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Effect of Soil Texture and Soil Sterilization on Susceptibility of Ovipositing Grasshoppers to *Beauveria bassiana*

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The effect of conidial concentration, soil texture, and soil sterilization on the efficacy of *Beauveria bassiana* against ovipositing grasshoppers (*Melanoplus sanguinipes*) was investigated in a controlled environment. In the first experiment, mortality of female grasshoppers ovipositing into a sterile loamy-sand soil containing conidia of *B. bassiana* was measured. The prevalence of mortality increased as the concentration of conidia in soil increased, and a median lethal concentration of 10^4 colony-forming units (CFU) per gram of soil (dry weight) was observed. Conidia ($10^{2.9}$ to 10^3 CFU per abdomen) were recovered from the abdomens of grasshoppers ovipositing into sand containing $10^{5.5}$ and 10^6 conidia per gram. Similar numbers of eggs were laid among treatments during the first oviposition period (1 to 7 days), but an effect of conidial concentration on eggs laid was observed during the second oviposition period (8 to 14 days). This was attributed to reduction in female numbers and not to reduction in fecundity independent of mortality. In a second experiment, grasshoppers oviposited into soils of three different textures (loamy-sand, sandy-loam, or clay-loam) that were amended with 10^5 *B. bassiana* conidia per gram and possessed either a viable or heat-killed microflora. There was no effect of soil texture on mortality of ovipositing grasshoppers, on the number of eggs laid, on positioning of egg pods in the soil profile, or on numbers of *B. bassiana* CFU recovered from female abdomens. However, a higher prevalence of mortality was observed for females ovipositing into the sterilized than nonsterilized sandy-loam and clay-loam soils. Substantial populations of fungi and bacteria were recovered from nonsterilized soils. The predominant fungi isolated from these soils were members of the genera *Chrysosporium, Fusarium, Gliocladium, Penicillium, Rhizopus,* and *Trichoderma,* whereas *Bacillus, Paenibacillus,* and *Pseudomonas* species were the most commonly isolated bacteria. This study demonstrates that ovipositing grasshoppers are susceptible to relatively low densities of conidia in soils of varying textures, but the soil microflora may have an adverse affect on the efficacy of *B. bassiana* in field soils.

Key Words: *Beauveria bassiana,* conidia, grasshopper, *Melanoplus sanguinipes,* oviposition, soil, texture, microflora, antagonism.

INTRODUCTION

Grasshoppers (Orthoptera: Acrididae) are major pests of crops in arid agroecosystems and existing control strategies rely almost exclusively on the use of chemical insecticides. However, recognition of the deleterious effects of pesticides has prompted the development of alternative, less obtrusive management strategies. The entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin has shown potential for the management of insects (Feng *et al.,* 1994) and is currently under investigation as a microbial control agent of acridids (Goettel *et al.,* 1995). In some field trials the application of *B. bassiana* conidia has resulted in substantial decreases in grasshopper populations (Johnson and Goettel, 1993). In others, results have been less promising (Johnson *et al.,* 1992; Inglis *et al.,* 1997b,c). The variable efficacy of *B. bassiana* in epigeal habitats has prompted investigation into alternative targeting strategies. *B. bassiana* is a soil-borne hyphomycete and its incorporation into soils has resulted in varying degrees of success for controlling other insect pests in field environments (Bajan *et al.,* 1977; Watt and LeBrun, 1984; Cantwell *et al.,* 1986; Forschler and Nordin, 1989; Gaugler *et al.,* 1989). Grasshoppers spend much of their life in contact with soil, and Inglis *et al.* (1995) found that ovipositing females and hatchling nymphs were susceptible to *B. bassiana* conidia that were uniformly incorporated into a sterile sand substrate at a relatively high density ($\approx 10^7$ conidia per gram). A number of abiotic and biotic properties of soil can influence the efficacy of entomopathogenic fungi (McCoy *et al.,* 1992), but the effect of these factors on the susceptibility of ovipositing grasshoppers to *B. bassiana* has not been investigated. Therefore, the objectives of this study were to: (1) determine the relationship between *B. bassiana* conidial concentration and mortality in females ovipositing into a sterile

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loamy-sand soil; (2) measure the effect of three soil textures on susceptibility of ovipositing grasshoppers to *B. bassiana;* and (3) evaluate the possible impact of the soil microflora on disease by comparing mortality in females ovipositing into sterilized and nonsterilized soils containing conidia of *B. bassiana.*

MATERIALS AND METHODS

Soils

The A horizon (the layer at the surface of a mineral soil in which the dominant acquired traits result from the accumulation of mineralized organic matter) of three Brown or Dark Brown Chernozemic soils from southern Alberta were used (Table 1). Although all soils were exposed to a minimum of agrochemicals, the clay-loam and sandy-loam soils had been treated with a broadleaf herbicide ca 6 months prior to their collection. Soils were sieved through a coarse mesh screen (0.3 × 0.3-mm squares) and maintained at 5°C until used (<1 month). To estimate field capacities, soils were dried in a drying oven at 110°C for 24 hr, 15-ml samples of soil were placed into a series of 20-ml glass vials, deionized water was placed on the soil surface in each vial, and the minimum volume of water required for the wetting front to reach the bottom of the soil profile was recorded. Soils were sterilized by air-drying and autoclaving thin layers (approximately 2 cm in depth) for 30 min at 125°C and 140 kPa.

