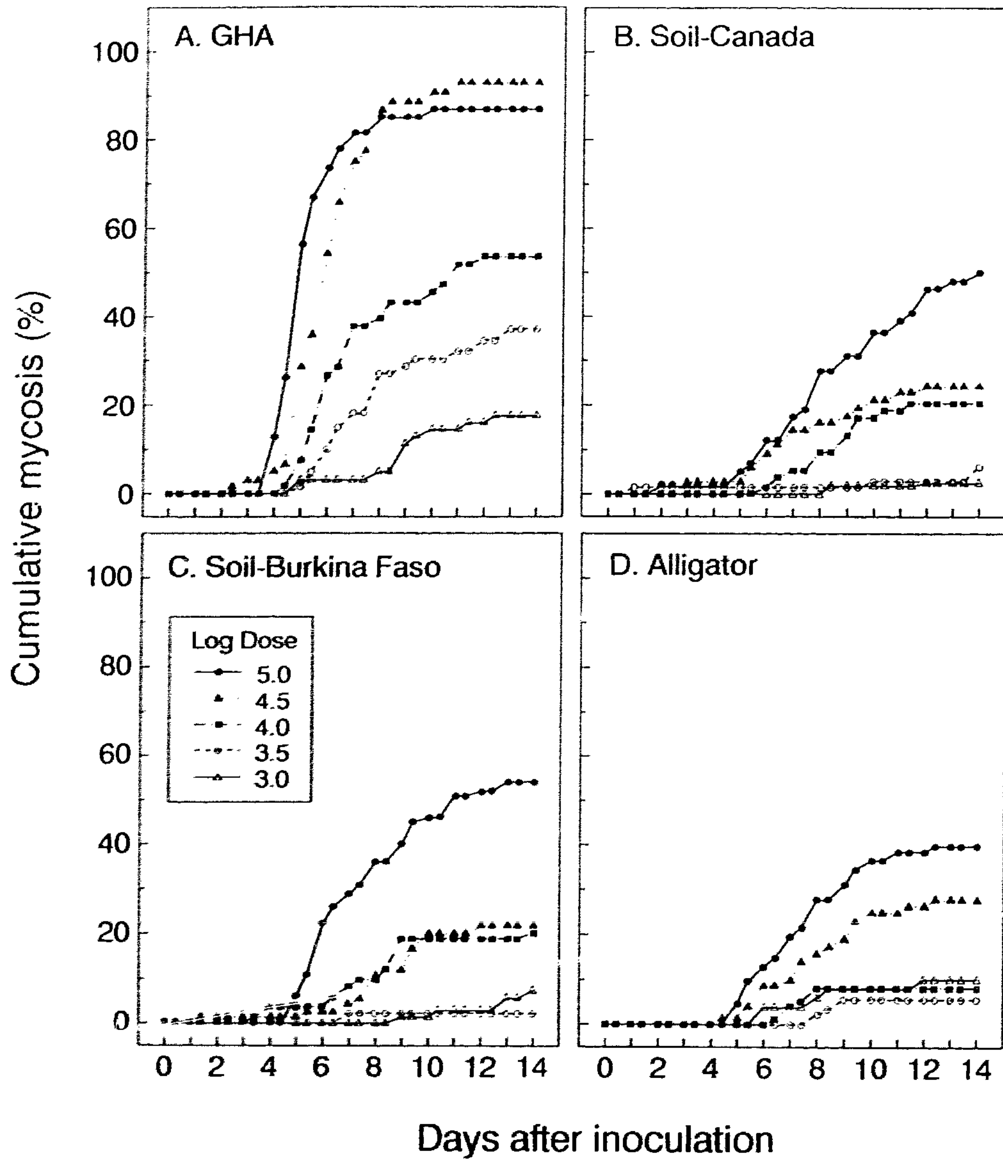

Figure 4.2.1 A-D. Disease progress curves for grasshopper (*Melanoplus sanguinipes*) nymphs ingesting lettuce disks inoculated with *Beauveria bassiana* conidia in oil containing either 3.0, 3.5, 4.0, 4.5 or 5.0 $\log_{10}$ conidia per nymph: A) Mycotech Corp. "GHA" isolate; B) soil isolate from Canada; C) soil isolate from Burkina Faso, Africa; and D) an alligator isolate.

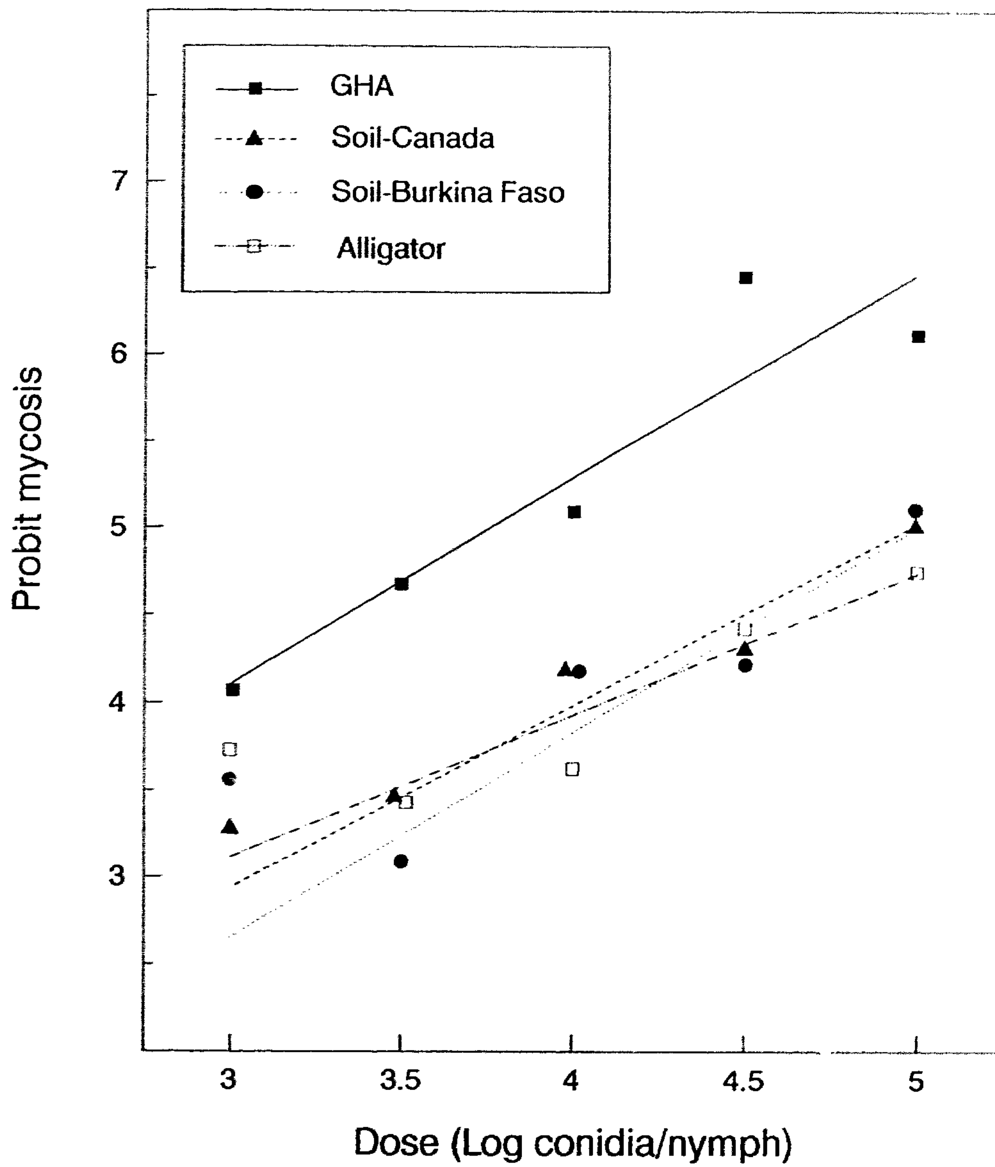

Figure 4.2.2 **Probit regressions of final mycosis (14 days) by isolate and dose.**

than the alligator and soil isolates. Since there were no differences ($F=2.0$; $df=3,57$; $P=0.12$) among replications, the mycosis data were combined for probit regression analysis. The median lethal dose observed for the GHA isolate was 5.8×10^3 conidia per nymph compared to $\geq 9.5 \times 10^4$ conidia per nymph for the soil isolates (Table 4.2.1); only 39.8 (SE=4.4) % mycosis was observed in nymphs treated with the alligator isolate of *B. bassiana*. Despite the lower LD_{50} of the GHA isolate, slopes of probit-transformed mycosis were similar ($\chi^2=2.5$, $df=3$, $P=0.48$) among isolates (Table 4.2.1), suggesting a similar mechanism of pathogenesis.

At the higher conidial doses ($\log_{10}$ 5.0 and 4.5), the rate of disease development also differed ($F=5.7$-18.1; $df=3,12$; $P\leq0.012$) among isolates (Figure 4.2.1). At these doses, disease progress was more rapid ($P<0.01$) for the GHA isolate; the LT_{50} for the GHA isolate was 5.0 (95% CL = 4.5 to 5.7) days at the $\log_{10}$ 5.0 dose compared to 12.0 (95% CL = 10.1 to 14.3) and 11.9 (95% CL = 10.0 to 14.2) for the Canadian and African soil isolates, respectively. The superiority of the GHA isolate is not surprising given its previous selection for activity against grasshoppers.

Infection of insects by *B. bassiana* most frequently occurs through the external integument (Ferron, 1978). Conidia formulated in oil on lettuce disks were more efficacious against grasshopper nymphs than those formulated in water (Inglis *et al.*, 1996a). Although infection by the alimentary canal could not be discounted, evidence suggested that the increased efficacy of the oil formulation was primarily due to enhanced surface-infestation of grasshoppers with conidia during contact with lettuce substrate (Inglis *et al.*, 1996a). The oil-bait bioassay method described herein expedited the inoculation of grasshopper nymphs with *B. bassiana* conidia, minimized the toxic effects of oil, resulted in a dose response of mycosis, and separated isolates on the basis of virulence. The oil-bait method will facilitate the identification of highly virulent genotypes and the elucidation of environmental factors influencing the activity of *B. bassiana* against grasshoppers.

Table 4.2.1 Probit analysis of four isolates of *Beauveria bassiana* against *Melanoplus sanguinipes* nymphs 14 days after inoculation[a].

Isolate	LD_{50} (95% Fiducial)[b]	a[c]	b (SE)[d]	X^2 heterogeneity
GHA	$5.8x10^3$ ($3.9x10^3$-$8.3x10^3$)	0.74a[e]	1.13 (0.14)	1.77
Soil-Canada (LRC 14)	$9.5x10^4$ ($5.8x10^4$-$2.3x10^5$)	-0.32b	1.07 (0.19)	1.47
Soil-Africa (LRC 30)	$1.0x10^5$ ($6.5x10^4$-$2.2x10^5$)	-0.78b	1.16 (0.20)	2.55
Alligator (LRC 82)	---[f]	0.82a	0.77 (0.17)	2.58

[a]Results from four replicates were combined.

[b]Median lethal dose (LD_{50}) with 95% fiducial limits in parentheses. Antilogs were calculated from the log_{10} doses obtained from probit analysis.

[c]Intercepts of probit regressions.

[d]Slopes of the probit regressions with standard errors (SE) of the slopes in parentheses.

[e]Intercepts not followed by the same letter are significantly different ($P \leq 0.05$).

[f]Maximum mycosis less than 50%.

4.3 FIELD EVALUATION OF *BEAUVERIA BASSIANA*[5]

Materials and Methods

Laboratory Assays

Viability assessments. Conidia were produced using solid-substrate technology (Bradley *et al.*, 1992). Dry conidia produced by Mycotech Corporation Inc. in 1992 (GHA 92; batch 921114), in 1994 (GHA 94; batch 940302), and a GHA standard (GHA STD) batch, known to be highly virulent against grasshoppers (Inglis *et al.*, 1996a), were suspended in sterile deionized water. Conidial viabilities were determined on PDA containing Benlate. After 6, 12, 18 and 24 h at 25 ± 1°C, germination rates were determined by the examination of a minimum of 500 conidia from each of three replicate cultures. The experiment was conducted three times ($n = 3$ cultures per time) and data from the three trials were combined after testing for homogeneity of variance using Bartlett's test. Germination percentages were compared among GHA strains over time as a split-plot in time with a Box correction (nine levels of replicate, three levels of conidial batch, and four levels of time). Comparisons among means at each sample time were made using lsmeans.

Virulence assessments. Non-diapause nymphs were inoculated with conidia and maintained in the CEC as described previously (section 4.2). Mean hourly ambient and within cage temperatures and relative humidities were recorded with a CR21X micrologger. The experiment was repeated in time with the exception of the log 5.5 dose. The total number of nymphs used was 859, consisting of 42 to 49 nymphs per treatment-dose combination (25 nymphs per cage).

[5]A version of this section has been published as: "Field and laboratory evaluation of two conidial batches of *Beauveria bassiana* against grasshoppers" Inglis, G.D., M.S. Goettel and D.L. Johnson. 1996. The Canadian Entomologist (in press).

The prevalence of disease at 8 and 14 days was analyzed as a RCBD with two levels of block, three levels of conidial batch, and five levels of dose; lsmeans was used to compare batches averaged over dose. In the absence of a block effect ($P \geq 0.05$), data was combined and probit-transformed regressions of mycosis at 8 and 14 days by log-dose were fitted by maximum likelihood using the S108 program.

Field Trials

Field application. The field experiment was conducted 7 km north-west of Barnwell, Alberta. The field site was native short-grass prairie rangeland. Dominant species were spear grass (*Stipa comata* Trin. & Rupr.), blue grama (*Bouteloua gracilis* (H.B.K.) Lag. *ex* Steud.), June grass (*Koeleria gracilis* Pers.) with subdominant low-growing broad-leaf plants and other grasses.

Treatments were GHA 92 conidia, GHA 94 conidia, a carrier control, and an untreated control, arranged in an RCBD with two blocks per treatment; each block consisted of four, 2-ha plots (141 x 141 m) separated by a minimum distance of 100 m. To eliminate contamination of the sprayer, the carrier control treatment was applied before the conidial treatments. Five, 4-m^2 square subplots were placed within each plot. Four of the subplots were inside the plot boundary, 30 m diagonally from the corners; whereas, the fifth subplot was at the center of the plot.

Conidia (2.5×10^{11} conidia/mL) were suspended in a 1.5% (v/v) emulsifiable oil-emulsion amended with 4% clay (w/v) for a target spore concentration of 2.5×10^{13} conidia/ha. Treatments were applied at a rate of 112 L/ha (207 kPa) with a tractor-mounted Hardi sprayer travelling at 12 kph. The boom was 9.5 m in length, equipped with 20 Tee Jet 110-2R flat-fan nozzles (50-cm spacing), adjusted to a height of 45 cm. Conidia were applied on the morning of July 12, 1994.

Application rates. Water-sensitive papers (Teejet Spraying Systems Co., Wheaton, IL) were placed on the soil surface in each of four peripheral subplots with the exception of

the untreated plots. Cards were collected 5 to 15 min after application. Droplet density, area, and size were assessed on the cards using a Tracor Northern 8502 Image Analyzer.

To quantify conidial deposition, five coverslips (13-mm diam) were positioned in a petri dish, and one dish was placed in each of the four peripheral subplots. The dishes were collected within 15 min of application, returned to the laboratory on ice and maintained at 5°C for 12 h. Conidia were isolated from the five coverslips per dish on oatmeal-dodine agar using the wash method. Subplot and treatment means were calculated from untransformed data.

Weather conditions. Mean hourly total solar radiation (300 to 2800 nm), temperature, and relative humidity were recorded at weather stations located at Lethbridge and Vauxhall; UVB radiation was also measured at the Lethbridge site. Temperatures and relative humidities recorded at the two weather stations were similar, and because the field site was located between them (36 km W and 30 km NE from Lethbridge and Vauxhall, respectively), it was concluded that the data recorded at Lethbridge were representative of the field site. Daily precipitation was recorded 15 km SE from the field site by the Taber Sugar Company.

Conidial persistence on leaves. Twenty grass leaves were arbitrarily collected immediately after, and 2, 5, 10, and 15 days post-application from each of the subplots, and returned to the laboratory in plastic bags on ice. Leaves were aseptically cut into pieces approximately 1-cm long, combined for each subplot and cfu isolated using the wash method. The total area of the leaves processed ranged from 3.6 to 12.9 cm^2. Subplot means were calculated from $\log_{10}$-transformed data, and treatment means were fit to linear models after $\log_{10}$-transformation of the time data. Untransformed means are presented in the text.

Conidial populations on nymphs. Samples of approximately 150 grasshoppers per plot were swept immediately after, and 2, 5, 10, and 15 days post-application. Nymphs

were transferred to cylindrical cages (45.5 cm high x 14.5 cm diam) and returned to the laboratory. With the exception of the first sample time, cages were separated from each other by a plastic barrier to prevent cross-contamination. Twenty, third- and fourth-instar nymphs were placed individually in sterile glass vials within 4 to 5 h of field collection, weighed, and maintained at 5°C for a maximum of 48 h. Individual nymphs were homogenized in 1 mL of buffer-Tween, the homogenate diluted and *B. bassiana* cfu enumerated on oatmeal-dodine agar. The mean weight of nymphs was 47.7 mg, and cfu populations per nymph were calculated per mg x 47.7 mg to account for variation in nymphal size.

Mycosis in field-collected grasshoppers. From the transport cages, 100 arbitrarily selected grasshoppers per plot were transferred to 51 x 23 x 23 cm wire-mesh cages randomly placed in a greenhouse. Grasshoppers were maintained on a diet of wheat seedlings, bran and a variety of grasses and broad-leaf plants collected from the field site (not exposed to *B. bassiana*). Cadavers were removed daily, and placed on moistened filter paper. Temperature and relative humidity in the greenhouse were measured with a hygrothermograph. Shades were drawn in the greenhouse to provide conditions of diffuse light. Daily mean temperatures and relative humidities recorded in the greenhouse were 3.7 °C (standard deviation=0.52) and 18.9 % (standard deviation=1.8) higher on average than the field environment. At each sample time, disease progress between conidial treatments was compared as a split plot with two levels of replicate and conidial batch, and ten levels of time. In addition, the prevalence of maximum mycosis at 10 days was compared over sample time as a split-plot with two levels of replicate and conidial batch, and five levels of sample time.

Grasshopper populations, species and age composition[6]. Grasshopper species and age composition (Brooks, 1958; Otte, 1981, 1984; Pfadt, 1988; Vickery and Kevan, 1983) were assessed from 200 random sweeps (180°, 38 cm-diam net) in two transects per plot on each sampling date. Grasshopper population densities were monitored by counting living grasshoppers in permanently situated, 0.25-m^2 sampling frames, on the day of application, and 1, 9 and 15 days after the application of *B. bassiana* conidia. Two types of frames were used (open squares and circles). The sampling frames were placed on the soil surface in the central sampling zones of the plots prior to the beginning of the experiment in three marked transects (20 rings, 18 squares and 20 rings, respectively) per plot. Rings and squares gave similar counts ($r=0.758$, $P<0.001$); the average ratio of mean counts in rings to mean counts in squares was 1.0 (SE=0.057). Logarithmically-transformed densities of grasshoppers in both types of frames were analyzed as a split-plot in time with four levels of treatment, two levels of block and four levels of time. In addition, reductions in treated plots were adjusted against reductions in the untreated plots relative to population densities at day 0 (Abbott, 1925; Schaalje *et al.*, 1986). To determine whether or not infection by *B. bassiana* affected sweepnet sampling efficiency, total sweep catches were adjusted against grasshopper density (sampling frames) by plot, date, and treatment; sweepnet collection has been used to determine grasshopper condition and activity (Johnson *et al.*, 1986).

Results

Laboratory Assays

Conidial viability. Germination of GHA 92, GHA 94 and GHA STD conidia exceeded 92% after 24 h (Figure 4.3.1). However, the rates of germination differed ($F=35.6$; $df=2,21$; $P<0.001$) among conidial treatments. There was no difference ($P>0.05$) in the prevalence of germination at 6 and 24 h, but a lower proportion ($P\leq0.05$) of GHA 94

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[6]Field population assessments, collection and identification of grasshoppers was performed by Craig Andrews. Abbott's adjusted analyses were conducted by Dr. D.L. Johnson.

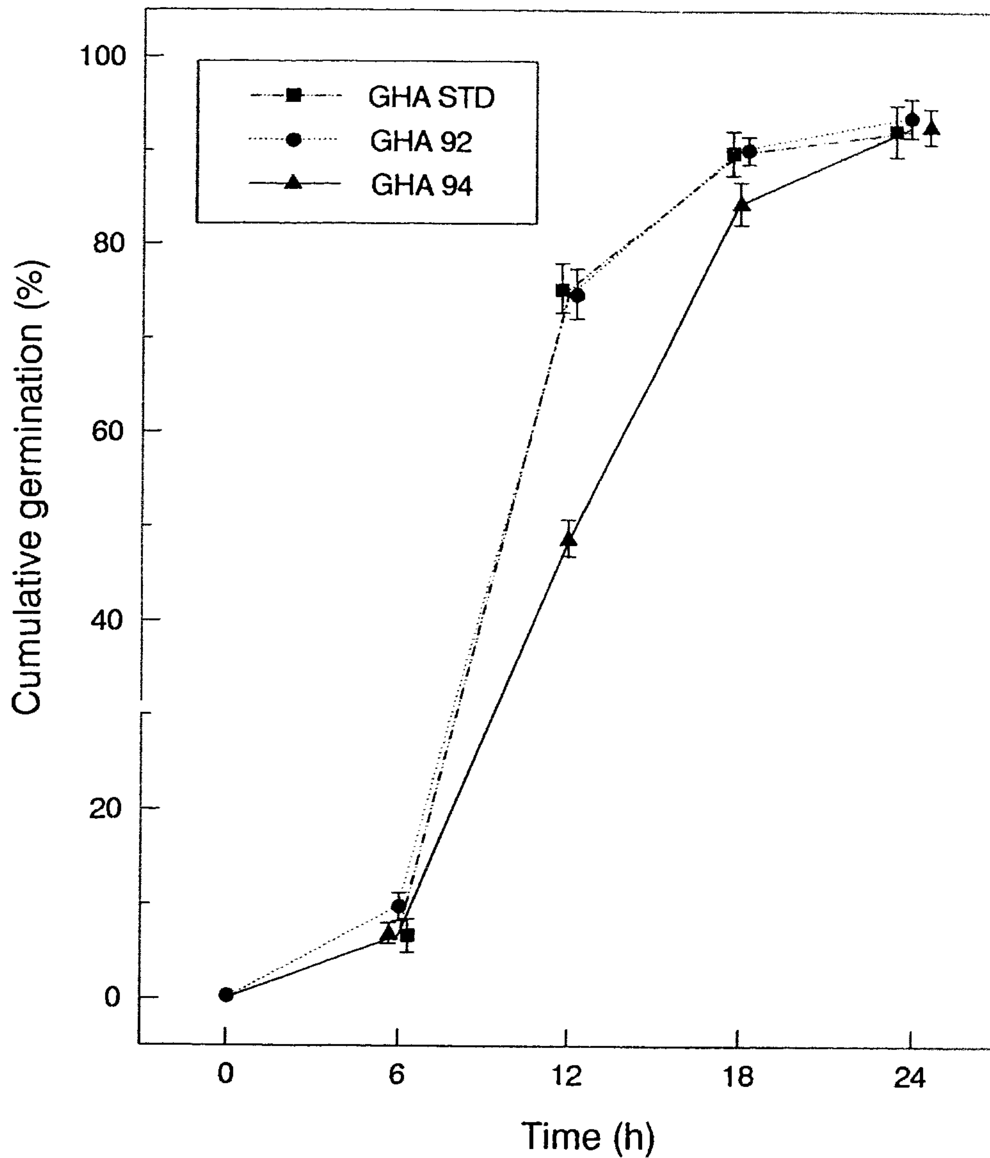

Figure 4.3.1 Germination of *Beauveria bassiana* conidia on potato dextrose agar at 25°C. Treatments consisted of *B. bassiana* (Mycotech strain GHA): conidia produced in 1992 (GHA 92); conidia produced in 1994 (GHA 94); and conidia of a standard batch (GHA STD) stored at -10°C.

conidia had germinated after 12 and 18 h.

Virulence assessments. The prevalence of mycosis was dissimilar ($F=6.4$ and 7.1; $df=2,14$; $P\leq0.011$) among conidial treatments after 8 and 14 days, respectively. Averaged over dose, mycosis was less ($P\leq0.05$) for GHA 94 than for GHA 92 and GHA STD. Correspondingly, the LD_{50} for GHA 94 was slightly larger than the other treatments (Table 4.3.1). Although there were no differences ($\chi^2=2.7$; $df=2$; $P=0.26$) in slopes of mortality 14 days after inoculation, slopes were dissimilar ($\chi^2=8.2$; $df=2$; $P=0.017$) among treatments 8 days after inoculation (Figure 4.3.2).

Field Trials

Application rates. There were no differences ($F=0.2$-4.7; $df=2,2$; $P>0.05$) among treatments in the deposition of spray droplets on water-sensitive papers (Table 4.3.2). The deposition of conidia on coverslips, leaves and nymphs were also similar between the two conidial treatments. From coverslips, 4.1×10^4 ($SE=0.33 \times 10^4$) and 3.4×10^4 ($SE=0.29 \times 10^4$) cfu/cm^2 were isolated for GHA 92 and GHA 94, respectively. From grass leaves, 2.9×10^4 ($SE=0.28 \times 10^4$) and 2.4×10^4 ($SE=0.19 \times 10^4$) cfu/cm^2 were isolated , respectively. All of the nymphs ($n=80$) processed after conidial application were infested with *B. bassiana*. Conidial populations on nymphs averaged 4.3×10^3 ($SE=0.39 \times 10^3$) and 3.5×10^3 ($SE=0.65 \times 10^3$) cfu/nymph for GHA 92 and GHA 94, respectively. Low levels of *B. bassiana* were isolated from carrier control nymphs (78.7 cfu/nymph), whereas *B. bassiana* was not isolated from coverslips or leaves from the carrier control treatment.

Weather conditions. In general, conditions were hot and sunny during the course of the experiment (Figure 4.3.3). Four periods of precipitation occurred, with approximately 16 mm of rain falling within 24 h of the conidial application.

Conidial persistence. Conidial survival on leaves declined logarithmically over time (Figure 4.3.4A), and slopes of conidial persistence were similar between GHA 92 and GHA 94 conidia (Table 4.3.3). No conidia were isolated from carrier control leaves.

Table 4.3.1 Probit analysis of mycosis in *Melanoplus sanguinipes* nymphs inoculated with conidia of *Beauveria bassiana* and maintained in a controlled environment[a].

Treatment[b]	Day 8			Day 14		
	LD_{50}	a	b (SE)	LD_{50}	a	b (SE)
GHA STD	3.65 (3.49-3.80)[c]	0.12	1.34 (0.16)	3.29 (3.00-3.49)[c]	1.54	1.05 (0.15)
GHA 92	3.30 (2.90-3.56)	2.42*	0.78 (0.14)*	2.85 (2.22-3.18)	2.73	0.79 (0.15)
GHA 94	4.02 (3.86-4.17)	0.13	1.24 (0.15)	3.79 (3.61-3.96)	0.72*	1.13 (0.15)

[a]Results from two trials were combined. The regression equation is probit mycosis = a + b (log_{10} dose).

[b]Conidial treatments consisted of *B. bassiana* (Mycotech strain GHA): conidia produced in 1992 (GHA 92); conidia produced in 1994 (GHA 94); and conidia of a standard batch of conidia (GHA STD) stored at-10°C.

[c]Numbers in parentheses following LD_{50} value means represent 95% fiducial limits.

*Slopes or intercepts significantly different ($P \leq 0.05$) from those of other treatments.

Table 4.3.2 Deposition of spray droplets on water sensitive cards placed on the soil surface within field plots sprayed with conidia of *Beauveria bassiana* or the carrier alone[a]

Treatment[b]	Droplet Area (mm^2)	Droplet Width (mm)	Coverage (%)	Number (per cm^2)
GHA 92	0.17 (0.028)	0.35 (0.013)	12.3 (3.6)	71.1 (7.1)
GHA 94	0.19 (0.011)	0.36 (0.031)	12.9 (3.4)	69.1 (21.1)
Carrier	0.10 (0.017)	0.27 (0.032)	9.9 (0.054)	112.1 (24.5)

[a]Droplets were analyzed in each of three randomly-selected areas (80 mm^2) per card; the droplet data from the three areas was combined and mean droplet area, width and density were determined. In addition, the ratio of total droplet area to total card area (% coverage) was calculated.

[b]Treatments consisted of *Beauveria bassiana* conidia produced in 1992 (GHA 92); conidia produced in 1994 (GHA 94); and a carrier control.

Table 4.3.3 Linear regression data of field persistence of *Beauveria bassiana* conidia on leaves and grasshopper nymphs[a]

Substrate/Treatment[b]	a	SE(a)	b	SE(b)	r^2
Grass Leaves					
GHA 92	4.21[c]	0.22	-2.77[c]	0.26	0.97
GHA 94	3.98	0.37	-2.76	0.45	0.92
Total Nymphs					
GHA 92	3.72	0.20	-2.75	0.25	0.98
GHA 94	3.51	0.18	-2.82	0.22	0.98
Infested Nymphs					
GHA 92	3.59	0.11	-2.18	0.14	0.99
GHA 94	3.36	0.18	-2.03	0.22	0.97

[a]The x-axis was $\log_{10}$-transformed and the regression equation is: $\log_{10}$ cfu/cm^2 or $\log_{10}$ cfu/nymph = a + b ($\log_{10}$ (days + 1)).

[b]Substrates consisted of: grass leaves; total nymphs; and nymphs from which $\geq$ 1 conidium of *B. bassiana* was recovered (infested). Treatments consisted of *B. bassiana* (Mycotech strain GHA): conidia produced in 1992 (GHA 92); and conidia produced in 1994 (GHA 94).

[c]All intercepts and slopes are significantly different from zero ($P \leq 0.01$).

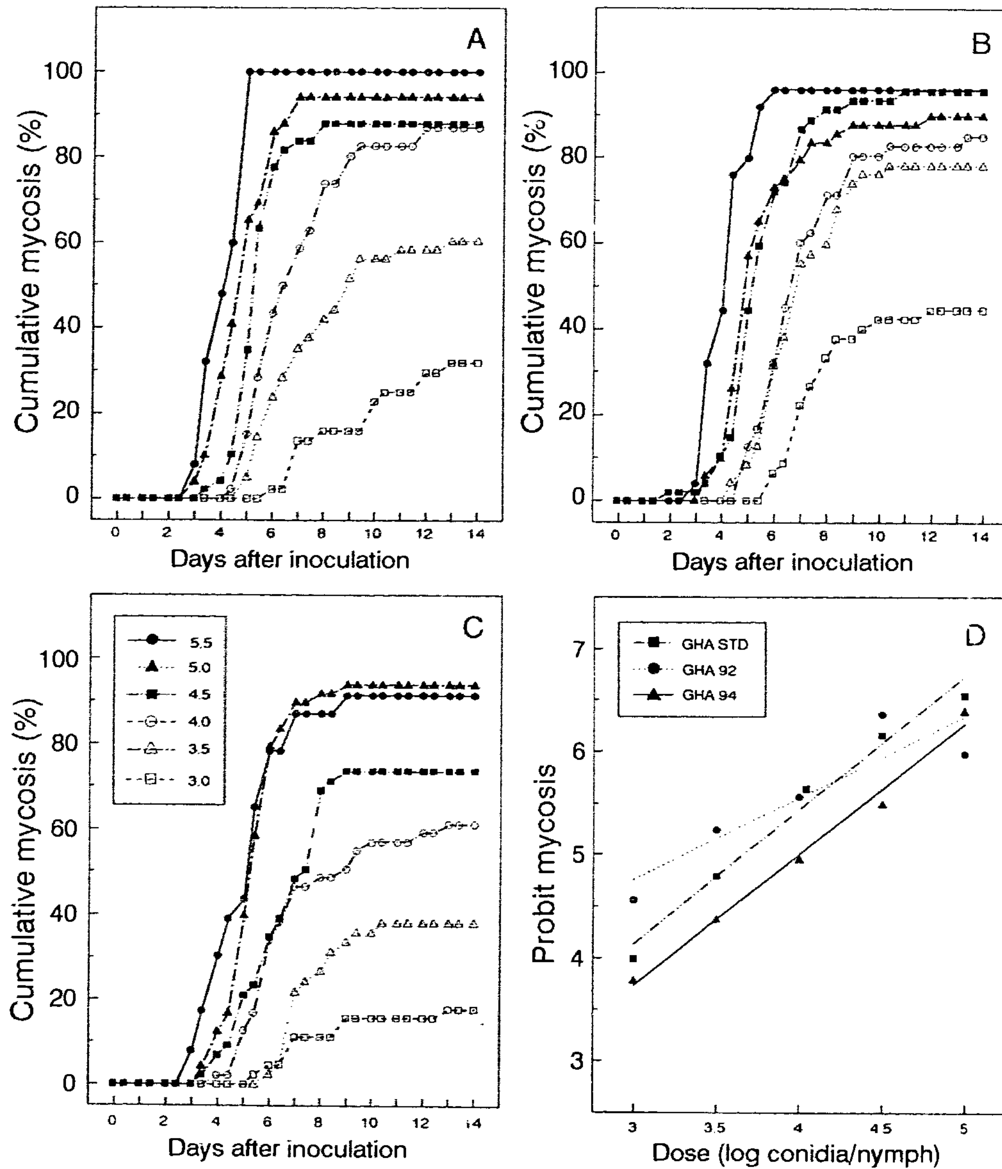

Figure 4.3.2 Disease progress curves for grasshopper (*Melanoplus sanguinipes*) nymphs ingesting lettuce disks inoculated with *Beauveria bassiana* conidia in oil containing either 0, 3.0, 3.5, 4.0, 4.5, 5.0 or 5.5 $\log_{10}$ conidia per lettuce disk: A) GHA standard (GHA STD); B) GHA 92; and C) GHA 94. D) Probit regressions of mycosis at 8 days for each treatment by dose.

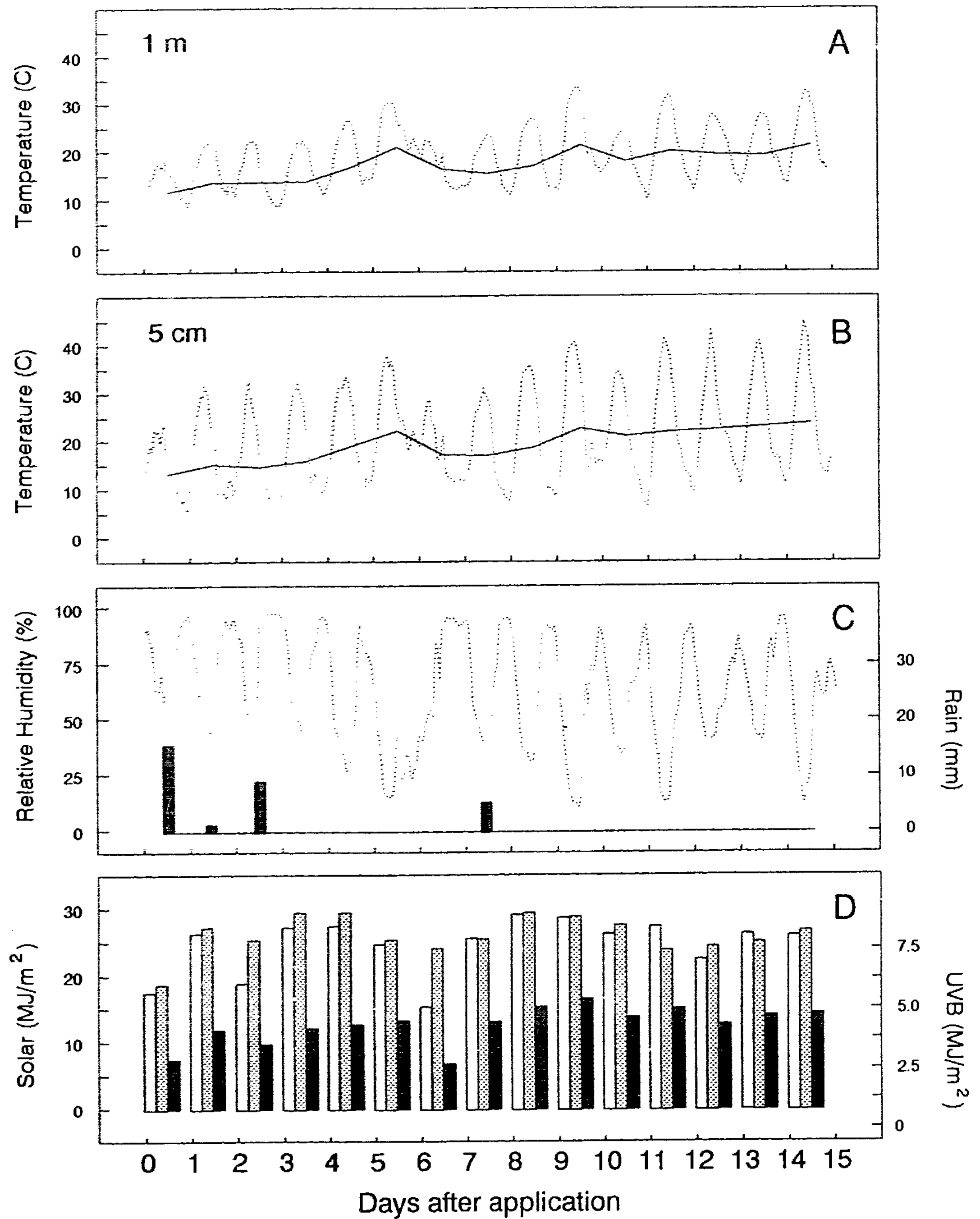

Figure 4.3.3 Weather during the field experiment (July 12 to 27, 1994). Hourly (dotted lines) and mean (solid lines) temperature at a height of 1 m and 5 cm, and relative humidity were recorded at Lethbridge. Daily precipitation (histograms) was recorded at Taber. Total daily solar radiation (300-2800 nm) was recorded at Lethbridge (open bars) and Vauxhall (stippled bars). Daily UV-B radiation (solid bars) at Lethbridge.

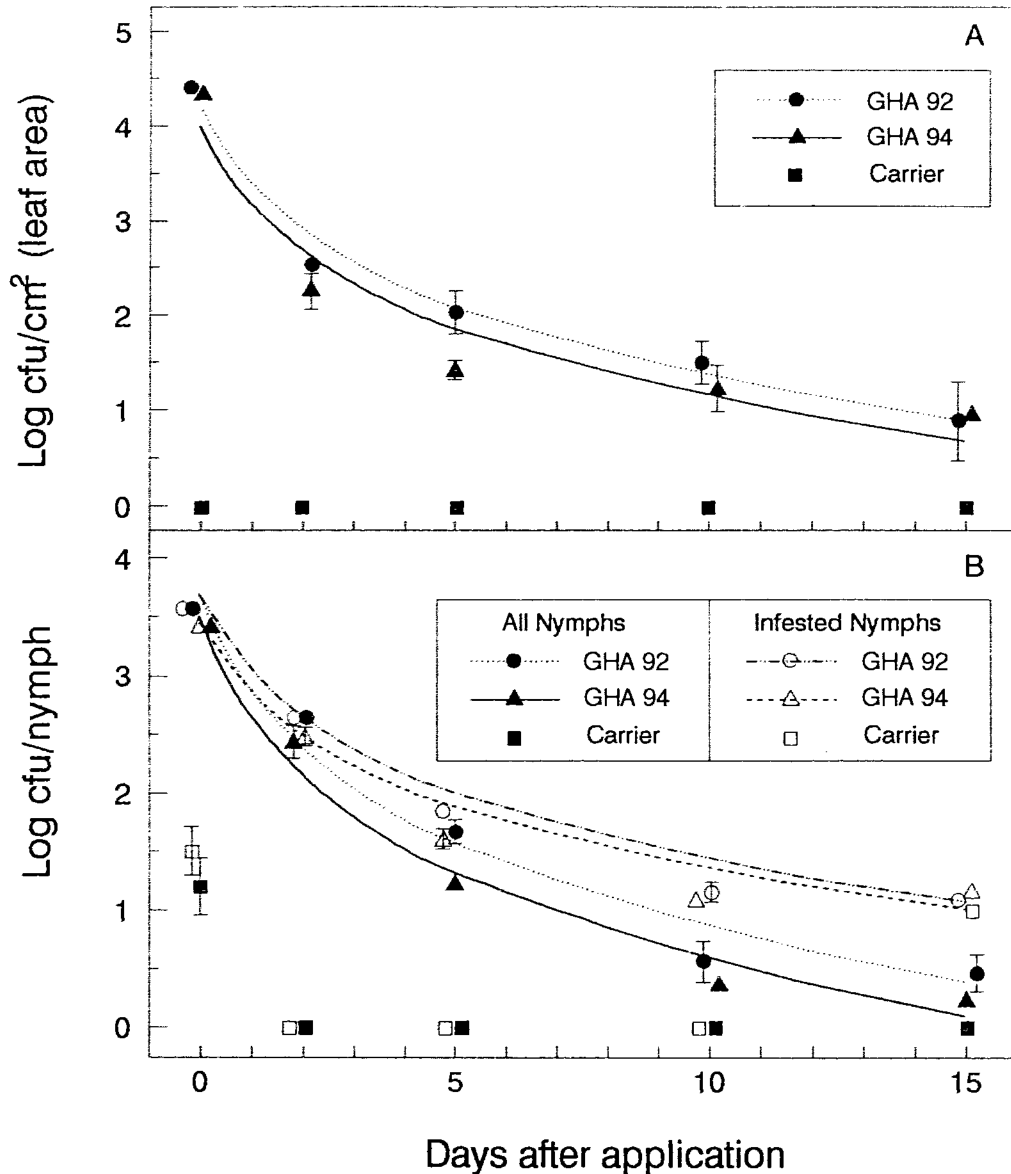

Figure 4.3.4 Persistence of *Beauveria bassiana* conidia. A) From grass leaves. Populations were quantified as $\log_{10}$ colony-forming units (cfu)/cm^2 of leaf area. B) From grasshopper nymphs. Populations were quantified as log cfu per nymph. To account for variation in nymph size, weights were standardized to 48 mg. Conidial populations were determined for total nymphs and for nymphs from which one or more cfu were recovered (classified as infested). Treatments consisted of GHA 92, GHA 94 and a carrier control. Vertical lines represent standard errors of means (n=2). To avoid superimposition of standard error bars, means are slightly offset along the x-axis.

Conidial survival on/in nymphs was similar to that observed on leaves (Figure 4.3.4B, Table 4.3.3). The prevalence of nymphal infestation also decreased with sample time to less than 30% by 15 days (Figure 4.3.5A). When non-infested nymphs were removed, slopes of conidial survival decreased from greater than 2.8 to less than 2.2 (Table 4.3.3). Of the carrier control nymphs collected immediately after application, 80 % (SE=0.05) were infested with *B. bassiana* at low levels ($\bar{X}$=49.0, SE=29.7 cfu/nymph). With the exception of day15 (1 of 40 nymphs), no viable conidia were isolated from nymphs at subsequent sampling times.

Mycosis in field-collected grasshoppers. Over 2800 grasshoppers were collected and confined in greenhouse cages for 10 days. At individual collection dates, the timing of mycosis (F=0.18-2.0; df=10,20; P≥0.090) was similar between GHA 94 and GHA 92 (Figure 4.3.6). For both conidial treatments, the prevalence of mycosis decreased (F=14.8; df=4,8; P<0.001) with sample time; beginning with the 5 day sample, less mycosis (P≤0.029) was observed (Figure 4.3.5B). However, the onset of mycosis was always 3 to 4 days after placement of the nymphs in cages regardless of the collection time. There was no difference (F=0.33; df=2,12; F=0.72) among treatments in the prevalence of non-*Beauveria* mortality.

Grasshopper populations, species and age composition. At the time of application, population densities were approximately 20 grasshoppers/m^2. None of treatments affected (F=0.64; df=9,12; P=0.74) field populations of grasshoppers (Figure 4.3.7). However, populations were consistently higher (P<0.05) in the unsprayed plots. The predominant grasshoppers (comprising more than 10% of the 4,333 individuals collected) were: *Ageneotettix deorum* (Scudder) (2-18%); *Melanoplus gladstoni* Scudder (10-28%); *M. infantilis* Scudder (14-36%); *M. packardii* Scudder (0-16%); *M. sanguinipes* (1-17%); and *Phoetaliotes nebrascensis* (Thomas) (24-47%). No remarkable shifts were observed in

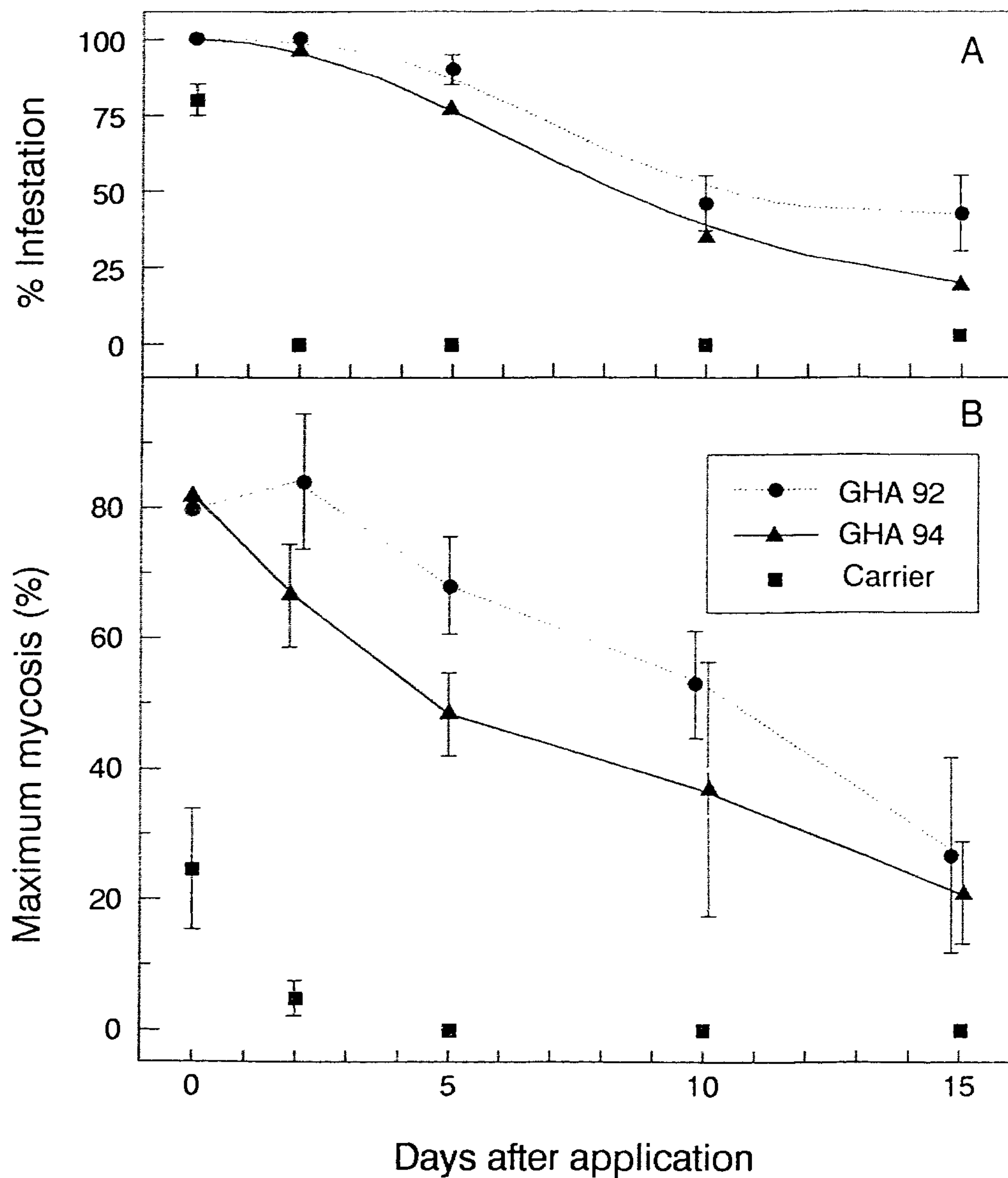

Figure 4.3.5 A) Infestation of grasshoppers nymphs with *Beauveria bassiana* following field application. The % infestation was calculated from 40 nymphs collected per sample time. B) Maximum mycosis in grasshoppers maintained in cages for 10 days per sample time. Treatments consisted of GHA 92, GHA 94 and a carrier control. Vertical lines represent standard errors of means (n = 2). To avoid superimposition of standard error bars, means are slightly offset along the x-axis.

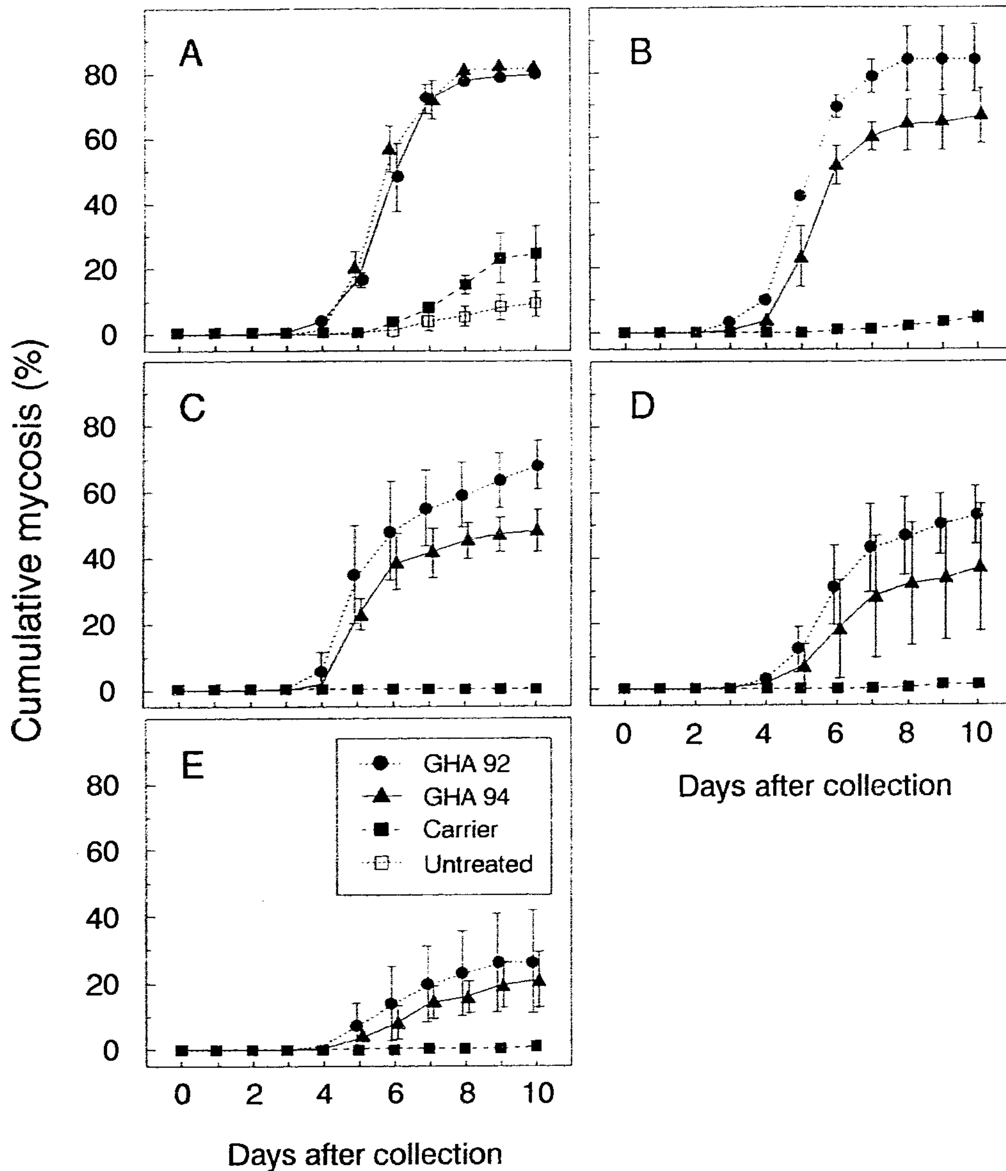

Figure 4.3.6 Cumulative mycosis of caged grasshoppers collected from field plots at various times after the application of *Beauveria bassiana* conidia. Grasshoppers for the GHA 92, GHA 94, a carrier control and untreated control treatments were maintained in a greenhouse for 10 days post-application, and hourly (dotted lines) and mean (solid lines) are temperature and relative humidity (RH) during this period. Grasshoppers were collected: A) immediately after the application of conidia; B) two days after application; C) five days after application; D) ten days after application; and E) fifteen days after application. Vertical lines represent standard errors of means (n = 2). To avoid superimposition of standard error bars, means are slightly offset along the x-axis.

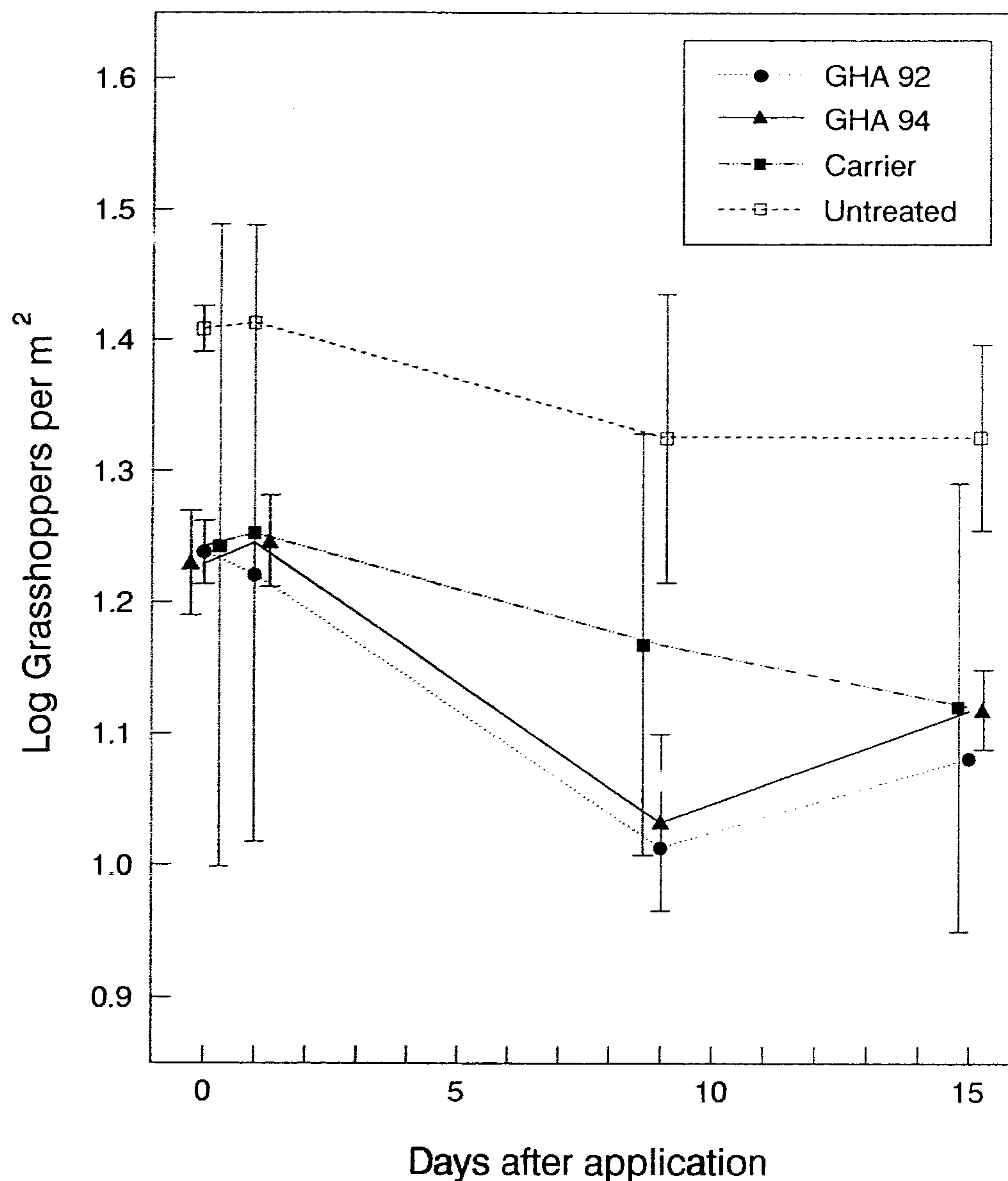

Figure 4.3.7 Grasshopper densities in sampling frames ($\log_{10}$ grasshoppers per m^2) for the GHA 92, GHA 94, carrier control, and untreated control treatments taken on the day of application of *Beauveria bassiana* conidia, and one, five and nine days after conidial application. Vertical lines represent standard errors of means (n=2). To avoid superimposition of standard error bars, means are slightly offset along the x-axis.

species composition in the treated or control plots over the four sampling dates (Table 4.3.4). Less than 9% of the grasshoppers at the field site were adults at the time of application and most of these were small, early-season, species (ie. *M. infantilis*). By 15 days, 25.8% of the grasshoppers collected were adults. Cooler than average weather in the spring and summer of 1994 accounted for the low proportion of adults at the time of conidial application.

The 200 sweeps per plot yielded an average of 135 grasshoppers. At each of the sample times, there were no differences ($F=0.06$-4.4; $df=3,3$; $P \geq 0.13$) among treatments in the catch per unit density (sampling frames). The ratio of grasshoppers caught in sweepnets to population densities ranged from 13.4 (SE=4.9) to 25.8 (SE=1.7) at the time of application. At the 1, 9 and 15 sample times, ratios ranged from 21.3 (SE=3.4) to 35.6 (SE=0.87), 33.4 (SE=0.26) to 51.6 (SE=7.3), and 32.0 (SE=0.10) to 46.1 (SE=1.8).

Discussion

The poor field efficacy of entomopathogens has frequently been explained, in many instances without corroborating evidence, as the result of either poor viability of propagules, reduced virulence, and/or on inadequate targeting of the host. The activity of two production batches of *B. bassiana* conidia (GHA 92 and GHA 94) were examined in laboratory and field environments, to determine if previous differences in field efficacy could be explained by changes in the pathogen or by other aspects of the pathogen-grasshopper-environment interaction.

The ability of *B. bassiana* conidia to germinate rapidly and synchronously is considered an important step in the infection process. Although GHA 94 conidia germinated slightly slower than did conidia of GHA 92, both were equally viable (> 92%) after 24 h. Both conidial batches were also highly virulent against *M. sanguinipes* nymphs in a controlled environment, with observed LD_{50} values of < 2.9 and 3.8 for GHA 92 and

Table 4.3.4 Proportion of prevalent grasshopper species in experimental plots[a]

Treatment[b] / Grasshopper Taxa	Days after application			
	0	1	9	15
GHA 92				
Ageneotettix deorum	18.0	7.3	13.3	9.0
Melanoplus gladstoni	21.6	16.7	9.8	14.7
Melanoplus infantilis	30.6	24.0	36.4	28.8
Melanoplus packardii	1.8	0.5	0.6	0.0
Melanoplus sanguinipes	0.9	4.2	1.2	1.7
Phoetaliotes nebrascensis	27.0	47.4	38.7	45.8
n	111	192	173	177
GHA 94				
Ageneotettix deorum	9.2	6.1	16.7	4.7
Melanoplus gladstoni	26.2	27.5	20.5	22.5
Melanoplus infantilis	15.6	17.8	24.2	27.1
Melanoplus packardii	15.6	6.1	2.6	2.3
Melanoplus sanguinipes	9.2	17.0	1.9	4.7
Phoetaliotes nebrascensis	24.1	25.5	34.2	38.8
n	141	247	269	214
Carrier				
Ageneotettix deorum	5.3	2.4	4.3	6.2
Melanoplus gladstoni	23.0	25.7	14.2	16.4
Melanoplus infantilis	18.1	17.8	23.5	14.2
Melanoplus packardii	5.6	4.4	1.1	1.5
Melanoplus sanguinipes	12.2	13.0	13.0	14.6
Phoetaliotes nebrascensis	35.9	36.8	43.9	47.3
n	304	253	353	275
Untreated				
Ageneotettix deorum	5.1	5.9	4.2	6.0
Melanoplus gladstoni	24.4	12.1	18.7	24.8
Melanoplus infantilis	19.0	19.3	13.7	14.5
Melanoplus packardii	4.4	3.1	6.5	3.1
Melanoplus sanguinipes	10.9	14.3	14.0	10.8
Phoetaliotes nebrascensis	36.3	45.3	43.0	40.8
n	295	322	337	351

[a]Samples of grasshoppers were collected on the day of application (0), and 1, 9 and 15 days after the application of *Beauveria bassiana* conidia. Prevalent species ≥ 1% frequency.

[b]Treatments consisted of: *B. bassiana* (Mycotech strain GHA) conidia produced in 1992 (GHA 92); conidia produced in 1994 (GHA 94); a carrier control; and an untreated control.

GHA 94, respectively. Some evidence suggests that the rate of conidial germination may be important (Ouedraogo, 1993) and this may explain why GHA 92 was slightly more virulent than GHA 94 against *M. sanguinipes* nymphs in the laboratory bioassay. In grasshoppers collected from field plots treated with GHA 92 and GHA 94 and subsequently held in cages in the greenhouse, I observed similar levels of mycosis for both treatments. Although non-significant, consistently less mycosis (excluding the day 0 sample) was observed in grasshoppers sprayed with GHA 94 than GHA 92, and this may reflect reduced virulence. Nevertheless, high levels of mycosis were observed in caged grasshoppers collected from plots sprayed with GHA 94, and the previous dissimilarity in the field efficacy of GHA 92 and GHA 94 cannot be explained by either differential virulence or conidial viability alone.

Adequate host targeting is imperative for successful inundative control of insects with entomopathogenic fungi. Spray droplets were relatively uniformly deposited onto cards and relatively large populations ($> 10^4$ conidia/cm^2) of both GHA 92 and GHA 94 conidia were isolated from coverslips and leaves. Furthermore, all of the grasshopper nymphs sampled following application were infested with *B. bassiana* (10^3 to 10^4 conidia per nymph). The homogenization method usually provides a conservative estimate of conidial populations on nymphs due to the strong attachment of conidia to insect integuments (Boucias *et al.*, 1988), to conidial aggregation, or both. However, some transmission of *B. bassiana* conidia may have occurred between nymphs and from infested foliage during their capture in sweepnets resulting in an overestimation of conidial populations. The wind direction at the time of conidial application would have precluded much wind contamination of control plots, yet a high prevalence (80%) of carrier control nymphs were infested with conidia, albeit at low levels (<80 cfu/nymph). These nymphs were inadvertently exposed to thatch from cages containing treated grasshoppers during their transport to the laboratory and they likely became contaminated at this time; after the

first collection time, cages were separated from each other with a plastic barrier and very few conidia (≤ 0.46 cfu/nymph) were subsequently isolated . In preliminary application tests using soluble dyes, excellent topical coverage of grasshoppers in sparse canopy grassland was observed (unpublished data). Therefore, it is highly probable that many of the conidia that were isolated were deposited on nymphs during the spray application. Despite the deposition of relatively large numbers of conidia on grasshoppers, no reductions in field populations were detected suggesting that factors other than targeting and pathogen virulence limited disease development.

For mycosis to occur, *B. bassiana* conidia must be deposited on grasshoppers in a suitable environment. After a cool and overcast application day, weather conditions were hot, dry and sunny during the field experiment. Although there was no evidence of disease in the field, substantial mycosis of grasshoppers treated with GHA 92 and GHA 94 was observed in greenhouse cages, a phenomenon observed by others (Johnson *et al.*, 1992; unpublished data in Mason and Erlandson, 1994). In the greenhouse cages, the prevalence of mycosis was observed to decrease with sample time, but the onset of disease was always 3 to 4 days after the placement of nymphs in cages regardless of when the grasshoppers were collected. Johnson and Goettel (1993) also observed a lag period of mycosis in caged grasshoppers, but since disease in cages corresponded to reductions in field populations of grasshoppers treated with *B. bassiana*, they speculated that the lag period was due to the differential collection of diseased and non-diseased grasshoppers; grasshoppers in an advanced stage of infection can become less active and therefore, less likely to be collected in sweepnets (Johnson, 1989a). However, the ineffectiveness of *B. bassiana* against field populations of grasshoppers in this study, indicates that the cage environment and not the differential collection of grasshoppers influenced disease development.

The influence of environment on mycosis of grasshoppers could be direct, indirect, or both. The survival of conidia exposed to UVB radiation on leaves in field environments is poor (section 3.3) and, conidial populations of GHA 92 and GHA 94 were reduced by more than 99% by 15 days. Conidial persistence on nymphs was similar to that on leaves suggesting that like on leaves, UVB radiation limits conidial survival on nymphs. This assumes that the majority of the conidia on nymphs remain exposed to sunlight. Although the homogenization technique does not discriminate between surface conidia and blastospores present in the hemolymph, comparisons of sweepnet efficacy relative to population densities (sampling frames) provided no evidence for reduced grasshopper activity due to infection. This would suggest that either conidia did not initiate infection and remain exposed to UVB on grasshopper integuments or infection occurred but was not debilitating, or both. Furthermore, in the diffuse light conditions of the greenhouse, it is probable that conidia on nymphs would be exposed to substantially lower levels of UVB radiation, prolonging their survival and thereby contributing to the development of disease.

Relative humidity was higher (0 to 40% on average) in the greenhouse than in field environment. Although there may be some effect of relative humidity on infection of acridids by entomopathogenic fungi (Bateman *et al.*, 1993), infection of grasshoppers by *B. bassiana* readily occurs at low humidities (Marcandier and Khachatourians, 1987). Another possibility is that the increased efficacy of *B. bassiana* in the greenhouse was due to temperature. Night-time temperatures in the greenhouse were slightly higher (approximately 4°C) than in the field but the upper cardinal temperature for *B. bassiana* (approximately 35°C) was surpassed for only short periods of time. However, behavioral thermoregulation by habitat selection and orientation to the sun allows grasshoppers to maintain their metabolic rates at temperatures near that which is optimal for development (approximately 42°C) (Hardman and Mukerji, 1982; Carruthers *et al.*, 1992), and body temperatures 18°C higher than ambient have been recorded for *M. sanguinipes* in natural habitats (Chappell and

Whitman, 1990; Kemp, 1986). The elevation of insect body temperature by thermoregulation has been shown to reduce the effects of disease (Carruthers *et al.*, 1992; Watson *et al.*, 1993), and it is possible that the poor light conditions, the inability of grasshoppers to sufficiently elevate their body temperatures by habitat selection in the greenhouse cages contributed to disease development. Investigations to determine whether the inability of grasshoppers to thermoregulate in the greenhouse has an affect on mycosis of grasshoppers are presented in chapter five.

A threshold of inoculum is required to cause disease and the quantities of viable *B. bassiana* conidia isolated from grasshoppers at later sampling dates would not normally be sufficient to cause mycosis. For example, only 20 to 43% of collected nymphs were infested at low levels (13-15 cfu/nymph) at day 15, yet 21 to 26% mycosis was observed. Confinement of grasshoppers in cages may have predisposed them to infection since a variety of factors, including crowding (Steinhaus, 1958), nutrition (Donegan and Lighthart, 1989), prior sublethal infection (Fargues *et al.*, 1991; Ferron, 1981), and agrochemical-exposure (Ferron, 1971) have been shown to predispose insects to infection by *B. bassiana*. Regardless of the reasons for the differential activity of *B. bassiana* between the field and greenhouse, it is clear from this study that mycosis in caged grasshoppers does not indicate field efficacy, and extreme care should be taken in predicting field efficacy on the basis of laboratory studies.

4.4 CONCLUSIONS

The oil-bait bioassay method expedited the inoculation procedure, minimizing the toxic effects of oil, and facilitated the identification of virulent genotypes. Using the oil-bait bioassay method, the two conidial batches of *B. bassiana* were observed to be highly virulent against grasshoppers despite previous reports of differential field efficacy. Although conidia from both treatments were uniformly deposited onto grasshoppers, no reductions in field populations of grasshoppers were detected. However, substantial

mycosis was observed in grasshoppers collected from the field and maintained in greenhouse cages. The prevalence of mycosis in the caged grasshoppers decreased with sample time, but the onset of disease was always 3 to 4 days after collection, suggesting that environmental conditions and not the quantity and quality of inoculum was responsible for the poor efficacy of *B. bassiana* in the field.

CHAPTER 5

GRASSHOPPER RESISTANCE TO *BEAUVERIA BASSIANA* BY THERMOREGULATION

5.1 INTRODUCTION

Evidence obtained in section 4.3 suggested that environmental conditions and not host targeting or pathogen virulence were responsible for the poor efficacy of *B. bassiana* in the field. Acridids elevate their body temperatures higher than ambient by habitat selection and/or orientation to solar radiation (Chappell and Whitman, 1990; Heinrich, 1993). Thermoregulation by grasshoppers has been shown to reduce disease in controlled environments (Boorstein and Ewald, 1987; Carruthers *et al.*, 1992) but the impact of grasshopper thermoregulation on *B. bassiana* mycosis has not been studied.

This chapter is divided into two sections. In section one, the effect of temperature and thermoregulation by grasshoppers on *B. bassiana* was investigated in controlled environments. The objectives of these experiments were to: 1) measure the effect of varying exposures to high temperatures on mycosis in grasshopper nymphs; 2) determine whether nymphs provided the opportunity would elevate their body temperatures sufficiently to inhibit disease development; and 3) ascertain if nymphs exhibit a "behavioral fever" response to *B. bassiana* infection. In section two, the objective of the study was to determine the effect of sunlight and temperature on mycosis of grasshoppers in a field setting. To accomplish this the following field experiments were conducted: 1) efficacy of *B. bassiana* against field populations of acridids; 2) prevalence of mycosis in field-collected grasshoppers placed in field cages (exposed to sunlight) and greenhouse cages (diffuse light); and 3) using three field cage environments (exposed to full spectrum sunlight, shaded from sunlight, and protected from UVB radiation) determine the effects of temperature, and light quality and quantity on mycosis of grasshoppers and on conidial survival.

5.2 CONTROLLED ENVIORNMENT STUDY[7]

Materials and Methods

Inoculation of grasshoppers. Viability of conidia was determined on PDA with Benlate. Within 12 h of use, conidia were suspended in sunflower oil using a micropestle, concentrations of conidia were estimated using a hemocytometer and adjusted to 2.0×10^5 viable conidia/mL. Nymphs of a non-diapause strain of *M. sanguinipes* were inoculated using the oil-bait bioassay method (section 4.2).

Continuous exposure. Following inoculation, a minimum of 10 to 12 nymphs were individually transferred to 240-mL clear plastic cups. Nymphs were placed at either 25, 30, 35 or 40°C for 10 days in CECs under a 16/8 h photoperiod provided by fluorescent lights. Conditions of ambient and within cup temperature and relative humidity were recorded with a CR21 micrologger. Nymphs were fed fresh wheat leaves and frass was removed from the containers daily. Dead nymphs were collected twice daily and cadavers were placed in petri dishes containing moistened filter paper at 25°C. Nymphs that survived the 10 day duration of the experiment were killed by freezing and placed on moistened filter paper. With the exception of the 25°C treatment (five replicates), the experiment was replicated four times (342 total nymphs).

Fluctuating exposures. Groups of 20 to 21 inoculated nymphs kept singly in 240-mL cups were exposed to 35°C or 40°C for 0, 2, 4, 6, 8, 12 or 24 h per day for 10 days in the CEC; a 1 h per day exposure to 40°C was also tested. The remainder of the day was spent at 25°C. Although the 35 and 40°C exposure experiments were conducted separately, both experiments were replicated four times (561 and 643 nymphs per temperature, respectively).

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[7]A version of this section has been published as: "Effects of temperature and thermoregulation on mycosis by *Beauveria bassiana* in grasshoppers" Inglis, G.D., D.L. Johnson and M.S. Goettel. 1996. Biological Control 7:131-139.

Conidial germination and colony development were examined after exposure to 35° and 40°C (times as above). For germination assessments, conidia were spread on PDA amended with Benlate, and germination rates were measured after 6, 12, 24 and 48 h for four replicate cultures conducted at different times. For colony growth determinations, 0.1 μl of a conidial suspension was centrally placed onto unamended PDA, and diameters were measured at 24-h intervals for 10 days by taking the mean of two perpendicular measurements. The experiment consisted of four replicate cultures per treatment.

Short duration exposures. Following inoculation, 20 to 22 nymphs per treatment were individually placed in 240-mL cups. Nymphs were exposed to 35°C for 24 h, immediately after inoculation, and at 1 day intervals until day four. The remainder of the 10 day incubation period was spent at 25°C with control treatment consisting of inoculated nymphs not exposed to 35°C. The experiment was replicated three times (310 total nymphs).

Basking nymphs. Nymphs inoculated with *B. bassiana* conidia were allowed to move vertically in a heat gradient provided by a 25-W incandescent light bulb (General Electric, Mississauga, ON) in 40 x 40 x 30 cm aluminum cages (Figure 5.2.1). Less than 0.3 W of ultraviolet-B radiation (280-320 nm) is emitted from incandescent bulbs representing 0.04% of the radiation output (General Electric, pers. comm.). The cages were equipped with a perforated metal floor to prevent contact with frass. The bulb was mounted on the back wall, 21 cm (bulb centre) from the bottom of the cage. A plastic mesh tube (8-cm diameter x 28-cm high) was placed upright on one side of the bulb to permit the vertical movement of nymphs. At various positions in the cage, temperatures were recorded with a CR21X micrologger equipped with eight wire thermistors (approximately 0.5 mm diameter).

Following inoculation, 47 to 50 arbitrarily selected nymphs per treatment were placed in each of eight cages kept in a CEC under a 16/8 h light/dark photoperiod provided by fluorescent bulbs. Nymphs were maintained on a diet of wheat seedlings (provided

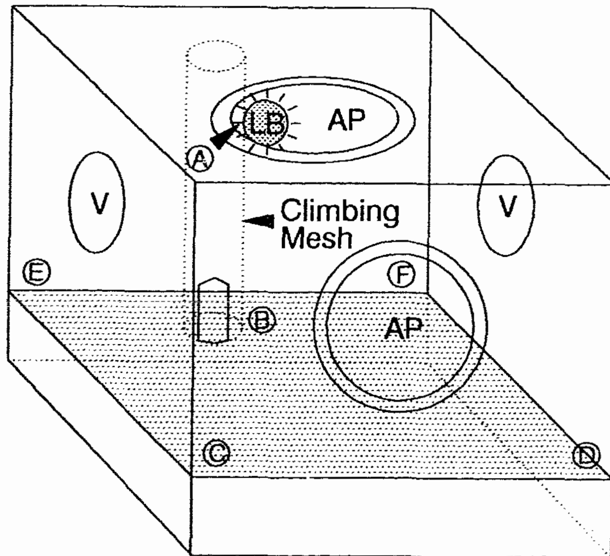

Figure 5.2.1 To elevate their body temperature by basking, nymphs had to climb a plastic mesh (8-cm diameter x 28-cm high) towards a heat source (25-W incandescent light bulb; LB) in a aluminum cage (40 x 40 x 30 cm). The cage was equipped with a perforated metal floor, and a clear plexiglass top and front. Air flow was enhanced by mesh vents (V) and access ports (AP), and temperatures were monitored at points A to F.

twice daily) and wheat bran for 10 days. Nymphs were exposed to the heat gradient generated by the light bulb for periods of 1, 2, 4 and 6 h per day; times of 12 and 24 h were also tested in two replicates but were not included in the analysis. The control treatment consisted of nymphs not exposed to a heat gradient. Cadavers were removed from the cages twice daily, and placed on moistened filter paper in petri dishes. The experiment consisted of four to six replicates (1200 total nymphs).

To determine the temperature of basking nymphs, a copper constantan thermocouple (0.127 mm diameter) was inserted into the hemocoel (thorax between the right meso- and meta-sternites) of each of five, third- and fourth-instar nymphs. These nymphs were positioned on the climbing mesh tube among thermoregulating nymphs (same height and orientation) and thoracic temperatures were output to a datalogger at 5 s intervals until they reached an asymptote. The thermocouple alone was also placed adjacent to nymphs on the mesh surface. In addition, the thoracic temperatures of nymphs were recorded at various positions on the floor of the cage.

Behavioral fever. Treatments consisted of *B. bassiana*-inoculated and oil-treated (control) nymphs. For each treatment, approximately 25 to 30 arbitrarily selected nymphs were grouped in 21 x 28 x 15 cm plexiglass containers equipped with a perforated metal floor and maintained at 25°C in the CEC under a 16/8 h photoperiod. Within 30 min of inoculation, and at 1-day intervals for 4 days, 20 nymphs per treatment were placed on a thermal gradient. With the exception of the time 0 nymphs which had been starved for 12 h, nymphs were fed fresh wheat leaves approximately 12 h prior to placement on the gradient. The gradient consisted of an aluminum tray (92 x 16 x 8 cm) divided into two equal compartments along its length with a plexiglass divider. One end of the tray was rested on a hot plate (Thermix, Model 310T, Fisher Scientific, Ottawa, ON), and eight surface temperature gradients (30° to 46°C) were marked in 2°C increments. To prevent nymphs from climbing the sides of the tray or on the plexiglass divider, cage walls were

coated with a Teflon spray (Super Lube, Permatex Industrial, Newington, CT). At 30 min intervals for 4 h, the number of nymphs in each gradient division were recorded; nymphs in each gradient were combined across observation times (n=8) for analysis. At the end of the observation period, nymphs were transferred back to the plexiglass cages and maintained on a diet of wheat for 10 days. Subsequent mycosis in these nymphs by day was compared to inoculated and control nymphs not placed on the gradient and kept at constant 25°C. The experiment was replicated four times (600 total nymphs), with the exception of the nymphs placed on the gradient within 30 min of inoculation (five replicates).

Statistical analyses. All experiments were arranged as RCBD, and with the exception of colony growth, replicates were conducted in time. There were no differences ($P>0.05$) in "other mortality" among treatments, and unless indicated otherwise, mortality ranged from 0 to 18%. Comparisons of disease progress and *B. bassiana* colony development among treatments were analyzed as split-plots in time with a Box correction. Since gradients were non-randomized (behavioral fever experiment), treatment and replicate effects were also analyzed as a split-plot. Subsequent to a significant F-test, means were compared using lsmeans. Conidial germination, colony size and mycosis at 10 days were compared at 35 and 40°C as a factorial experiment with replicates nested in temperature.

Results

Continuous exposure. Temperatures in the 240-mL cups were always similar (≤1°C) to ambient. In contrast, relative humidity in the cups was higher (≤ 36%) than ambient after the addition of wheat leaves each morning. The greatest rate of water loss from leaves occurred in cups maintained at the hotter temperatures. At all temperatures, humidity in the cups decreased as the leaves dried.

The rate of disease development ($F=18.0$; $df=3,10$; $P<0.001$) and cumulative mycosis after 10 days ($F=33.0$; $df=3,6$; $P<0.001$) differed among temperature treatments

(Figure 5.2.2). Disease progress ($F=1.2$; $df=1,6$; $P=0.32$) and the final prevalence of disease ($P=0.27$) were similar between 25° (93.9 %, SE=3.6) and 30°C (77.5 %, SE=13.8). In contrast, considerably less ($P<0.001$) mycosis (≤ 7.4 %) was observed for the 35° and 40°C treatments after 10 days. In nymphs maintained at 40°C, a loss of cuticle pigmentation occurred resulting in an "albino" appearance to the nymphs. Mortality not attributed to *B. bassiana* averaged 35.5 % (SE=16.7) for the 40°C treatment and less than 15.1% for the others.

Fluctuating exposures. Increasing exposures to 35° and 40°C for varying periods each day influenced both the rate of disease development ($F=34.8$-110.3; $df=6$-$7,21$-24; $P<0.001$) and the prevalence of final mycosis ($F=42.7$-191.4; $df=6$-$6,18$-21; $P<0.001$) (Figure 5.2.3). Less ($F=30.7$; $df=6,36$; $P<0.001$) mycosis was observed in inoculated nymphs exposed to 40°C than 35°C, and minimum exposures of 1 and 6 h per day affected ($P<0.04$) disease for the two temperature treatments, respectively.

Colony development was inhibited by daily exposures to 35° ($F=76.9$; $df=6,28$; $P<0.001$) and 40°C ($F=145.7$; $df=7,24$; $P<0.001$) (Figure 5.2.4), but exposure to 40°C was more ($F=22.2$; $df=6,42$; $P<0.001$) inhibitory. Hyphal growth was inhibited ($P<0.05$) in cultures maintained at 35° for 4 h or longer per day and at 40°C for 1 h or more per day. Although a minimal increase (1.5 mm, SE=0.24) was observed in colonies kept at constant 35°C, conspicuous aerial tufts of white hyphae were observed. No growth was observed on cultures exposed to 40°C for ≥ 12 h per day. The effect of high temperatures on colony growth was highly correlated ($r \geq 0.95$) with mycosis in grasshopper nymphs (Figure 5.2.5). The best relationship was observed after logit-transformation of the mycosis data. The logit model used was $\ln((p + 0.005) / 1.005 - p)$ where p is the proportion of final mycosis (%).

Daily exposure to 35° ($F=67.4$, $df=18,117$; $P<0.001$) and 40°C ($F=50.4$; $df=21,62$; $P<0.001$) delayed conidial germination on PDA; 40°C was more ($F=9.3$-52.8; $df=6,30$-40; $P<0.001$) inhibitory than 35°C (Figure 5.2.6 A-B). With the exception of

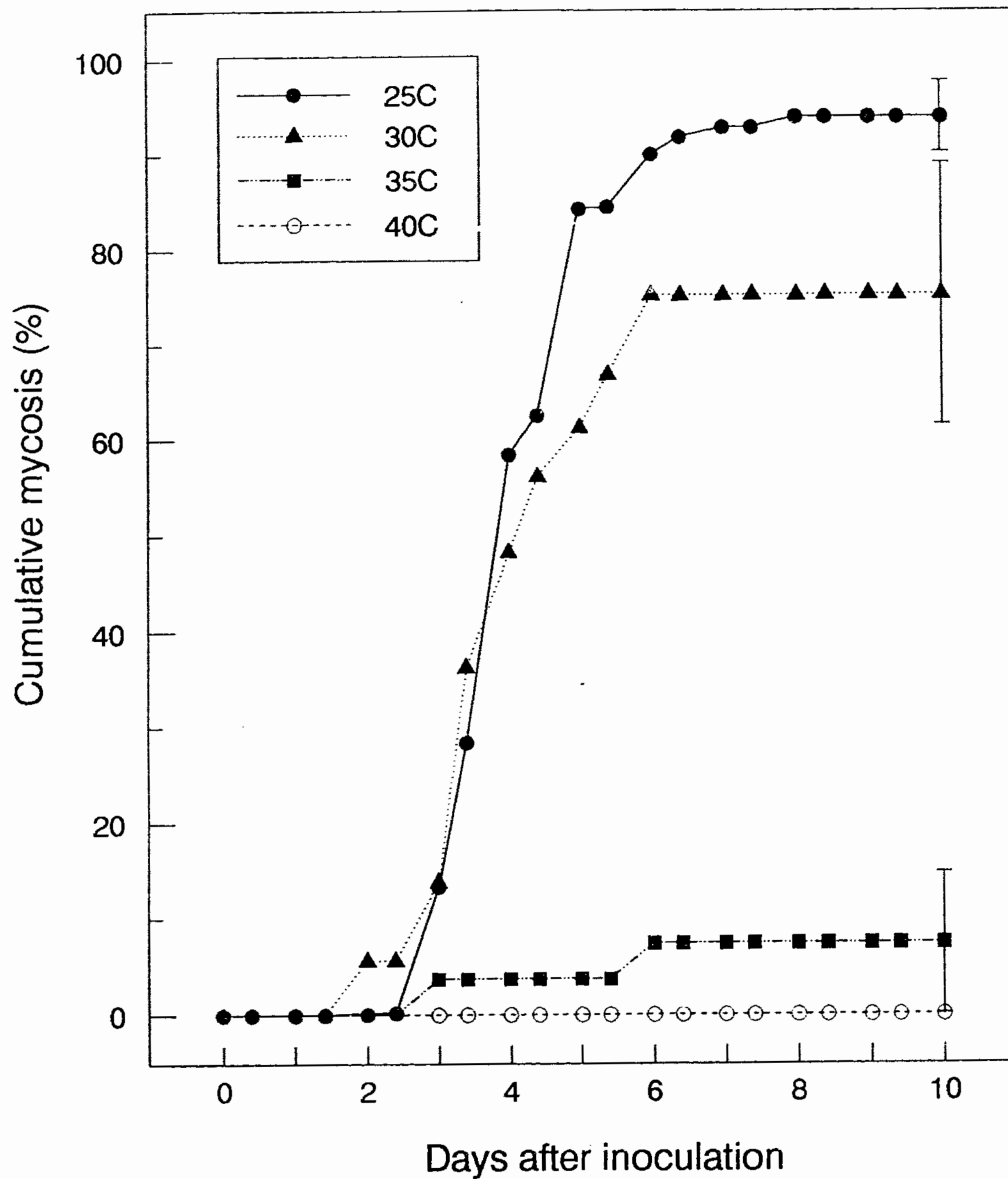

Figure 5.2.2 Disease (*Beauveria bassiana*) progress curves for *Melanoplus sanguinipes* nymphs maintained at 25°, 30°, 35°C and 40°C. Vertical lines represent standard errors of means for mycosis at 10 days (n = 4 to 5).

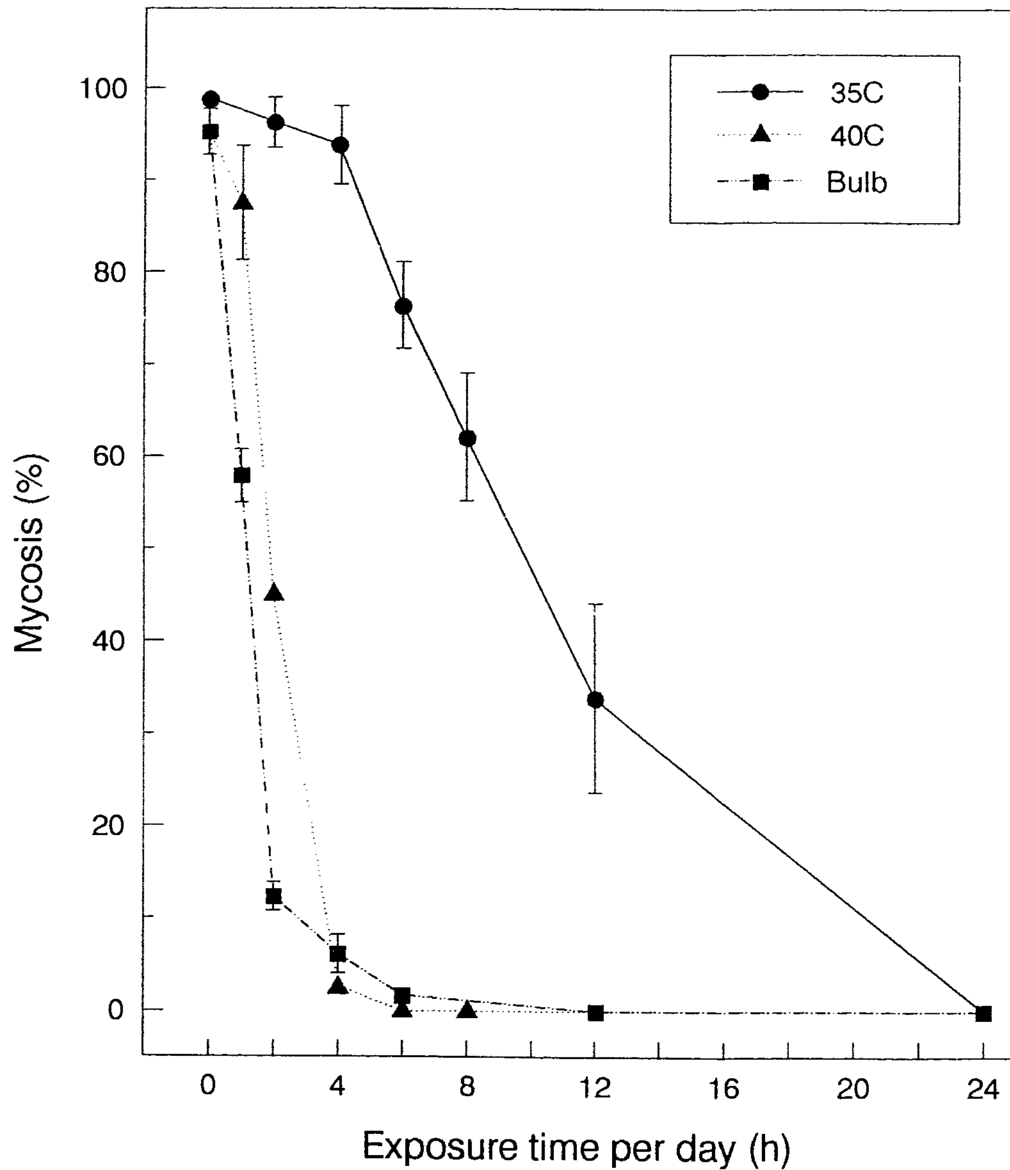

Figure 5.2.3 Prevalence of mycosis (*Beauveria bassiana*) in *Melanoplus sanguinipes* nymphs (10 days) exposed to 35°, 40°C or a vertical heat gradient (25-W incandescent light bulb) for varying times per day. Vertical lines represent standard errors of means (n = 4 to 6).

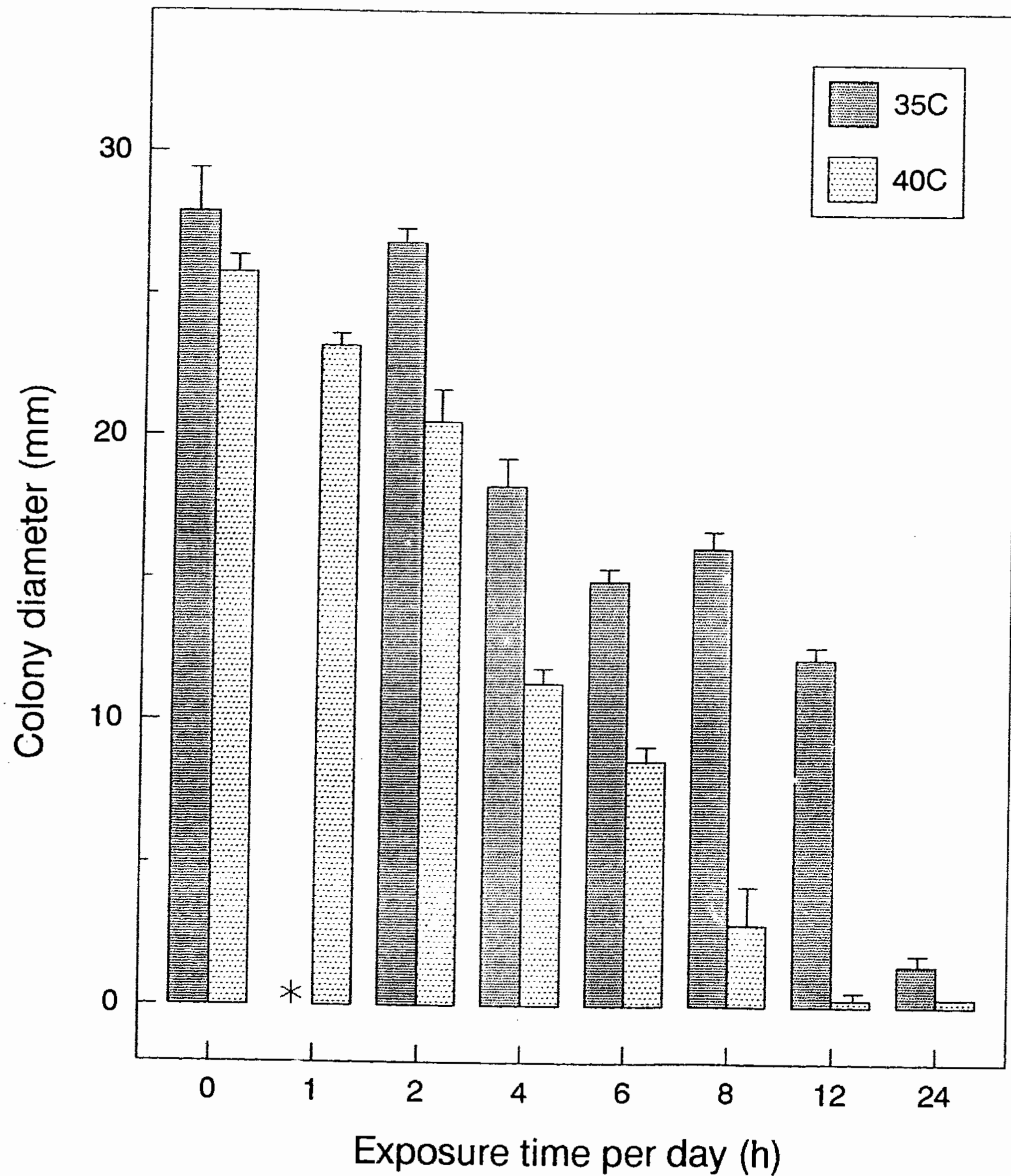

Figure 5.2.4 Diameter (mm) of *Beauveria bassiana* colonies on potato dextrose agar (10 days) exposed to 35° or 40°C for various times per day. The asterisk indicates a missing datum point, and vertical lines represent standard errors of means (n = 4 to 5).

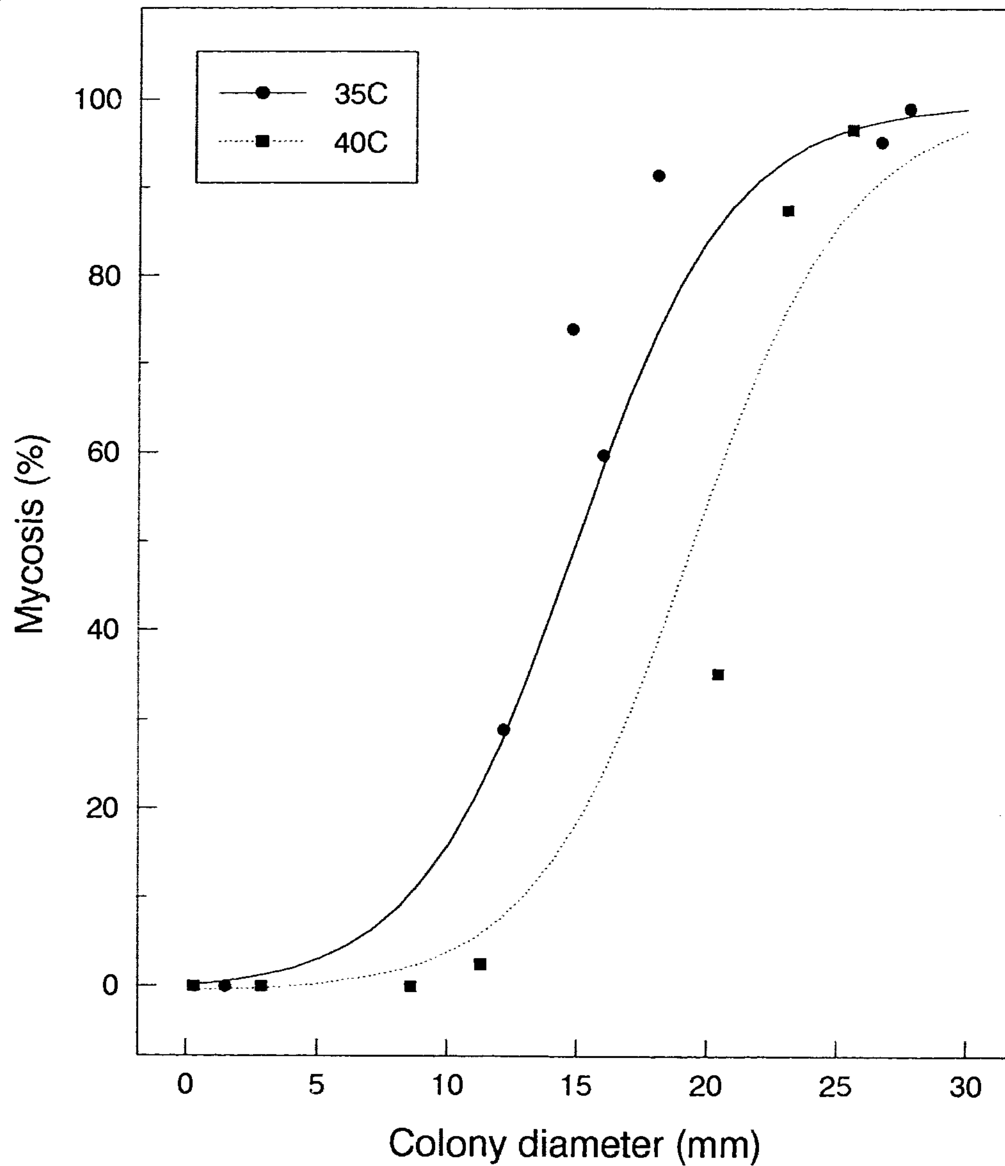

Figure 5.2.5 Logistic relationship between *Beauveria bassiana* colony diameter (mm) on potato dextrose agar and mycosis of *Melanoplus sanguinipes* nymphs (%) exposed to 35° or 40°C for various durations each day.

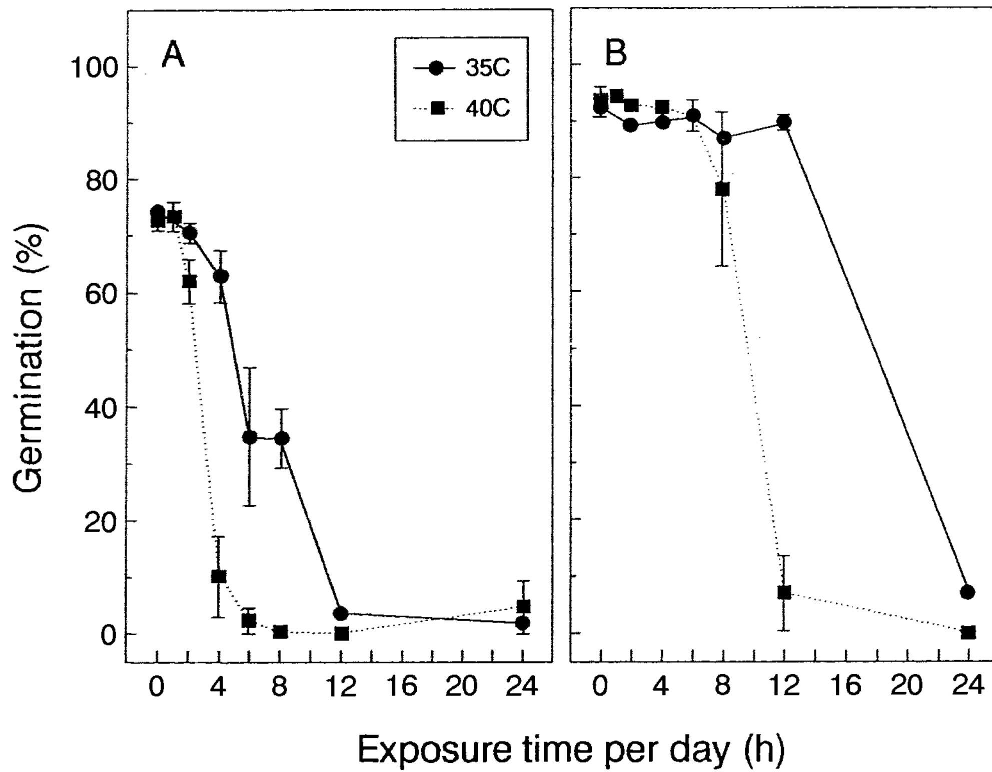

Figure 5.2.6 Germination (%) of *Beauveria bassiana* conidia after various periods of exposure to 35° or 40°C per day: A) germination at 12 h; B) germination after 24 h. Vertical lines represent standard errors of means (n = 4 to 6).

conidia placed at constant 35°C, there were no differences ($P>0.05$) in conidial germination at 24 h among exposure treatments. In contrast, exposure to 40°C for ≥ 8 h per day reduced ($P\leq0.05$) conidial germination at 24 h. Relative to colony growth, poorer relationships ($r = 0.47$ to 0.77) were observed between conidial germination after 24 h and mycosis in nymphs exposed to high temperatures.

Short duration exposure. Exposure of nymphs to 35°C for 24 h reduced the prevalence of final mycosis ($F=3.9$; df=4,8; $P=0.048$) and slowed ($F=3.7$; df=4,10; $P=0.043$) the rate of disease development. However, mycosis was only reduced ($P=0.006$) in nymphs exposed to 35°C between 1 and 2 days post-inoculation (46.7 %, SE=9.3). In nymphs exposed to 35°C at other times after inoculation, final mycosis ranged from 71.2 % (SE=15.8) to 81.9 % (SE=9.1). The prevalence of mycosis was 92.1 % (SE=4.1) in nymphs kept at constant 25°C.

Basking nymphs. While the 25-W incandescent light bulb was on, temperatures within the cage ranged from 46 to 49°C on the climbing mesh at the closest proximity to the bulb (Figure 5.2.1A), 26 to 27°C at the base of the mesh (Figure 5.2.1B), and near ambient (25 to 26°C) at the other positions in the cage (Figure 5.2.1 C-F). When the bulb was off, temperatures throughout the cage were similar to ambient (≈ 1°C). When switched on, nymphs quickly (*ca* 1-5 min) climbed towards the heat source positioning themselves in a circle around the bulb; the internal thoracic temperature of both third- and fourth-instar nymphs ranged from 38 to 42°C. Basking affected both disease development ($F=149.8$; df=6,19; $P<0.001$) and the prevalence of mycosis at 10 days ($F=385.5$; df=4,12; $P<0.001$) (Figure 5.2.3). In nymphs allowed to bask for only 1 h per day, 46.0% less ($P<0.001$) disease was observed relative to non-basking nymphs.

Behavioral fever. Nymphs starved for 12 h and placed on the gradient immediately after inoculation were more active than nymphs placed on the gradient at later times. By 3 days, a higher ($F=14.1$; df=1,6; $P=0.009$) frequency of nymphs inoculated with *B.*

bassiana selected hotter positions on the heat gradient than nymphs treated with oil alone (Figure 5.2.7). However, when the number of nymphs observed at the 40°C to 42°C surface temperature gradient or greater were combined, a difference ($F=6.9$; $df=4,28$; $P<0.001$) between the control and *B. bassiana* treatments was detected ($P=0.003$) in nymphs placed on the gradient by 2 days (Figure 5.2.8).

Of the nymphs inoculated with *B. bassiana* but not placed on the gradient, 92.9 % (SE=3.1) died of mycosis after 10 days at 25°C. The prevalence of mycosis was also high in nymphs placed on the thermal gradient for *ca* 4.5 h; cumulative mycosis ranged from 71.5 % (SE=7.0) to 88.2 % (SE=8.3). Although the rate of disease development was similar ($F=0.1$; $df=5,20$; $P=0.99$) among treatments, time affected ($F=3.8$; $df=5,15$; $P=0.020$) mycosis and the prevalence of final mycosis was less ($P\leq0.018$) for nymphs placed on the thermal gradient 2 to 4 days post-inoculation relative to those inoculated with *B. bassiana* but not placed on the gradient. Low levels of mycosis (≤7%) were observed in nymphs treated with oil alone.

Discussion

Using behavioral mechanisms, acridids elevate their body temperatures by either directly or indirectly intercepting solar radiation (Chappell and Whitman, 1990; Heinrich, 1993). Although acridids are capable of raising their temperature substantially higher than ambient given the appropriate conditions (Kemp, 1986; Carruthers *et al.*, 1992), the influence of thermoregulation by grasshoppers on the impact of entomopathogens has not been extensively studied. I observed that mycosis in grasshoppers treated with *B. bassiana* was severely inhibited by continuous exposures to high temperatures. This finding is not surprising given the optimal (18° to 30°C) and upper thermal limit (approximately 35° to 38°C) for conidial germination and/or vegetative growth of *B. bassiana* (Roberts and Campbell, 1977; Fargues *et al.*, 1992). However, the impact of continuous high temperatures on mycosis is unrealistic since acridids elevate their body temperatures

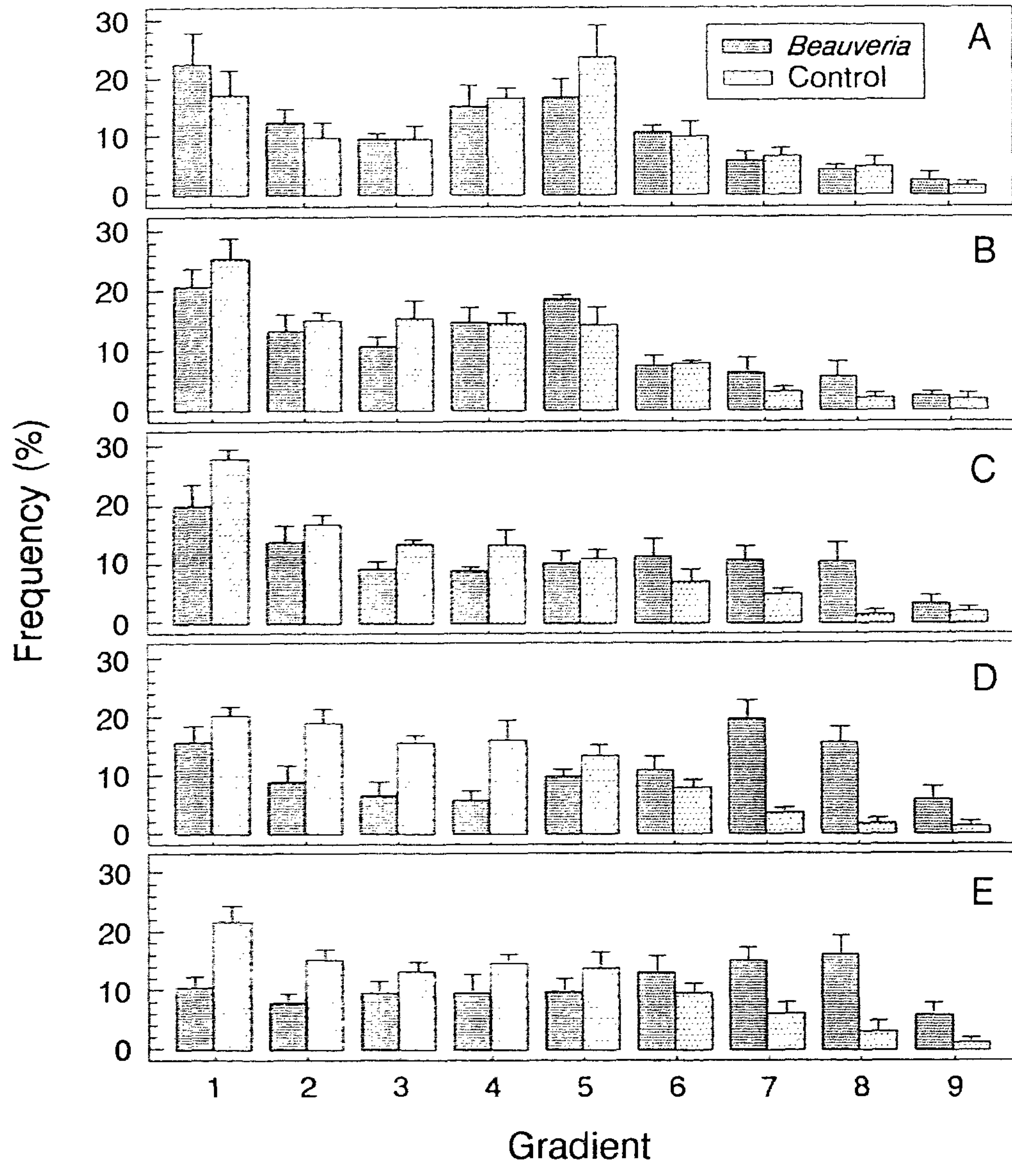

Figure 5.2.7 Frequency distribution of *Beauveria bassiana*-inoculated and control *Melanoplus sanguinipes* nymphs (oil alone) on a thermal gradient. Gradients were marked at 2°C intervals (surface temperature) and ranged from 30 to 32°C (gradient 1) to greater than 46°C (gradient 9). Observations were made at 30 min intervals for 4 h, and nymphs per gradient per observation time were combined for final analysis. Positioning of nymphs on the gradient were analysed at various times post-inoculation (different nymphs were used): A) immediately after inoculation; B) 1 day after inoculation; C) 2 days after inoculation; D) 3 days after inoculation; and e) 4 days after inoculation. Vertical lines represent standard errors of means (n = 4 to 5).

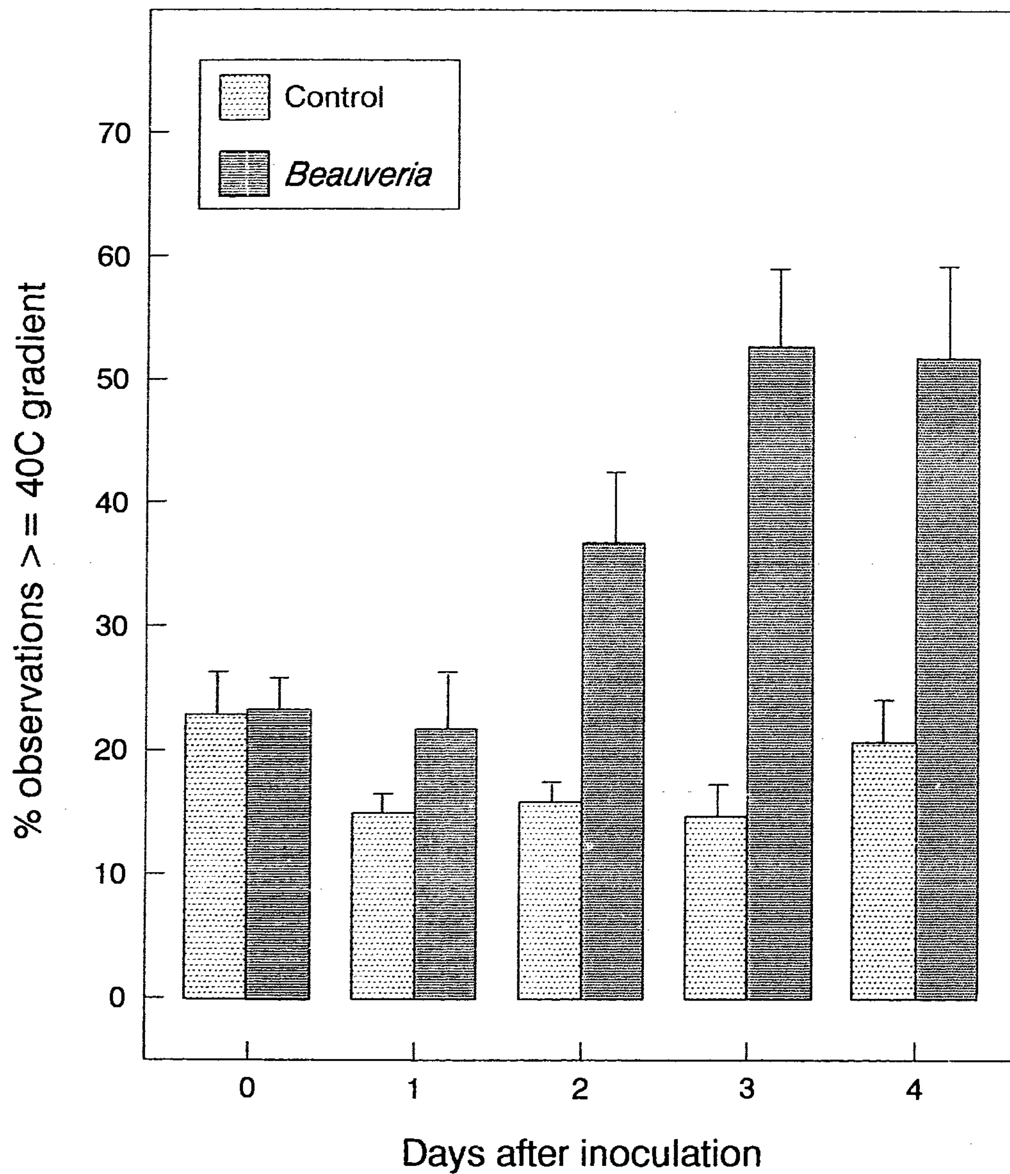

Figure 5.2.8 Prevalence of *Beauveria bassiana*-inoculated and control *Melanoplus sanguinipes* nymphs (oil alone) observed on a thermal gradient at surface temperatures greater than or equal to 40°C ($\geq$ gradient 6). Vertical lines represent standard errors of means (n = 4 to 5).

primarily during periods of sunlight.

Grasshoppers maximize the interception of solar radiation by habitat selection and/or by basking (Chappell and Whitman, 1990). To mimic basking, nymphs were exposed to a 25-W incandescent light bulb (heat source) placed adjacent to a climbing mesh. Nymphs quickly positioned themselves on the mesh in a ring around the bulb (Lactin and Johnson, 1996). The internal thoracic temperatures of these grasshoppers ranged from 38 to 42°C, which is consistent with reports of optima for development (Hilbert and Logan, 1983) and feeding (Lactin and Johnson, 1995) of *M. sanguinipes*. I observed that basking by grasshoppers severely inhibited mycosis caused by *B. bassiana*. In nymphs permitted to bask for only 1 h per day, 46% less mycosis was observed, and as the basking period increased, so did the inhibition of disease. Although there are costs associated with basking in nature (e.g., lost foraging time, increased parasitism and predation), the benefits accrued may outweigh the costs. This study demonstrates that by elevating their body temperature by basking, grasshoppers can reduce the impact of disease caused by *B. bassiana*.

Grasshopper thermoregulation models indicate that in the prairie provinces of Canada and the northern states of the USA, days occur (cool and/or overcast) when grasshoppers are not able to achieve a body temperature at or near their developmental optimum (Kemp, 1986; Carruthers *et al.*, 1992). To determine the effects of suboptimal body temperatures on disease and to corroborate the results obtained from the basking experiment, disease in nymphs exposed to 35°C and 40°C for varying periods each day were compared. Exposures to 35°C were substantially less detrimental than exposures to 40°C. However, an exposure time of 6 h or greater per day at 35°C inhibited mycosis suggesting that even when conditions do not permit optimization of body temperature, thermoregulation by grasshoppers can influence mycosis. In nymphs exposed to 40°C, results were similar to those observed in the basking experiment. This would be expected

given the supposition that acridids optimize their body temperature (*ca* 40°C) by basking. In both the 35° and 40°C fluctuation experiments, conditions of relative humidity varied between treatments. This was attributed to differences in ambient relative humidity in the CEC and by the rate of water loss from wheat leaves (unpublished data). Although relative humidity represents a potential confounding factor in these experiments, Marcandier and Khachatourians (1987) showed no effect of relative humidity on the efficacy of *B. bassiana* against grasshoppers. I observed that disease development was similar between nymphs exposed to 40°C in cups and those allowed to bask (conditions of humidity increased ≤ 5% in the basking cages following feeding) further suggesting that temperature and not relative humidity was the primary factor affecting disease development.

In an attempt to elucidate the mechanism(s) by which temperature inhibits mycosis, the effects of temperature on vegetative growth and conidial germination on disease was compared. Colony growth on PDA was more inhibited by exposures to 40° than 35°C, and strong correlations were observed between hyphal growth and mycosis at both temperatures. This suggests that the direct effects of temperature on growth of *B. bassiana* is important. In addition, 30 to 47% of nymphs inoculated with *B. bassiana* and kept at 25°C for 4 days were cured by transfer to a high temperature (≥ 35°C) environment (data not presented). It is uncertain whether the direct effects of temperature on *B. bassiana* alone are responsible for arresting disease development in nymphs at such an advanced stage of infection. The influence of high temperature on the physiology of the pathogen-host interaction has not been extensively studied, and it is seems highly probable that temperature also influences the immune response of insects.

The ability of conidia to germinate rapidly and synchronously is believed to be important in the infection process. The surface temperature of the cuticle in thermoregulating grasshoppers would be expected to be as high or higher than the temperature in the hemocoel. Although conidial germination was delayed by exposures to

high temperatures, the effect of temperature on germination was observed to be a poor predictor of mycosis. I also observed a reduction in mycosis only in grasshoppers placed at 35°C between 1 and 2 days post-inoculation. Since the majority ($\geq$ 92%) of *B. bassiana* conidia had germinated by 24 h at this temperature, this would seem to suggest that the early stages of pathogenesis (penetration and early internal proliferation) and not conidial germination, are most affected by high temperatures. However, the germination assessments were made on PDA, and results obtained *in vitro* do not always correspond to those observed *in vivo*. Furthermore, conidia exposed on the surfaces of grasshoppers are rapidly deactivated by UVB radiation in field environments (section 3.3). Since the experiments were conducted under fluorescent lights, the consequences of delayed germination would be underestimated relative to a field environment.

Differential behavioral activity due to infection ("behavioral fever") has been reported in bacteria-infected cockroaches (Bronstein and Conner, 1984), rickettsia-infected crickets (Louis *et al.*, 1986), microsporidian-infected grasshoppers (Boorstein and Ewald, 1987), and recently in house flies infected with the entomopathogenic fungus, *Entomophthora muscae* (Watson *et al.*, 1993). A behavioral fever response to infection was also observed in *M. sanguinipes* nymphs infected with *B. bassiana* in the present study. The positioning of the nymphs on the gradient does not provide an accurate measure of internal body temperature because nymphs can alter their body temperature by changing postures. Nevertheless the observations between infected and healthy nymphs were conducted simultaneously, and as infection progressed, a higher proportion of *B. bassiana* infected *M. sanguinipes* nymphs selected hotter positions on a heat gradient than non-infected nymphs.

A number of factors other than infection by entomopathogens affect acridid behavior on thermal gradients. Chapman (1955) noted that starved nymphs (21-26 h) were more active than recently fed nymphs. This is consistent with the observations of

increased activity in *M. sanguinipes* nymphs placed on the gradient immediately after inoculation (time 0); these nymphs had been starved for *ca* 12 h to facilitate ingestion of the lettuce bait. Subsequently, nymphs (times 1 to 4 days) were considerably less active on the thermal gradient than the time 0 nymphs. However, the frequency distributions were, in general, wider than that reported by Chapman (1955) for locust nymphs (*Schistocerca gregaria*). Similar to Chapman (1955) a disproportionate number of nymphs were observed to become "trapped" at the cool end of the gradient. Whether these nymphs became lost or were attempting to maintain a lower body temperature is uncertain (Heinrich, 1993). Observations of grasshopper behavior on the thermal gradient suggests that nymphs infected with *B. bassiana* will differentially elevate their body temperature in response to the infection. Most reports of behavioral fever in insects have been demonstrated on a thermal gradient and the validity of my observations of differential thermoregulation in *M. sanguinipes* nymphs infected with *B. bassiana* should be confirmed in basking experiments.

This study demonstrates that high temperature and thermoregulation by grasshoppers can inhibit/or prevent disease caused by *B. bassiana,* and the ability of grasshoppers to elevate their body temperature may explain the variable efficacy of this entomopathogen in field environments. The ability of acridids to elevate their body temperatures is consistent with observations of successful control of grasshoppers with *B. bassiana* during cool overcast periods (Johnson and Goettel, 1993) and unsuccessful control during hot sunny periods (section 4.3). Furthermore, the inability of acridids to thermoregulate in greenhouse cages may explain their increased susceptibility to *B. bassiana* in this environment (section 4.3). It is also possible that high temperature affects the efficacy of *Metarhizium flavoviride* against acridids (Lomer, 1994). Although the detrimental effects of high temperatures and thermoregulation by grasshoppers on mycosis

caused by *B. bassiana* have been demonstrated, confirmation of this phenomenon in a field environment is necessary.

5.3 FIELD STUDY[8]

Materials and Methods

Field Efficacy

Field site. The rangeland site was located 28 km north of Coaldale, AB (SEC 1, TWP14, RGE20, W4M) on native short-grass prairie rangeland. Dominant species were spear grass, blue grama, and June grass intermixed with various low-growing forbs and other grasses. Twelve plots were established. Each plot was 3 ha (173 x 173 m), and plots were separated from each other by ≥ 100 m. Treatments consisted of *B. bassiana* conidia, a carrier control and an untreated control arranged as an RCBD with four blocks. Within each of the *B. bassiana* and carrier control plots, four, 4-m^2 subplots were established for assessments of conidial deposition and persistence; subplots were located 70 m in diagonally from the corners of the plot boundary.

Conidial application. Dry conidia were suspended in a 1.5% (v/v) oil emulsion amended with 4% clay (w/v) immediately before application (2.5×10^{11} conidia per mL). The target concentration was 2.5×10^{13} conidia per ha. Treatments were applied on the morning of July 12, 1995. The *B. bassiana* and carrier control treatments were sprayed at 112 L per ha using a tractor-mounted Hardi sprayer (section 4.3).

Conidial deposition. To quantify the deposition of conidia per cm^2, four sterile round glass coverslips (13-mm diam) were evenly spaced on the bottom of a petri dish with double-sided tape. Dishes were placed randomly on the soil surface in each of four subplots in the *B. bassiana* and carrier control plots. Within 5 to 30 min of application, the

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[8]A version of this section has been published as: "Effects of temperature and sunlight on mycosis (*Beauveria bassiana*) (Hyphomycetes: Sympodulosporae) of grasshoppers under field conditions" Inglis, G.D., D.L. Johnson and M.S. Goettel. 1996. Environmental Entomology (in press).

dishes were collected and placed on ice until they could be returned to the laboratory where they were placed at 5°C for 12 h. Conidia were isolated from the four coverslips per dish on oatmeal-dodine agar using the wash method.

Droplet deposition was assessed using water-sensitive cards. Cards were set in 9-cm diam petri dishes and the dishes were set out at the same time and in the same manner as the coverslips. Cards were collected within 5 to 30 min of application. Droplet density, area, and size were assessed using a Tracor Northern 8502 Image Analyzer equipped with a Dage 68 video camera for image acquisition. Droplets were analyzed in each of two areas (6.2 cm^2) per card; the droplet data from the two areas were combined and mean droplet area, width and density was determined. Total droplet area per card was calculated and converted to percent coverage.

Conidial persistence on leaves. Ten grass leaves were collected arbitrarily from each subplot and combined in plastic bags. Times of sampling were immediately after (time 0), and 2, 5, 10, and 15 days following application. Leaves were returned to the laboratory on ice and maintained at 5°C for a maximum of 48 h. All leaves were cut aseptically into pieces approximately 1 cm long, and conidia were isolated on oatmeal-dodine using the wash method. Sample leaf areas ranged from 1.2 to 5.0 cm^2. Subplot means were calculated from $\log_{10}$-transformed data, and treatment means were fit to linear models after $\log_{10}$-transformation of the time axis.

***Beauveria* associated with grasshoppers.** Grasshoppers were collected in sweepnets from *B. bassiana* and carrier control plots immediately after treatment (time 0), then 5, 10, and 15 days thereafter. Grasshoppers from each plot were placed in 44 x 58 x 46 cm cages and returned to the laboratory. Within 4 to 5 h of field collection, 30 and 15 nymphs per plot were selected arbitrarily from the cages containing grasshoppers from the *B. bassiana*-treated and carrier control plots, respectively. Nymphs were placed individually in vials, weighed, and placed at 5°C for a maximum of 48 h. Fifteen nymphs from each of the

B. bassiana-treated and carrier control plots were homogenized, the homogenate diluted and spread on oatmeal-dodine agar as described previously. The remaining 15 nymphs from the *B. bassiana* sprayed plots were surface-sterilized in ethanol for 1 min, followed by two rinses in sterile deionized water prior to maceration. The number of cfu per nymph was then determined on oatmeal-dodine agar. To account for variation in the size of nymphs, cfu per nymph were calculated per mg x the mean weight of nymphs (44.7 mg).

To test the efficacy of the sterilization procedure, 20 *M. sanguinipes* nymphs (fifth-instar) were killed by freezing. Nymphs were weighed, sprayed with 100 μL of a suspension of *B. bassiana* conidia in water (10^9 viable conidia per mL) using an airbrush (section 3.4). One half of the nymphs were surface-sterilized in ethanol as above, then all were macerated individually, and the cfu per insect were enumerated as detailed previously.

Grasshopper populations, species and age composition[9]. Grasshopper population densities were monitored by counting living grasshoppers in sample areas delimited by 0.25-m^2 sampling frames one day before treatment (time 0), and 4, 11, and 15 days after the application of conidia. The sampling frames were open circles of 0.5-cm diam white plastic tubing placed on the soil surface in the central sampling zones of each plot (20 per plot). Mean grasshopper densities per plot were analyzed as a split plot in time with a Box correction (four levels of block, three levels of treatment, and four levels of time). Comparisons between means at each sample time were made using the lsmeans function.

To determine grasshopper species and age composition, two samples of 100 sweeps (180°, 38-cm diam net) were collected arbitrarily from each plot at time 0, and 4 and 15 days after conidial application. Grasshoppers were returned to the laboratory, frozen, counted, and determined to species and age class.

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[9]Field population assessments and collection of grasshoppers were carried out by Steve Van Wilgenberg. Identification of rangeland grasshoppers was performed by Craig Andrews.

Weather data. Mean hourly solar radiation (300 to 2800 nm), temperature, and relative humidity were recorded at AAFC weather stations located at Lethbridge and Vauxhall; UVB radiation (280-320 nm) was also measured at the Lethbridge site. The Lethbridge and Vauxhall stations are located 49 km south-west and 33 km south-east from the field site, respectively. Conditions of temperature and relative humidity were similar at the two weather stations, and data for Lethbridge alone are presented. In contrast, differences were observed in radiation between the Lethbridge and Vauxhall sites, so data for both are presented. Daily precipitation and mean hourly wind speeds (height of 65 cm) at the field site were also recorded.

Mycosis in Field and Greenhouse Cages

To determine if previous observations of mycosis in greenhouse cages (section 4.3) was due to the predisposition of grasshoppers (e.g., stress) or the result of a more conducive environment for disease development, mycosis in grasshoppers placed in the field and greenhouse cages were compared; cages consisted of a wood-frame (46 x 61 x 48 cm) covered with fibreglass netting (49 filaments per cm^2). Grasshoppers were collected from each of the *B. bassiana*-treated and carrier control plots by sweeping within 1 to 3 h of application (time 0), and 5, 10 and 15 days thereafter. From each plot at each sample time, 100 grasshoppers (primarily melanopline species) were placed into two cages. One of the cages was placed adjacent to the field plot, the other was placed in a greenhouse located at the AAFC Research Centre. All grasshoppers were maintained on a diet of wheat (cv. Norstar) seedlings (10 to 14 day-old in 10-cm diam pots), bran, and a variety of grasses and forbs collected from the field site but not exposed to *B. bassiana*; wheat seedlings, and rangeland grasses and forbs were replaced daily. Relative humidity, temperature and solar radiation (400 to 1100 nm) were recorded in a cage maintained in the field and in the greenhouse using CR21X microloggers.

Cadavers were removed from cages each morning, placed on moistened filter paper. None of the nymphs that died within 2 days of placement in cages (1 to 21%) were colonized by *B. bassiana*; these were considered to have died from mechanical damage by sweeping or from natural causes, and were excluded from subsequent analyses. At each sample date, comparisons of disease progress between field and greenhouse cages were conducted as split plots in time with cage type nested within blocks. The prevalence of final mycosis (12 d) were compared, by sample date as a split plot (cage type nested in blocks) with four levels of block, two levels of cage and four levels of sample time. A Box correction was used in both analyses. When the F-test for the treatment by time interaction was significant, means were compared using lsmeans.

Effect of Temperature and Sunlight

To elucidate the influence of temperature and/or light quantity and quality on mycosis, disease was compared in grasshoppers placed in different field cages. Cage treatments included: 1) exposed to full spectrum sunlight; 2) shaded from sunlight by a black plastic screen; and 3) protected from UVB radiation by a UVB-absorbing plastic film (< 355 nm; Dura Film 3, AT Plastics Inc., Edmonton, AB). Plastic screens (1.8 x 3.0 m) were attached to a wood frame. The front of the frame (facing south) was situated 0.9 m above the soil surface and the back of the frame was 1.2 m above the soil; this arrangement shaded the cages for most of day (*ca.* 0900 to 1700 hr) and had a minimal impact on air movement. Throughout the experiments, conditions of light, temperature and relative humidity were recorded within each of the cage types using a CR21X micrologger.

Rangeland site. Grasshoppers were collected from each of the four, *B. bassiana* sprayed plots (time 0) by sweeping. Grasshoppers from individual field plots were allocated to 3 cages (100 grasshoppers per cage) and cages were arranged adjacent to the field plots as a RCBD with 4 blocks, each containing 3 cage treatments per block. Grasshoppers were

maintained on a diet of wheat seedlings and rangeland grasses, cadavers were removed daily and processed for internal *B. bassiana* as detailed previously.

Lethbridge site. In a parallel trial, *M. sanguinipes* nymphs (F_1 laboratory generation) (n=1644 nymphs) were inoculated with *B. bassiana* using the oil-bait method (section 4.2). Inoculated nymphs were allocated to 12 cages and cages were placed in a field of crested wheatgrass at the AAFC Research Centre. Cages were arranged as RCBD with 3 cage treatments per block.

Conidial persistence. Conidial survival on wheat leaves was measured in both trials. In trial one, 10- to 14-day-old wheat seedlings in 10-cm diam pots were placed in rangeland plots before conidial application. Within 5 to 15 min of application, wheat plants were moved to locations on the soil surface under each UVB screen or adjacent to it in an exposed position (approximately 5 m distant to prevent any shading). In trial two, conidia in water were applied to wheat seedlings using an airbrush (section 3.4). The water carrier was allowed to dry for 15 min, and the plants were located adjacent to each of the cages. Wheat leaves in trial two, particularly those in the exposed and UVB protected environments, were subject to clipping by resident grasshoppers. Three days after conidial application, pots exposed to full sunlight were transferred to cages to protect them.

At time 0, and 2, 5 and 10 days after conidial application, 10 leaf segments were collected from each pot. At later sampling times care was taken to sample older leaves and to use segments near the leaf tips. Populations of viable *B. bassiana* conidia were quantified using the wash method as described previously. Conidial populations on leaves were calculated as $\log_{10}$ cfu per cm^2, and were analyzed as a split plot in time with four levels of block, four levels of environment and four levels of time. A Box correction was used for the time and time by environment interactions. Comparisons between means at each sample time were made using Tukey's studentized range test ($\alpha = 0.05$).

Results

Field Efficacy

Conidial deposition. From coverslips and leaves sprayed with *B. bassiana*, 2.1 x 10^4 (SE=0.11 x 10^4) and 1.7 x 10^4 (SE=0.26 x 10^4) cfu per cm^2 were isolated , respectively. No conidia were isolated from leaves or coverslips collected from the carrier control plots. The densities of droplets on water-sensitive cards were similar ($t=-2.4$; $df=6$, $P=0.06$) between the *B. bassiana* and carrier control treatments (Table 5.3.1). Droplet coverage was also similar ($t=-1.8$, $df=6$, $P=0.13$) between the two treatments.

Conidial persistence on leaves. Conditions were hot and sunny, and eight periods of precipitation ($\leq$ 10 mm per event) were recorded during the course of the experiment (Figure 5.3.1). Conidial survival was poor and populations declined logarithmically over time (Figure 5.3.2).

***Beauveria* associated with grasshoppers**. *Beauveria bassiana* was isolated from all nymphs collected from plots sprayed with conidia, regardless of whether they were surface-sterilized or not. However, substantially less ($t=38.4$; $df=6$; $P<0.001$) cfu were isolated from surface-sterilized (4.3 x 10^2 cfu per nymphs, SE=0.87 x 10^2) than from unsterilized nymphs (6.7 x 10^3 cfu per nymph, SE=0.15 x 10^3) collected immediately after conidial application. Conidial populations isolated from unsterilized grasshopper nymphs declined logarithmically over time (Figure 5.3.2) at a rate similar to that of conidia on grass leaves (Table 5.3.2). In contrast, small populations of *B. bassiana* (< 5 cfu per nymph) were isolated from surface-sterilized nymphs at subsequent sample times. Low numbers of *B. bassiana* cfu (< 30 cfu per nymph) were isolated from nymphs collected from the carrier control plots at all sample times.

To determine if the cfu isolated from surface-sterilized grasshoppers from rangeland was due to inadequate sterilization, laboratory-reared nymphs were sprayed with *B. bassiana* conidia and sterilized in ethanol. From nymphs submerged in ethanol for 1 min,

Table 5.3.1 Deposition of spray droplets on water sensitive cards placed on the soil surface within field plots sprayed with conidia of *Beauveria bassiana* or the carrier alone[a]

Treatment[b]	Droplet Area (mm^2)	Droplet Width (mm)	Coverage (%)	Number (per cm^2)
Beauveria	0.37 (0.01)	0.55 (0.01)	15.4 (1.1)	42.5 (3.0) a[c]
Carrier	0.38 (0.04)	0.54 (0.03)	19.9 (2.3)	51.9 (2.4) b

[a]Droplets were analyzed in each of two randomly-selected areas of 6.2 cm^2 per card; the droplet data from the two areas was combined and mean droplet area, width and density were determined. In addition, the ratio of total droplet area to total card area (% coverage) was calculated.

[b]Treatments consisted of *B. bassiana* (Mycotech strain GHA) and a carrier control.

[c]Means not followed by the same letter are significantly different from each other according to a t-test ($P=0.05$).

Table 5.3.2 Parameter estimates from logarithmic regressions of the persistence of *Beauveria bassiana* conidia on grass leaves and grasshopper nymphs[a]

Substrate[b]	a[c]	SE(a)	b[c]	SE(b)	r^2
Leaves	4.32	0.21	-2.92	0.26	0.98
Nymphs	3.76	0.26	-2.82	0.30	0.98

[a]The regression equation is: $\log_{10}$ cfu per cm^2 or $\log_{10}$ cfu per nymph = a + b ($\log_{10}$ (days + 1)).

[b]Substrates consisted of grass leaves and nymphs from rangeland.

[c]All intercepts and slopes are significantly different from zero ($P \leq 0.01$).

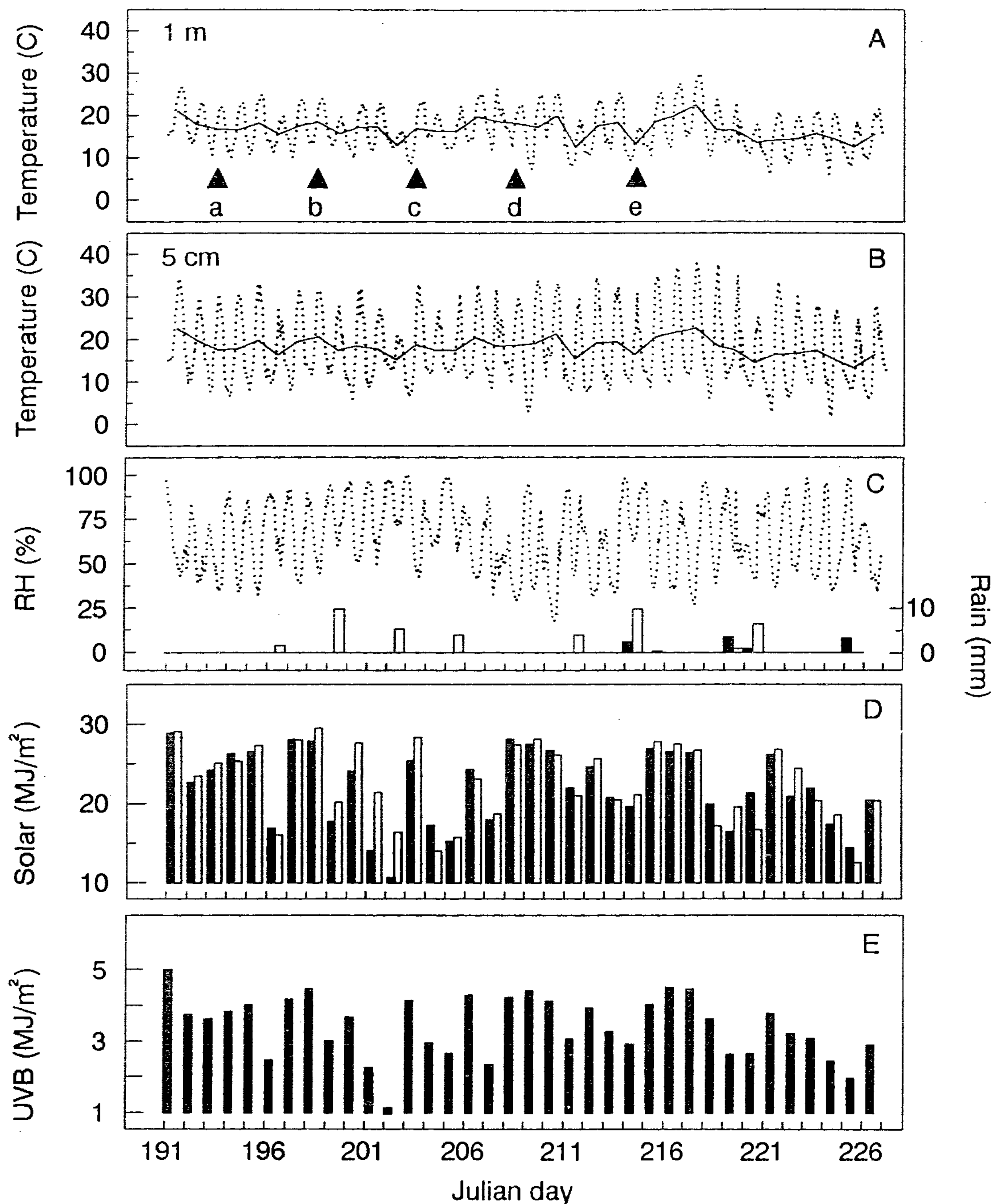


Figure 5.3.1 Weather data from the field experiments (July 10 to August 14, 1995). Hourly (dotted lines) and mean (solid lines) temperatures at heights of 1 m (A) and 5 cm (B), and hourly relative humidity (C) were recorded at Lethbridge. Arrows represent times of conidial application or sample collection (days). a) conidia application in rangeland and collection time 0; b) grasshopper collection time 5; c) grasshopper collection time 10; d) grasshopper collection time 15; e) laboratory inoculation of *Melanoplus sanguinipes* nymphs and placement in field cages. Daily precipitation (histograms) were recorded at the rangeland (open bars) and Lethbridge (solid bars) field sites. Total daily solar radiation (D; 300-2800 nm) was recorded at Lethbridge (solid bars) and Vauxhall (open bars). Daily UV-B radiation (E) was measured at Lethbridge.

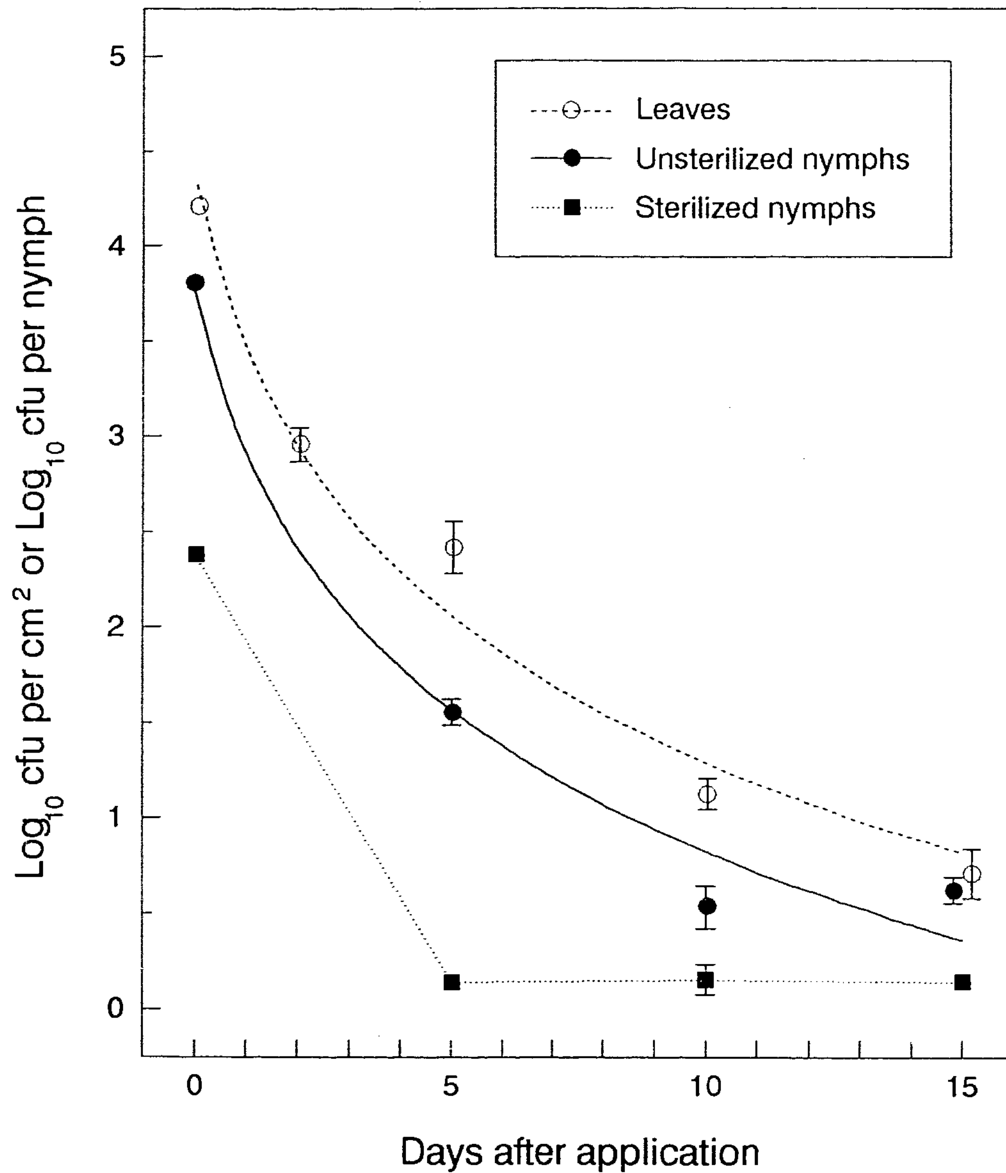


Figure 5.3.2 Populations of *Beauveria bassiana* recovered from grass leaves and grasshopper nymphs collected from field plots sprayed with conidia. From grass leaves, populations were quantified as $\log_{10}$ colony-forming units (cfu) per cm^2 of leaf area. From grasshopper nymphs, populations were quantified as log cfu per nymph; to account for variation in nymph size, weights were standardized to 44.7 mg. Nymphs were either unsterilized or sterilized prior to maceration. Vertical lines represent standard errors of means (n=4). To avoid superimposition of the standard error bars, some means are offset along the x-axis.

1.3×10^2 (0.73×10^2) cfu per nymph were isolated compared to 1.6×10^6 (0.13×10^6) cfu per nymph from unsterilized nymphs, representing > 99.9% reduction in conidial populations due to the sterilization treatment.

Grasshopper populations, species and age composition. At the time of application, mean population densities ranged from 7 to 10 grasshoppers per m^2, and 67% of the individuals (n=605) were third or fourth instars. After 15 days, 31% of the grasshoppers collected (n=835) were adults. The application of *B. bassiana* did not affect ($F=2.3$; df=2,9; $P=0.16$) densities of grasshoppers observed in the sampling frames relative to the other treatments (Figure 5.3.3). The predominant grasshoppers collected were: *M. infantilis* Scudder (67%, n=1509); *Aeropedellus clavatus* (Thomas) (7%, n=153); *M. sanguinipes* (5%, n=111); *Ageneotettix deorum* (Scudder) (4%, n=91); *M. gladstoni* Scudder (4%, n=84); *Philibostroma quadrimaculatum* (Thomas) (3%, n=70); *Phoetaliotes nebrascensis* (Thomas) (3%, n=64); and *M. packardii* Scudder (3%, n=59). There were no conspicuous shifts in species composition of dominant grasshopper taxa collected in sweepnets between the *B. bassiana* and control treatments.

Mycosis in Field and Greenhouse Cages

A total of 6400 grasshoppers were collected from *B. bassiana*-treated and carrier control plots over the 15-day sampling period. In nymphs collected from the carrier control plots, 0 to 7% mycosis was observed after 12 d; this was judged to be negligible and excluded this treatment from all subsequent analyses of mycosis. Disease progressed more rapidly ($F=12.4$-189; df=1,6; $P \leq 0.013$) and attained a higher prevalence of final mycosis ($F=226$, df=1,6; $P<0.001$) in nymphs from *B. bassiana* plots placed in greenhouse than in field cages (Figure 5.3.4). In the greenhouse cages, disease levels deceased ($F=7.5$; df=3,9; $P=0.008$) with sample time; less mycosis ($P \leq 0.02$) was observed in grasshoppers collected 10 and 15 days after conidial application. However, mycosis first occurred 3 to 4 days after placement of the nymphs in cages greenhouse regardless of the collection time.

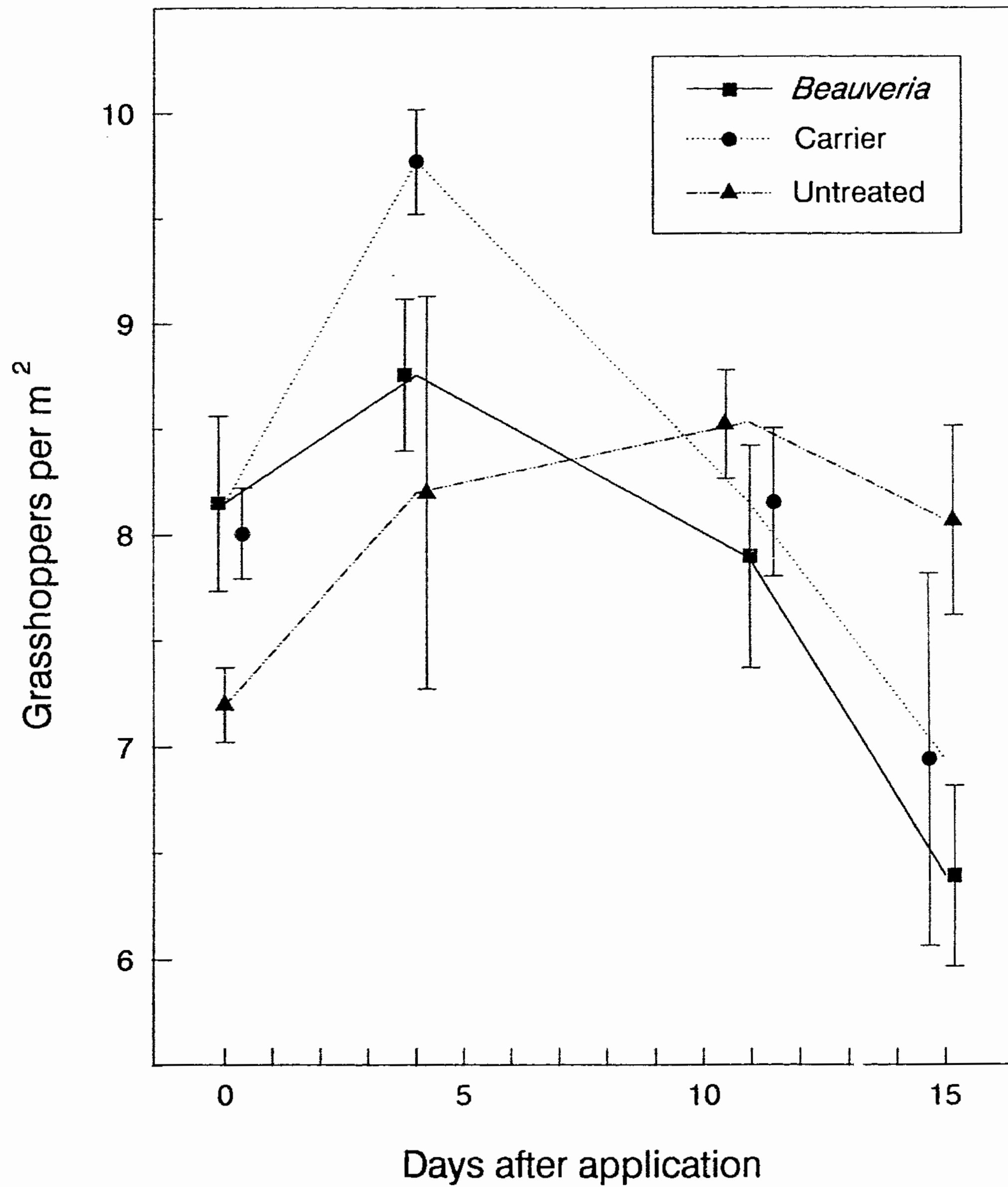


Figure 5.3.3 Grasshopper densities (grasshoppers per m^2) for the *Beauveria bassiana*, carrier control and unsprayed treatments taken prior to the application of *Beauveria bassiana* conidia (time 0), and 2, 11 and 15 d after conidial application. Vertical lines represent standard errors of means (n=4). To avoid superimposition of standard error bars, means are slightly offset along the x-axis.

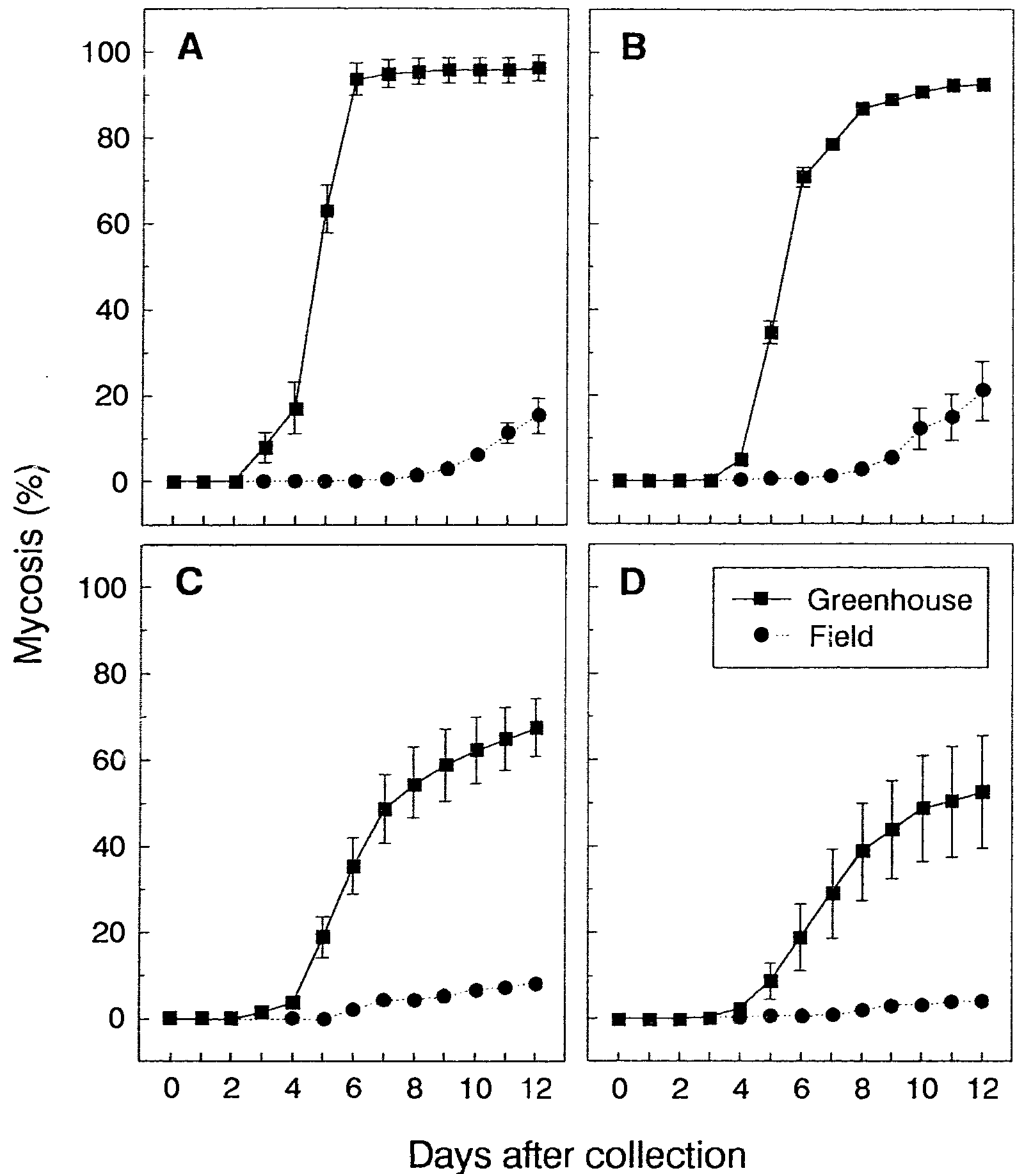


Figure 5.3.4 Disease progress for rangeland grasshoppers collected from *Beauveria bassiana*-treated field plots maintained in greenhouse and field cages. Grasshoppers were collected: A) immediately after conidial application, B) 5 d after application, C) 10 d after application, and D) 15 d after application. Vertical lines represent standard errors of means (n=4).

Disregarding grasshoppers that died within 2 days of placement in cages, mortality not attributed to *B. bassiana* ranged from 1.1 (SE=1.1) to 19.7 % (SE=2.6) by 12 days. Non-*B. bassiana* mortality was not influenced by either sample time ($F=0.66$; $df=1,12$; $P=0.43$) or cage environment ($F=1.6$; $df=1,3$; $P=0.26$).

During the day, temperatures in the field and greenhouse cages differed by ≤ 5°C (Figure 5.3.5); mean maximum temperatures were 33°C (n=26 d). At night temperatures in the greenhouse cages were up to 8°C higher on average than in the field cages; mean minimum temperatures were 19°C in the greenhouse and 9°C in the field. Shades were drawn in the greenhouse to prevent overheating and 74% less solar radiation was recorded in the greenhouse than in the field cages. During the day, relative humidities in the two types of cages differed by less than 19%. However at night, humidities in the greenhouse cages were substantially (24 to 33%) lower than in the field cages.

To determine the degree to which the cage itself influenced environment, conditions of light, temperature and relative humidity in a field cage were compared to that outside the cage over a 8 days period; sensors outside of the cage were situated at the same height and orientation as in the cage. Temperature (± 3°C) and relative humidity (± 5%) were similar inside and outside of the cage. However, the cage mesh caused considerable shading, and 57% less solar radiation was recorded in the cage.

Effect of Temperature and Sunlight

Rangeland and Lethbridge sites. Grasshoppers were observed to congregate in areas of high sunlight (basking behavior) in the exposed and UVB-protected cages. Congregation of grasshoppers was not observed in the shaded cages. The rate of disease development ($F=146$ and101; $df=2,9$; $P<0.001$) and the prevalence of final mycosis ($F=109$-3340; $df=2,9$; $P<0.001$) differed among the three cage environments (Figure 5.3.6); disease development was more rapid ($P<0.05$) and the prevalence of final mycosis (83-89%) was higher ($P<0.001$) in the shaded than in the exposed (0-15%) and UVB-

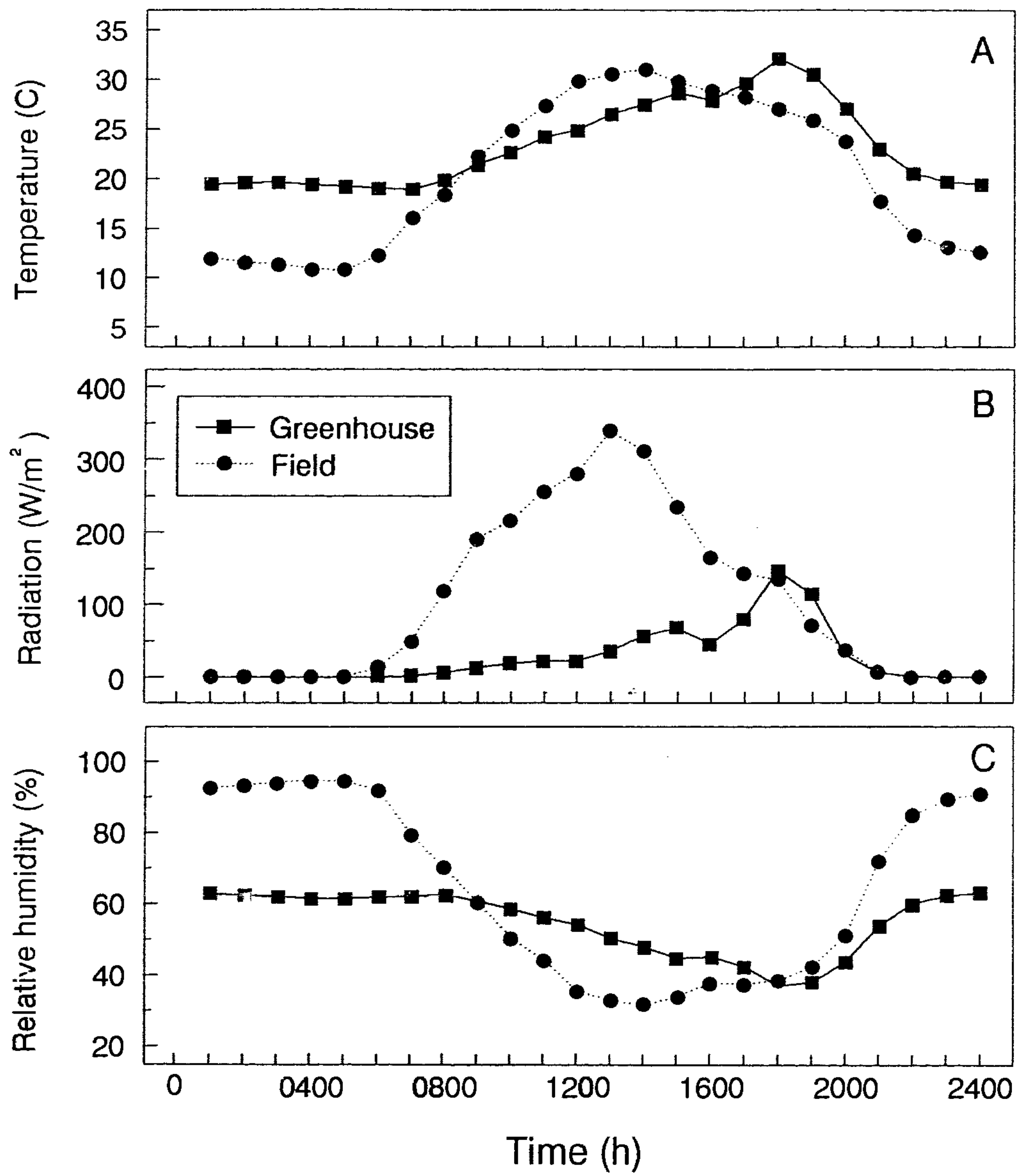


Figure 5.3.5 Mean hourly conditions of temperature, solar radiation and relative humidity in cages maintained in the field and greenhouse environments.

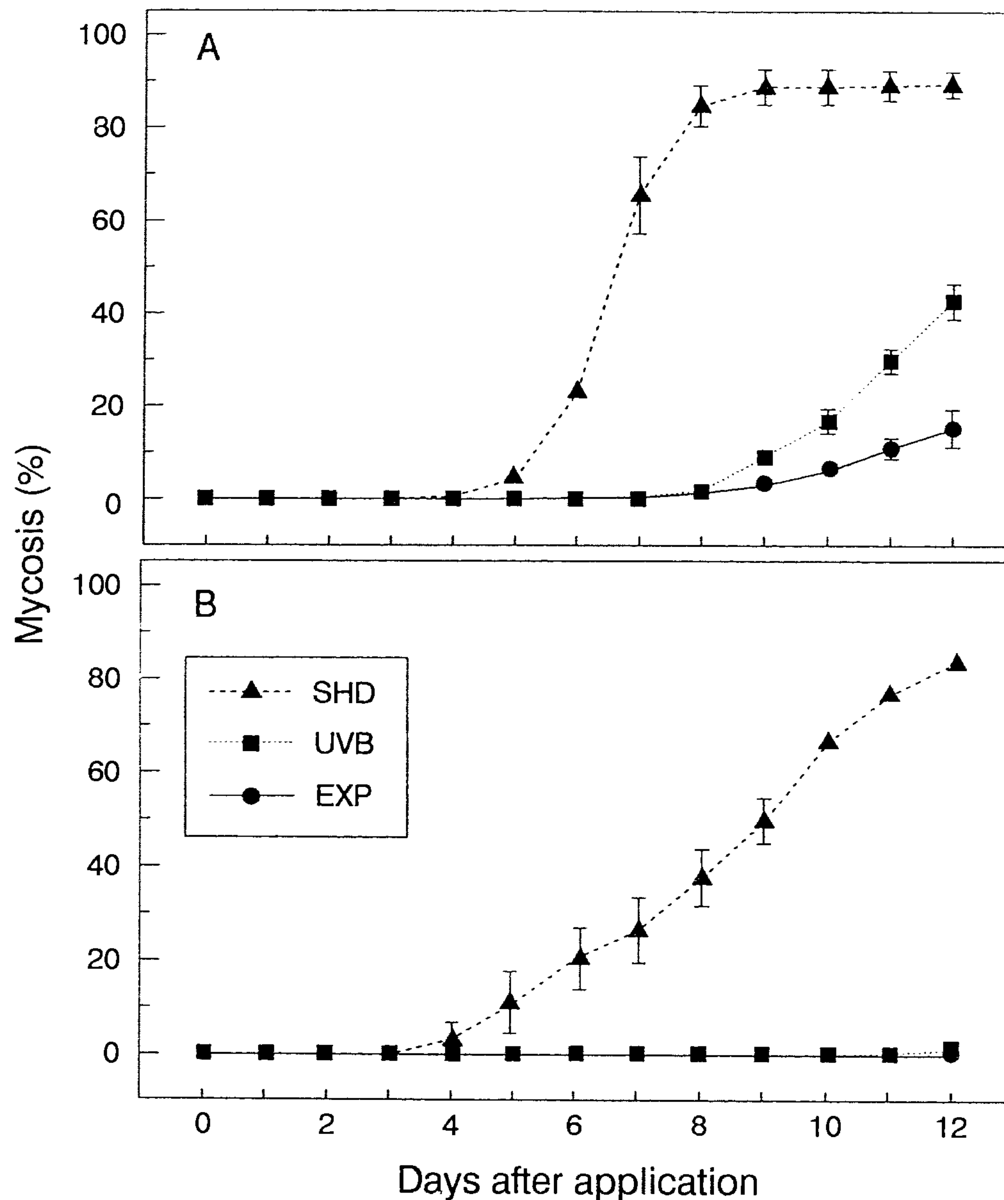


Figure 5.3.6 Disease progress for grasshoppers maintained in field cages that were shaded (SHD), exposed to UVB-filtered sunlight (UVB), or to full spectrum sunlight (EXP). A) Rangeland grasshoppers collected from field plots sprayed with *Beauveria bassiana* conidia; and B) *Melanoplus sanguinipes* nymphs inoculated with conidia. Vertical lines represent standard errors of means (n = 4).

protected (1-43%) field cages. At the rangeland (Figure 5.3.6A) but not the Lethbridge (Figure 5.3.6B) site, substantial mycosis (≥ 15%) was observed in grasshoppers placed in the UVB-protected and exposed field cages. Mycosis was first observed in these cages 8 days after inoculation (Figure 5.3.6) during a relatively cool overcast period (Figures 5.3.7A and 5.3.7C). At the end of the experimental period, more ($P<0.001$) grasshoppers had died of mycosis in the UVB-protected (42.5 %, SE=3.9) than in the exposed (15.2 %, SE=4.0) cages. The prevalence of "other mortality" ranged from 10.0 (SE=2.8) to 17.7 (SE=7.8) % at the rangeland site, and from 1.5 (SE=0.06) to 10.7 (SE=1.3) % at the Lethbridge site.

Temperatures, relative humidities, and solar radiation (> 400 nm) were similar in the exposed and UVB-protected cages (Figure 5.3.8 and 5.3.9). Conditions were generally hot and sunny; temperatures reached or exceeded 35°C in the exposed and UVB-protected cages for relatively short periods of time during 5 days in each trial (Figure 5.3.7). In contrast to exposed and UVB-protected cages, relative humidities were up to 17% higher, temperatures were up to 6°C cooler, and visible light was reduced by 59 to 80% in the shaded cages during the day.

Conidial persistence. The environment in which the wheat plants were maintained significantly affected ($F=6.8\text{-}21.2$; df=1-2, 6-8; $P\leq0.040$) conidial survival. At both field sites, more ($P\leq0.05$) cfu were isolated from wheat leaves placed under the UVB-screen than those exposed to full spectrum sunlight (Table 5.3.3). At Lethbridge, the persistence of conidia on shaded wheat leaves was also measured. There was no difference ($P>0.05$) in numbers of cfu isolated from the shaded and UVB-protected environments 2 and 5 days after conidial application; at the 10 day sample time, most of the leaves under the UVB-film had been consumed by resident grasshoppers.

Table 5.3.3 Populations of *Beauveria bassiana* conidia on wheat leaves[a]

Environment[b]	Days after application 0	2	5	10
Rangeland site				
Shaded	---	---	---	---
UVB	4.17 (0.09)	4.23 (0.06) a[c]	3.46 (0.10) a	1.95 (0.30) a
Exposed	4.41 (0.16)	3.56 (0.08) b	2.88 (0.09) b	0.42 (0.42) b
Lethbridge site				
Shaded	5.24 (0.03)	4.46 (0.07) a	3.42 (0.12) a	2.40 (0.23)
UVB	5.32 (0.07)	4.10 (0.17) a	2.99 (0.07) a	---[d]
Exposed	5.37 (0.07)	3.44 (0.10) b[e]	1.83 (0.30) b	0.0

[a]Conidia were recovered on a semi-selective oatmeal-dodine agar and populations were calculated as $\log_{10}$ cfu per cm^2 of leaf tissue.

[b]Wheat plants were placed under black plastic (shaded), placed under a UVB absorbing film (UVB), or exposed to full spectrum solar radiation (exposed).

[c]Means at each site and within each column that are not followed by the same letter are significantly different from each other according to Tukey's studentized range test ($\alpha = 0.05$).

[d]Wheat leaves were extensively clipped by resident grasshoppers.

[e]Wheat plants were transferred to cages to prevent foraging by indigenous grasshoppers.

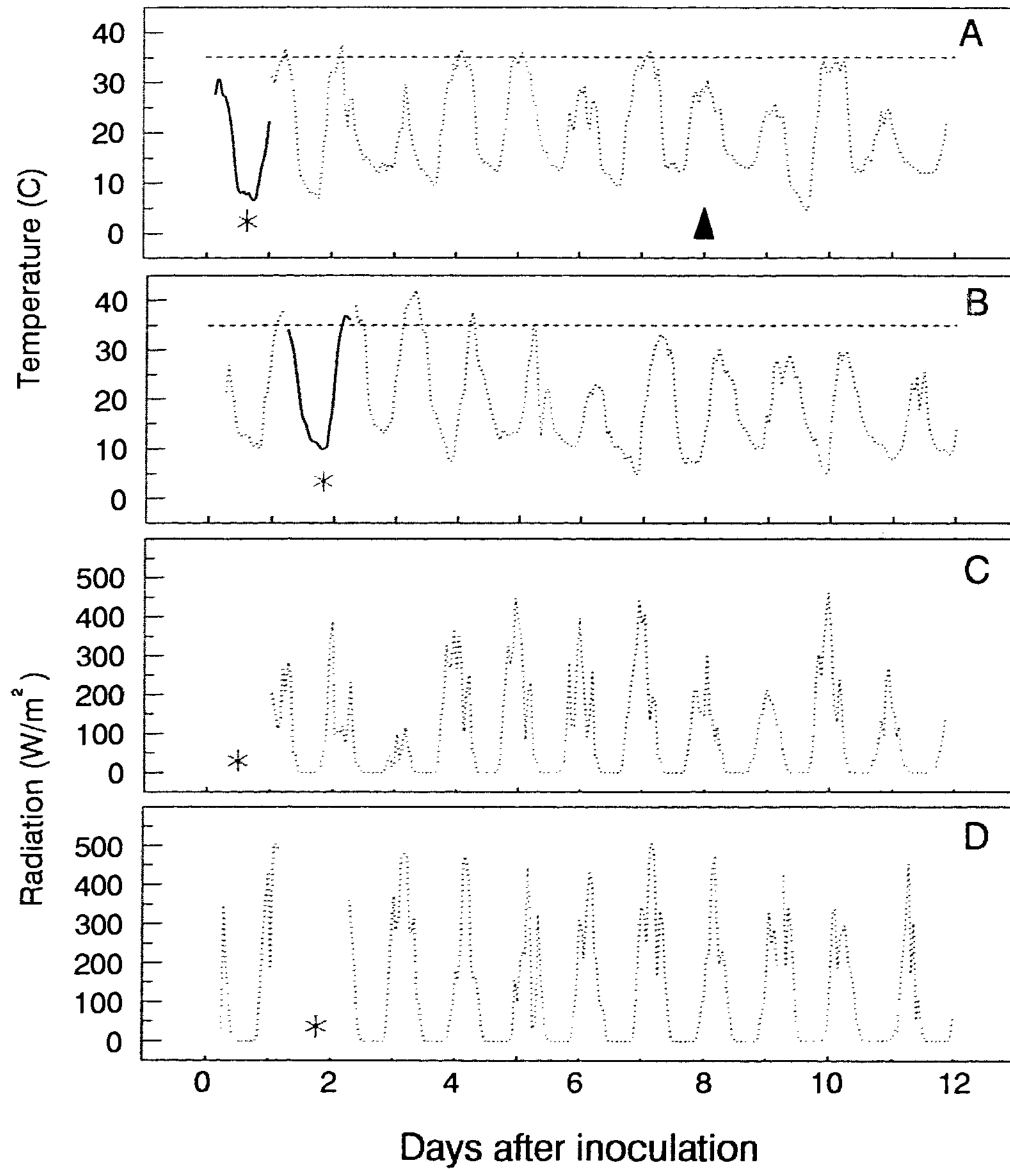


Figure 5.3.7 Conditions of temperature and light by hour in exposed cages at the rangeland (A & C) and Lethbridge (B & D) field sites. Asterisks represent missing data and solid lines are temperature data recorded at a height of 5 cm at Lethbridge. The arrow indicates the time at which mycosis was first detected in the exposed and UVB-protected cages at the rangeland site, and the dashed line indicates 35°C.

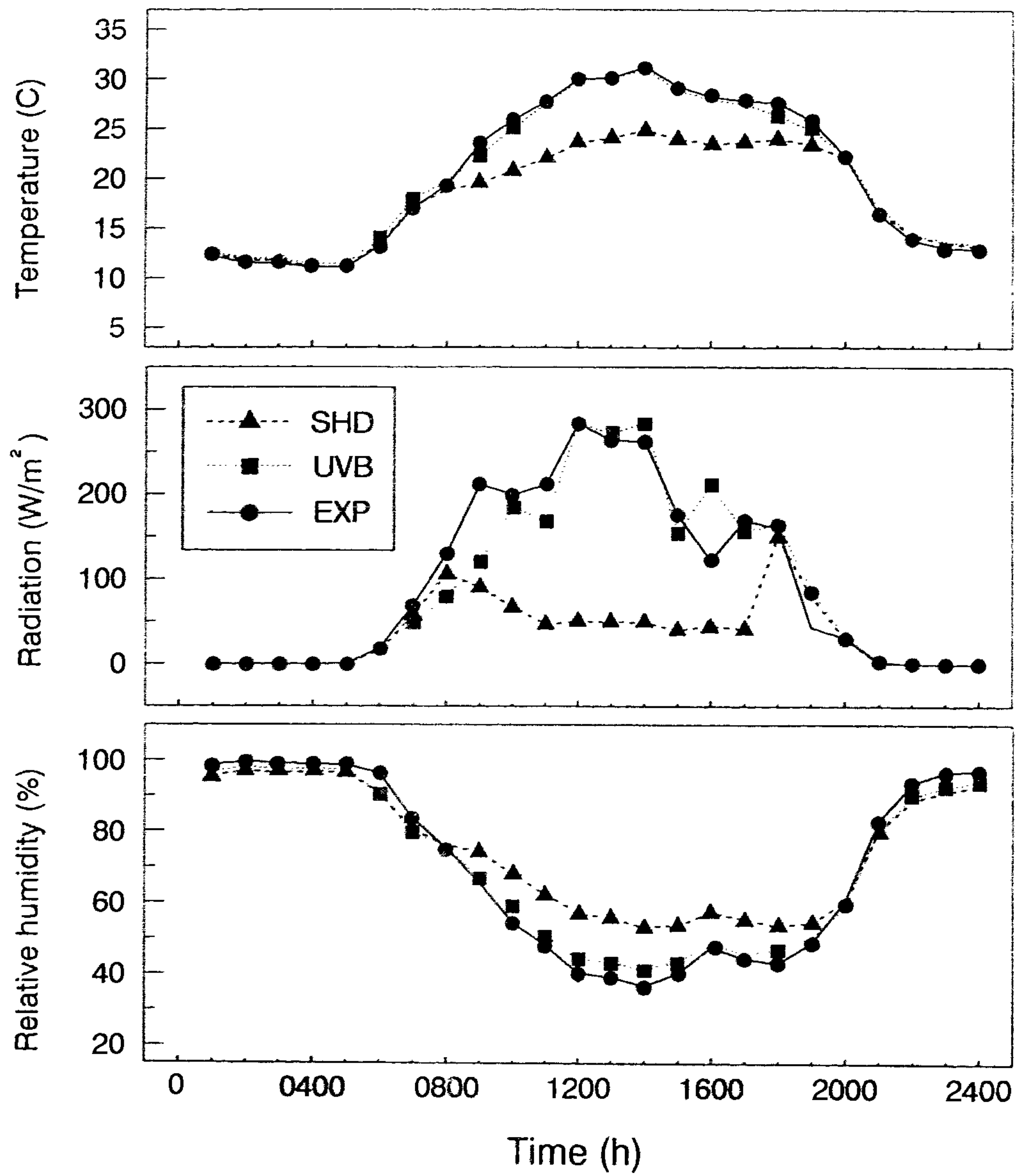


Figure 5.3.8 Mean hourly conditions of temperature, solar radiation and relative humidity in cages at the rangeland field site. Cages were shaded (SHD), exposed to UVB-filtered sunlight (UVB), or to full spectrum sunlight (EXP).

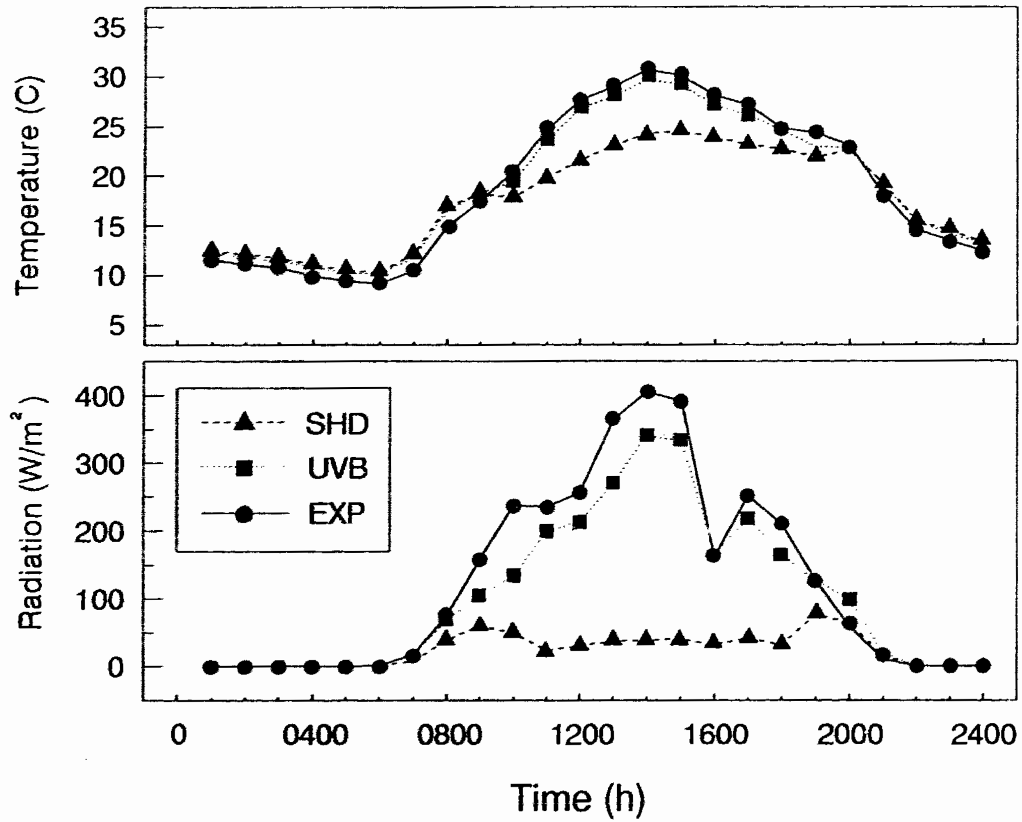


Figure 5.3.9 Mean hourly conditions of temperature and solar radiation in cages at the Lethbridge field site. Cages were shaded (SHD), exposed to full spectrum sunlight (EXP) or to UVB-filtered sunlight (UVB).

Discussion

Field Efficacy and Environment

Despite the deposition of substantial quantities of conidia onto grasshoppers, no reductions were detected in field populations. While some disease was observed in grasshoppers maintained in cages adjacent to the field plots, substantially higher levels of mycosis were observed in grasshoppers maintained in greenhouse cages. The prevalence of mycosis in greenhouse cages decreased with sample time but the onset of disease was always 3 to 4 day after the placement of nymphs in cages, regardless of when the grasshoppers were collected; this observation agrees with earlier reports (Johnson and Goettel, 1993; section 4.3). Predisposition of insects to infection by entomopathogens has been reported and grasshoppers were exposed to a variety of factors that may have caused stress (e.g., collection in sweepnets, confinement in cages and/or altered diet). However, with exception of cage environment, they were treated similarly suggesting that the differences observed in susceptibility of grasshoppers in the two cage types were due to environmental conditions, and not due to stress independent of environment. Although our results indicate that a conducive environment is essential for disease development, the mechanism(s) by which environment influences mycosis was uncertain.

Temperature and Sunlight. Conditions of temperature, light exposure and relative humidity differed between the field and greenhouse cage environments. Relative humidity has a negligible effect on infection of grasshoppers by *B. bassiana* (Marcandier and Khachatourians, 1987), and mean hourly temperatures were only slightly cooler in the greenhouse for most of the day. Nighttime temperatures were higher (≈8°C) and light levels were substantially reduced (≈74%) in the greenhouse cages, and these factors could have negatively affected disease development in grasshoppers maintained in the field cages. While no reductions were detected in field populations of acridids, some disease (15-21%) was observed in grasshoppers collected within 5 days of conidial application and maintained

in cages adjacent to the field plots. The mesh covering the field cages had a minimal affect on temperature and relative humidity but caused considerable shading (≈55%) further implicating light as a factor influencing the susceptibility of grasshoppers to *B. bassiana*.

Grasshopper Thermoregulation and Conidial Survival. Grasshoppers elevate their body temperature higher than ambient by directly or indirectly intercepting solar radiation (Chappell and Whitman, 1990; Heinrich, 1993). Given the opportunity, grasshoppers optimized their body temperature (≈38 to 40°C) by basking, and mycosis (*B. bassiana*) was reduced by 46% in grasshoppers that were allowed to bask for only 1 h per day (section 5.2). Levels of light were 61 to 80% less, daytime temperatures were up to 6°C cooler, and mycosis was substantially greater in the shaded (>80%) than in unshaded field cages (0-15%). Temperatures in the unshaded cages reached or exceeded 35°C in only 5 days per trial, and then for relatively short periods of time (generally less than 4 h per day). Since exposures to 35°C for ≤4 h per day had no effect on mycosis (section 5.2), cage temperatures alone cannot explain the differences in disease between the two cage environments. Furthermore, basking behavior (e.g., congregation in areas of intense sunlight) was only observed in grasshoppers in the unshaded cages. The detrimental effects of grasshopper thermoregulation on mycosis is consistent with observations of successful suppression of field populations of grasshoppers with *B. bassiana* during cool overcast periods (Johnson and Goettel, 1993) but not hot sunny periods (section 4.3).

Exposure to solar radiation negatively affects the survival of *B. bassiana* conidia in epigeal habitats (sections 3.2 and 3.3), and conidia deposited on grass leaves were rapidly killed. In section 4.3, it was observed that the persistence of *B. bassiana* associated with field-collected grasshoppers was similar to that on leaves, an observation substantiated in the present study. In section 4.3, it was not possible to distinguish between conidia on the surface of nymphs and blastospores and hyphae in the hemocoel. In the present study, populations of *B. bassiana* from surface-sterilized (internal cfu) and unsterilized nymphs

(internal and external cfu) were compared at various times after conidial application. From surface-sterilized nymphs, *B. bassiana* was isolated in substantial numbers only from grasshoppers collected immediately after conidial application. Conidia ingested by grasshoppers survive passage through the alimentary tract (Inglis *et al.* 1996a), and *B. bassiana* cfu isolated from surface-sterilized nymphs immediately following application likely represent conidia deposited onto foliage that were ingested by nymphs. The low numbers of conidia isolated from surface-sterilized grasshoppers at subsequent collection times indicates that the majority of *B. bassiana* conidia deposited on grasshoppers remain exposed on the surface of the integument, and if infection occurs, the fungus does not proliferate in the hemocoel.

To determine the relative importance of grasshopper thermoregulation and the deactivation of conidia by UVB radiation on mycosis, disease development and conidial survival in UVB-protected and shaded environments was compared. Conidial survival was similar between the two environments, but substantially more disease was observed in the shaded (>80%) than in the UVB-protected (1-43%) cages at both sites. These observations indicate that the indirect effects of temperature and light on the susceptibility of grasshoppers to *B. bassiana* (i.e., behavioral thermoregulation) had a greater influence on disease development than did the rapid deactivation of conidia by UVB radiation.

While the ability of grasshoppers to elevate their body temperature influenced mycosis, evidence also suggested that conidial survival had an impact on disease. Conditions of visible light were similar between the UVB-protected and unshaded environments (i.e., grasshoppers had similar opportunity to thermoregulate), but conidial survival was enhanced in the former. At the rangeland site, mycosis was substantially higher in the UVB-protected (43%) than in the unshaded (15%) cages, and disease was first observed in both cage types, 8 days after conidial application during a relatively cool, overcast period. These observations suggest that by prolonging conidial survival until

conditions are conducive for disease development (i.e., during a period when grasshoppers were incapable of thermoregulation), the field efficacy of *B. bassiana* may be enhanced. Since conidia formulated in sunscreens survive longer than unprotected conidia in field settings (section 3.3), it may be possible to use sunscreen formulations to study the relationship between conidial deactivation and disease development.

5.4 Conclusions

An understanding of the factors that limit the development of epizootics is imperative if *B. bassiana* is to be used to manage insects. Grasshoppers that were permitted to bask elevated their body temperatures to a point that was optimal for feeding and development. Basking for as little as 1 h per day reduced the prevalence of mycosis by 46%. Furthermore, grasshoppers exhibited a "behavioral fever" response to infection. By studying conidial survival and disease development in different environments, it was demonstrated that solar radiation and temperature, and not pathogen virulence or host targeting, limited the efficacy of *B. bassiana* against acridids in field settings. The low levels of disease that were observed in unshaded and UVB protected cages is consistent with the ability of grasshoppers to thermoregulate. However, conidial deactivation by UVB radiation appeared to influence disease development in one of two trials. The relationship between conidial survival and the ability of grasshoppers to elevate their body temperature is necessary, as is determination of the timing and likelihood of weather conditions that may affect these variables. Furthermore, the profound effect that the cage environment has on mycosis emphasizes that caged insects not be used for assessing the field efficacy of entomopathogenic Hyphomycetes against grasshoppers (see also section 4.3), and possibly against many other insects including non-target arthropods. Ultimately the success of *B. bassiana* against acridids in field settings will depend on the development of biorational strategies that overcome the light and temperature constraints on *B. bassiana*.

CHAPTER 6

GENERAL DISCUSSION

6.1 INTRODUCTION

The efficacy of *B. bassiana* against acridids in field environments has yielded inconsistent results. Although substantial reductions in acridid populations have been observed in some trials (e.g., Johnson and Goettel, 1993), in the vast majority of field experiments a minimal impact on field populations has been detected (e.g., Schaefer, 1936; Johnson *et al.*, 1992; Lobo Lima *et al.*, 1992; Jaronski and Goettel, 1996; N. Foster, pers. comm.; sections 4.3 and 5.3). The primary objective of my research was to identify factors that limit the efficacy of *B. bassiana* in field settings. If *B. bassiana* is going to be developed as a microbial control agent, it is essential that the factors that restrict the onset and development of epizootics be understood. Once identified, it may be possible to overcome constraints through the biorational selection and deployment of *B. bassiana*.

6.2 FINDINGS AND IMPLICATIONS

Most of the research conducted on *B. bassiana* against insects has focused on the interaction between the host and the fungus, and has largely ignored the interaction between these variables and the environment (Figure 1.1.2). My research demonstrated that sunlight, and in particular the UVB portion of the solar spectrum is a major factor limiting the survival of *B. bassiana* conidia on leaves. Other researchers have also demonstrated that exposure to solar radiation kills conidia of *B. bassiana* and propagules of other hyaline entompathogenic Hyphomycetes (Zimmerman, 1982; Daoust and Pereira, 1986a; Fargues *et al.*, 1988). However in contrast to previous reports, my research measured reductions in conidial populations over time in a variety of natural field settings. Monitoring viable conidial populations in these habitats allowed me to determine the relative importance of temperature, relative humidity, precipitation and solar radiation. In addition, I was also able to use this method to study the fate of conidia on grasshoppers in field

settings. My findings indicate that most of the conidia deposited on grasshoppers remain exposed on their integument and are killed at the same rate as *B. bassiana* conidia on leaves (logarithmically). If infection occurs, it does so at a relatively low rates, and the fungus is unable to proliferate in the hemocoel of grasshoppers under sunny and hot conditions.

Grasshoppers elevate their body temperatures directly or indirectly by intercepting solar radiation (Chappell and Whitman, 1990; Heinrich, 1993). My research demonstrated that given the opportunity, grasshoppers optimize their body temperature (38 to 40°C), and basking has a detrimental effect on mycosis; in grasshoppers allowed to bask for as little as 1 h per day for 10 days, mycosis was reduced by ≈ 50 %. The detrimental effects of elevated grasshopper body temperature on disease incited by *E. grylii* (Carruthers *et al.*, 1992) and *Nosema acridophagus* (Boorstein and Ewald, 1987) has previously been demonstrated in the laboratory, but my research provided additional evidence for this phenomenon in a field setting utilizing different cage environments. Many reports of field efficacy of *B. bassiana* have been based on disease observed in confined grasshoppers. My research clearly indicates that mycosis in caged grasshoppers is not necessarily correlated with field efficacy. Cages may provide a microclimate that is very different from that of the natural environment and extreme care should be exercised in extrapolating from cage data. Rather than using cage environments as an indicator of field efficacy, I demonstrated that they can be used to provide information on the environment-host-pathogen interaction. However, it must be stressed that findings based on cage efficacy must ultimately be tested against acridids in natural habitats.

While my research indicated that the direct (conidial persistence) and indirect (grasshopper thermoregulation) effects of solar radiation are the important factors limiting the efficacy of *B. bassiana* in field environments, the relative importance of these two factors on disease are uncertain. Using field cage environments (exposed to full spectrum solar radiation, protected from UVB radiation and shaded from direct sunlight) I attempted

to elucidate how these two factors interact. The ability of grasshoppers to thermoregulate would be similar in the exposed and UVB-protected environments, but grasshoppers in the shaded cages would be incapable of elevating their body temperatures substantially higher than ambient. Furthermore, conidial survival was similar in the shaded and UVB-protected but less in the exposed environment. Results from this experiment indicated that while conidial persistence is important, the ability of grasshoppers to elevate their body temperatures limits disease development in field settings. In one of two trials substantially more disease was observed in the UVB-protected than the exposed cage environments and the onset of disease occurred during a relatively cool overcast period. This observation supports the hypothesis that by increasing the time that conidial populations exceed the threshold (e.g., until conditions conducive to disease development) will enhance disease. A threshold of inoculum is required to incite disease in insects (Figure 6.2.1 A) but inoculum thresholds are dynamic, and during periods where grasshoppers can optimize their body temperatures by basking, the threshold may be sufficiently elevated that disease is prevented (Figure 6.2.1 B). However during cool overcast periods when grasshoppers were incapable of thermoregulating, the inoculum threshold may have dropped sufficiently to permit disease initiation.

6.3 STRATEGIES TO OVERCOME CONSTRAINTS

Once the factors that limit the efficacy of *B. bassiana* in field environments have been identified, it may be possible to reduce the detrimental impact of these parameters on disease development. Strategies that may be used to accomplish this include: selection of isolates; use of biorational formulations; utilization of novel targeting strategies; and the ability to predict the efficacy of *B. bassiana* under a variety of conditions.

Selection of isolates. There is considerable phenotypic and genotypic variability among isolates of entomopathogenic hyphomycetes. Differential responses of *B. bassiana* isolates to temperature, UVB radiation and relative humidity have been demonstrated

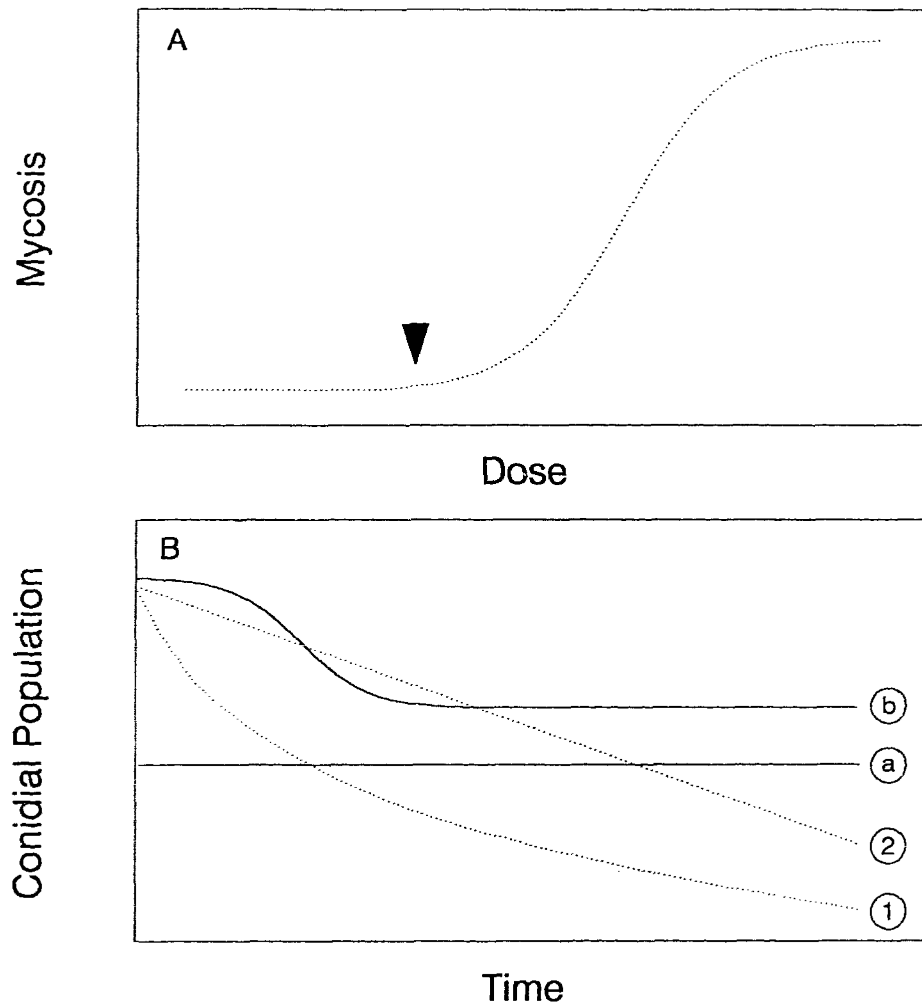


Figure 6.2.1 A) A threshold of inoculum (arrow) is required to incite disease in insects. B) The time that conidial populations exceed the inoculum threshold may contribute to disease development. Line "a" indicates a situation where the inoculum threshold is static over time. Although *Beauveria bassiana* populations in both treatments (represented by dotted lines) exceed the inoculum threshold, more disease would be expected for line "2" since the time in which populations exceed the threshold is prolonged. Inoculum thresholds are dynamic influenced by various aspects of the disease model and line "b" represents a situation where the inoculum threshold changes non-linearly over time. When grasshoppers are able to bask the inoculum threshold is elevated, but during cool-overcast periods when grasshoppers are unable to thermoregulate, the threshold decreases. In this scenario, the population represented by line "1" never exceeds the inoculum threshold and therefore no disease occurs. By prolonging the conidial survival (i.e. by using sunscreens) until conditions prohibit thermoregulation by grasshoppers, populations exceed the inoculum threshold for a period of time and disease is initiated.

(Ouedraogo, 1993; Hywel-Jones and Gillespie, 1990; M.S. Goettel, pers. comm.); this is not surprising given the genetic diversity contained within this group (St. Leger *et al.*, 1992). It may be possible to use *in vitro* selection methods (e.g., cardinal growth temperatures) to identify isolates that can overcome the effects of elevated grasshopper body temperatures. Ouedraogo (1993) observed that several isolates of *B. bassiana* from Burkina Faso soils germinated at higher temperatures than did isolates from North America. However, an understanding of the mechanism by which body temperatures impacts on *B. bassiana* may increase the likelihood for success by isolate selection. Evidence suggested that the direct effects of temperature on *B. bassiana* growth but not germination may explain its poor efficacy at high temperatures (section 5.2). Although there is very little known about the interaction between temperature and the immune response of invertebrates, it seems probable that temperature affects *B. bassiana* indirectly by enhancing immunity. Whether temperature impacts directly or indirectly on *B. bassiana* will determine what criteria should be used in the selection of an isolate (e.g., ability to grow at high temperature or the ability to overcome the defense response at elevated temperatures). While *in vitro* selection methods may identify prospective isolates, the efficacy of isolates should be further tested against insects in bioassays that incorporate as many pertinent environmental parameters as possible. inoculation techniques and the environmental conditions chosen should mimic as much as possible the natural situation and more specifically, conditions at the level of the host microhabitat.

While it may be possible to select individual genotypes to overcome the temperature constraints, a more successful strategy likely will involve the use of pathogen "cocktails". This may include isolates within the same species or from different species. An example of how entomopathogen cocktails may be used is demonstrated by Inglis *et al.* (1996c). *Metarhizium flavoviride* possesses a relatively high cardinal temperature (≈40°C), and in contrast to *B. bassiana*, basking by grasshoppers has a minimal impact on mortality caused

by *M. flavoviride*. Temperatures can be quite cool in prairie agroecosystems, particularly at night, and cool temperatures substantially reduce the activity of *M. flavoviride* (Inglis et al., 1996c). By co-applying both pathogens, the prevalence of mortality was greater for the combination treatment than for *M. flavoviride* alone in a simulated hot temperature environment, and equal to *B. bassiana* in a simulated cool temperature environment. While the simulation used by Inglis *et al.* (1996c) was relatively simple, it demonstrates the potential value of utilizing isolates with different characteristics in combination.

Formulation. While differential susceptibility of isolates of entomopathogenic fungi (e.g., with hyaline propagules) to UVB radiation have been demonstrated (Fargues *et al.*, 1996), it seems unlikely that natural tolerances alone will be sufficient to increase efficacy. The use of formulation strategies (in combination with isolate selection) may be used to enhance survival. I demonstrated that the persistence of *B. bassiana* conidia formulated in a sunlight blocker (clay) and in a UVB-absorbing compound (Tinopal) was enhanced in a field setting (section 3.3). All of the seven formulations tested protected conidia from artificial UVB radiation in the laboratory but only two were effective under field conditions. While I demonstrated that conidial survival could be increased in the field, the use of superior formulations strategies, such as encapsulation (McGuire and Sasha, 1992), may be necessary to increase persistence sufficiently to enhance the onset and development of epizootics.

Formulation of entomopathogens as baits is receiving increasing attention, particularly with pathogens (e.g., *Nosema locustae*, *Steinernema feltiae*, and entomopox virus) that are active *per os* (Henry, 1971; Capinera and Hibbard, 1987; Johnson, 1989a; McGuire *et al.*, 1991). Encapsulation of conidia can increase survival in epigeal habitats and formulation of *B. bassiana* in baits may be used to protect conidia from sunlight. Although infection of insects by *B. bassiana* usually occurs through the external integument, grasshopper nymphs were highly susceptible to infection by conidia formulated

on bait substrates (Inglis *et al.*, 1996a). Evidence indicated that grasshoppers became surface-contaminated with conidia during handling and ingestion of the bait, and future development of bait formulations should focus on baits that promote tactile handling by the grasshoppers thereby enhancing transfer of conidia to the integument surface.

Formulation need not be restricted to increasing the persistence of conidia. Formulation may also be used to modify the behavior and/or act as a physiological stressor. A variety of factors (e.g., insecticides, crowding, sublethal infection) have been shown to predispose insects to infection. Although this possibility has not been investigated, it may be possible to use stressors, behavioral modifiers, or both, to overcome the detrimental effects of thermoregulation by grasshoppers on *B. bassiana*.

Novel targeting. The preferred breeding habitats of most economically-important grasshopper species are field margins and roadsides (Criddle 1918, 1933; Johnson, 1989b). *Beauveria bassiana* is a soil-borne hyphomycete, and conidia applied over vegetation typically observed in roadsides, penetrated the canopy and exhibited good persistence over winter (less than one order of magnitude decrease in populations) in an Alberta soil (Inglis *et al.*, 1996b). High densities of conidia in sterile sand ($\approx 10^7$ conidia per g) caused extensive mortality in ovipositing females, associated males, and subsequently, in emergent grasshopper nymphs (Inglis *et al.*, 1995). Recently, an LD_{50} response of approximately 10^4 conidia per g of soil (dry weight) was observed in females allowed to oviposit into a sterile sandy-loam soil (unpublished). Soil texture, water availability, pH, exposure to agrochemicals and the presence of a microflora can influence the efficacy of entomopathogenic hyphomycetes (McCoy *et al.*, 1992). While, soil texture had a minimal effect on *B. bassiana,* the soil microflora impacted negatively on *B. bassiana* against ovipositing grasshoppers (data not presented). The soil microflora has previously been shown to decrease the efficacy of *B. bassiana* against soil-dwelling insects (Groden and Lockwood, 1991; Pereira *et al.*, 1993). Whether soil inoculation of oviposition sites will be

an efficacious method of applying *B. bassiana* for the integrated management of grasshoppers will depend on the degree to which a number of potentially detrimental biotic and abiotic factors influence the host-pathogen interaction.

Predictive models. The ability to predict the efficacy of *B. bassiana* under various environmental conditions may be possible as the factors that contribute to the development of epizootics become better understood. Using bioassay methods in which single or multiple factors are varied, it may be possible to develop models that will facilitate the deployment of entomopathogens under suitable conditions. For example, the effect of high temperatures on mycosis of grasshoppers are easily measured (section 5.2). Using Kemp's (1986) empirical model (relationship between air temperature and body temperature) or more sophisticated models (sunlight, wind, insect mass, orientation and height in the canopy) to predict grasshopper body temperatures, it may be possible to develop a model that predicts the success of *B. bassiana* (Campbell, 1977). This type of model is presently being developed at AAFC Research Centre, Lethbridge (D. Lactin, pers. comm) and it may allow us to deploy *B. bassiana* when the likelihood of success is high (e.g., based on weather forecasts). As our knowledge of the grasshopper-pathogen-environment interaction improves, the model can be altered accordingly.

6.4 SUGGESTIONS FOR FUTURE RESEARCH

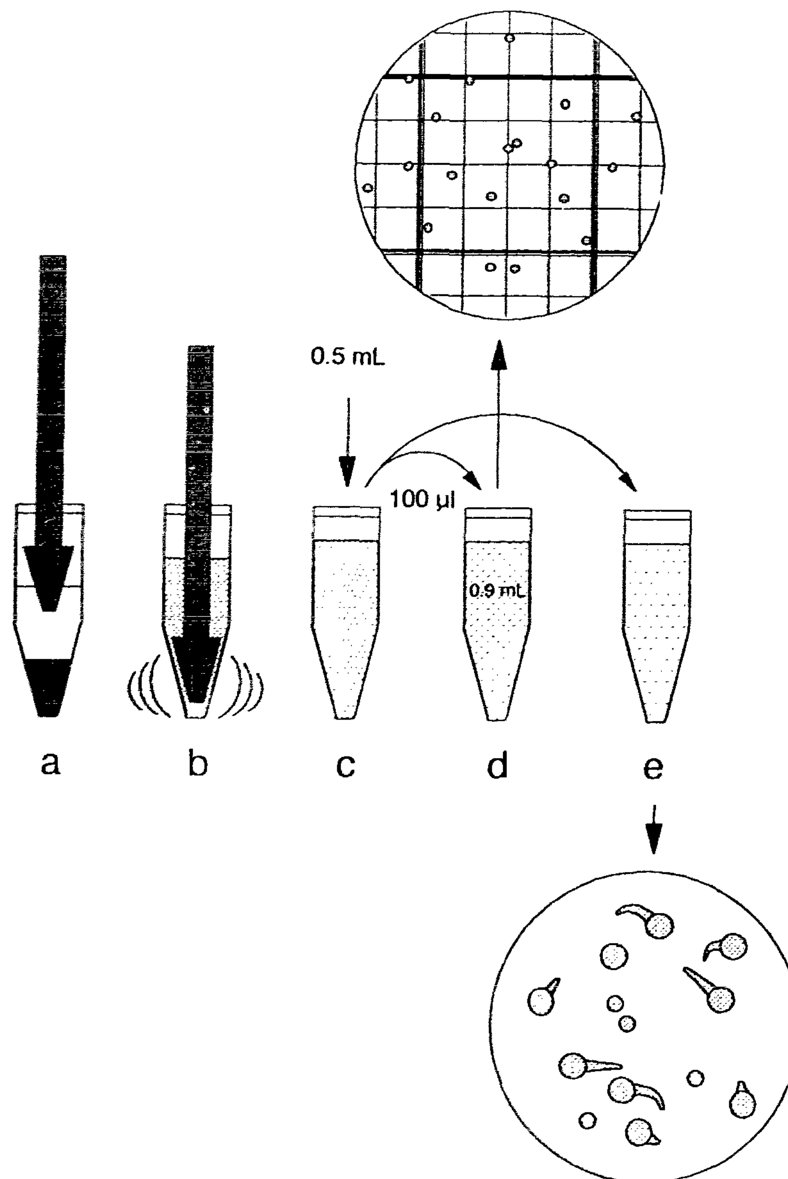
In light of the findings from my research, I would like to make the following suggestions for future research with *B. bassiana* against acridids:

1) Repeated and large scale field trials are required to better understand the interaction between environment, the pathogen and the host, and how we can influence this interaction to increase the efficacy of *B. bassiana* as a microbial control agent against grasshoppers and other insect pests.

2) Development of methods to overcome constraints of temperature on entomopathogenic fungi. These may include: selection of isolates possessing higher

and lower cardinal temperatures; deployment of pathogens in combination (e.g., cocktails); and the use of grasshopper physiological stressors and/or behavioral modifiers to reduce the detrimental effects of basking by acridids on *B. bassiana*.

3) The development of predictive models that will facilitate the efficacious deployment of entomopathogens and the identification of factors important in the environment-pathogen-host-man interaction. Grasshopper thermoregulation models are presently being developed and these can be adapted to include the impact of *B. bassiana*; models should include the effects of canopy architecture and microclimate.

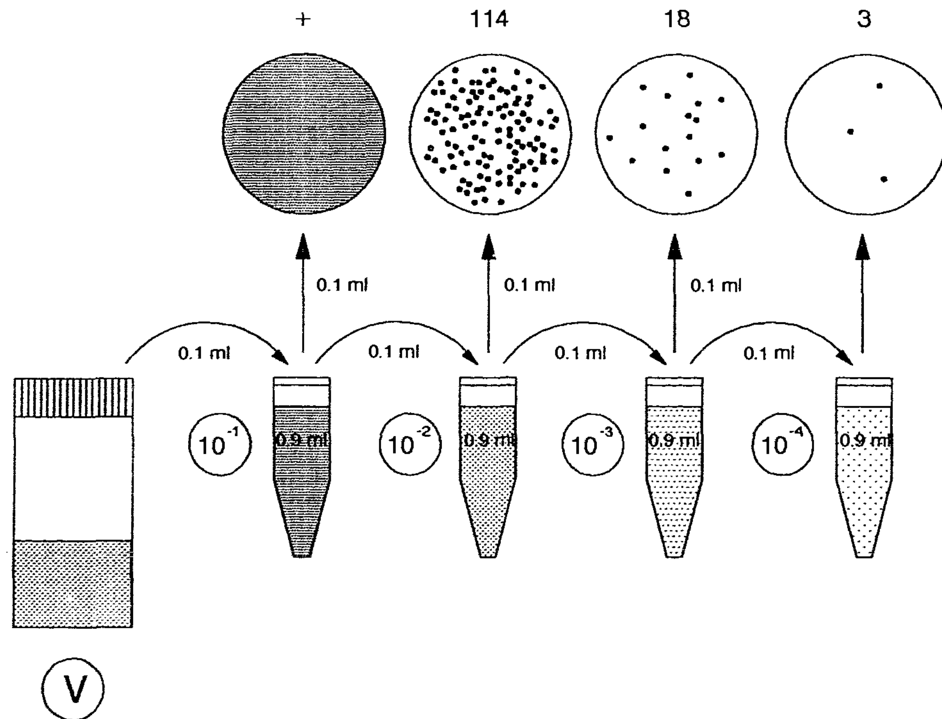


Appendix 1.1 Suspension, enumeration, density adjustment and assessment of *Beauveria bassiana* conidial germination: a) conidia at the bottom of a 1.5 mL microcentrifuge tube are gently agitated in ≈ 0.5 mL of water using a sterile micropestle; b) clumps of propagules are separated by vigorous agitation of the suspension aided by the use of a pestle motor; c) the volume of the water carrier is increased to ≈ 1 mL; d) the suspension is diluted 10 X, 10 μl is loaded into a hemocytometer, and the number of propagules per mL calculated; e) the original suspension is diluted accordingly to obtain the desired target concentration, 100 μl of the suspension is spread onto potato dextrose agar (Difco) containing 0.005% Benlate 50WP (a.i. benomyl), incubated for *ca* 24 h, and the number of germinated conidia enumerated.

Appendix 1.2 Oatmeal-dodine medium

- 5.55 g oatmeal
- 0.55 g magnesium sulphate
- 0.83 g Potassium phosphate
- 0.55 g sodium nitrate
- 12.5 g agar
- 0.45 g Cyprex (dodine)
- 2.5 mg crystal violet (stock solution = 0.10 g in 200 mL water)
- 0.2 g Penicillin G
- 0.5 g Streptomycin
- 500 mL deionized water

Grind rolled oats in a Waring blender. Once ground, add agar to the oatmeal homogenate. Salts and dodine are ground into a fine powder using a mortar and pestle and added to the oatmeal-agar mixture. Place 20.45 g of the mixture into 500 mL of water and bring to boil (note: the medium should be stirred vigorously). Add 5 mL of the crystal violet stock solution to the infusion and autoclave at 15 PSI for 20 min. Cool to approximately 55°C and adjust pH to 5.6 with NaOH if necessary. The antibiotic stock solution is prepared by dissolving 4.0 g of Penicillin G and 10.0 g of streptomycin sulphate in 40 mL of deionized water. The solution is then filter sterilized using a 0.2 μm filter. Add 2 mL of the antibiotic stock solution to the medium, swirl and pour 15 mL into 15 x 100 mm petri dishes. The surface of the medium should be flamed to remove bubbles.



Appendix 1.3 Recovery and enumeration of *Beauveria bassiana* conidia on oatmeal-dodine agar using the wash, dilution plating method. Leaves or coverslips are placed in 5 mL of phosphate buffer (0.01 M) amended with 0.05% Tween in a 20-mL vial (V). The samples are washed at 300 rpm for 2 h and the original suspension is diluted in a 10-fold dilution series by placing 100 μl in 0.9 mL of buffer-Tween; each dilution is vortexed ($\approx$ 10 sec) before the transfer is made. An aliquots of 100 μl from each suspension is spread on the surface of the oatmeal-dodine agar. After 6 to 7 days at 25°C, the number of colony-forming units (cfu) are counted at the dilution yielding 30 to 300 cfu per dish. The culture from the 10^{-1} dilution contains too many colonies to obtain an accurate count; colonies were confluent and crowded each other. The 10^{-2} dilution culture contained 114, well-distributed and easily counted colonies. The culture from the 10^{-3} dilution contained 18 colonies, seven more than would be predicted from the 10^{-2} culture. The percentage error in each colony at the 10^{-3} dilution makes such a count unreliable. Therefore, the 10^{-2} dilution is selected and the number of viable propagules per mL = number of colonies x tube dilution factor x plating dilution factor. Using the 10^{-2} culture, propagules per mL = 114 x 500 x 10 = 5.7×10^5 propagules per mL. The density of propagules per mL is then divided by the area of the samples (i.e. 10 cm^2) = 5.7×10^5 propagules per mL / 10 cm^2 = 5.7×10^4 propagules per cm^2.

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