Conidial Inoculum

Conidial viability of a strain of *B. bassiana* (GHA batch 921114, Mycotech Corp., Butte, MT), known to be highly virulent to grasshoppers (Inglis *et al.,* 1995), was determined on potato dextrose agar amended with 0.005% Benlate, 0.04% Penicillin G, and 0.1% streptomycin sulfate (Inglis *et al.,* 1996). Within 24 hr of use, dry conidia were suspended in sterile deionized water, and concentrations of viable conidia per milliliter were estimated with a hemocytometer and adjusted accordingly. With the exception of the clay-loam soil, moisture contents were adjusted to field capacity. Conidia were incorporated into 360 g (dry weight) of soil by applying 6 ml of the conidial suspension, in 1-ml aliquots, onto the surface of the soil in a 23-cm diameter mixing bowl with an airbrush (Artek, Rockford, IL) operated at 100 kPa. The soil was mixed thoroughly between conidial applications with a plastic spatula. Due to initial difficulties in mixing, the clay-loam soil was adjusted to 27% moisture (w/w) prior to conidial incorporation and subsequently adjusted to field capacity. Following incorporation of conidia, soils were dispensed into cylindrical oviposition containers (400 ml). The containers had a gravel bottom and were equipped with a central tube that permitted addition of water to the soil (Goettel and Inglis, 1997). A thin layer (approximately 5 mm in depth) of sterile soil was placed on the soil surface. Additional containers prepared in the same manner as above but without *B. bassiana* conidia were prepared as moisture controls. The moisture control soils were weighed daily, water loss was calculated, and the appropriate amount of sterile water was added to all soils at 24 hr intervals.

To quantify *B. bassiana* in soil, two cores of soil (before the addition of the sterile overlay) were removed from each container (for each replicate container) using a 3.5-mm diameter cork borer and weighed. Core samples were individually vortexed for 60 sec at high speed in 10 ml of 0.01 M phosphate buffer with 0.05% Tween 80 (buffer Tween). The suspension was diluted three times in a 10-fold dilution series, and 100-µl aliquots for each dilution were spread in duplicate onto a semi-selective oatmeal–dodine medium (Inglis *et al.,* 1995). The cultures were incubated at 25°C in the dark for 5 to 6 days, and where possible, the number of colonies were counted at the dilution yielding 30 to 300 CFU per dish. The number of CFU per gram (dry weight) of soil was determined and the mean value for the two cores per sample were calculated.

Grasshoppers

Nymphs of a nondiapause strain (Pickford and Randell, 1969) of *Melanoplus sanguinipes* (Fabricus) were reared on a diet of lettuce, bran, and wheat seedlings under a 16/8 hr light/dark photoperiod at room temperature; a vertical heat gradient was produced in the cages by a 25 W incandescent light bulb. Fifth-instar nymphs were separated according to sex and adult grasshoppers were maintained in cages for 7 to 10 days to attain sexual maturity.

Conidial Concentration

Thirty-four virgin adult females and 20 virgin adult males were placed into each of seven aluminum cages (40 × 40 × 30 cm) equipped with a clear Plexiglass front and top and a perforated metal floor to reduce contact with frass. The floor was provisioned with a hole for an oviposition container and 24 hr after placement of the grasshoppers in the cages, a container filled with loamy-sand soil from Purple Springs was positioned in each cage so that the soil surface was level with the floor of the cage. Soil contained 0, $10^{3.5}$, 10^{4}, $10^{4.5}$, 10^{5}, $10^{5.5}$, or 10^{6} viable conidia per gram of soil (dry weight); the cage in which each container was placed in a given replicate was randomly chosen. The moisture control soil was placed in an eighth cage. Cages were situated in a controlled environment chamber at 25/20°C day/night temperature with a 16/8 hr light/dark photoperiod; light was provided by fluorescent bulbs. Wheat seedlings were placed in all cages

twice daily throughout the 14-day experimental period. Conditions of ambient and within-cage relative humidity and temperature were recorded with a CR21 micrologger. Temperatures in cages were within 1°C of ambient throughout the experiment, and relative humidities were 5 to 10% higher than ambient (14 to 41%) for 1 to 2 hr following the placement of wheat in the cages.

The experiment was separated into two oviposition periods (7-day intervals) and at the end of the first oviposition period, soil containers were replaced. Soils from each oviposition period were sieved, and the number of eggs per oviposition container and their vertical positions were recorded. The relative prevalence of mating activity, defined as coupling and copulatory attempts by males, was observed three times daily at the time of feeding (800 and 1700 hr) and at 1230 hr. Dead adults were removed twice daily with forceps dipped in 70% ethanol between cages. Surviving individuals were killed by freezing at the end of the experimental period. All cadavers were placed on moistened filter paper in the dark at 25°C, and cadavers that produced hyphal growth of *B. bassiana* were recorded. Three replicates of the experiment were conducted on separate occasions.

Texture and Sterilization

Conidia (5.0 log viable conidia per gram) were incorporated into the three test soils (Table 1) that were either sterilized or unsterilized, and the soils were placed in oviposition containers. The test soils were randomly positioned in each of six cages, and the sterile and unsterilized moisture control soils were placed in two adjacent cages. All other experimental conditions were identical to those described previously for the conidial concentration experiment.

To isolate bacteria and fungi, duplicate samples were collected from the moisture control soils before placement in the cages and at the end of each oviposition period. Soil samples (10 g dry weight) were individually homogenized for 30 sec in 90 ml of sterile deionized water using a blender (Osterizer, 10 speed model set at "purée" speed). Suspensions were diluted (10-fold) five times in buffer Tween and 100 µl were spread in duplicate per dilution onto nutrient agar (NA) for the isolation of bacteria, and a medium containing 5 g peptone, 10 g dextrose, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 50 mg rose bengal, 100 mg streptomycin sulfate, 50 mg Penicillin G, and 20 g agar in 1 liter of water for the isolation of fungi. Cultures were maintained in the dark at 25°C and bacterial and fungal CFU were counted after 3 and 4 days, respectively. Predominant fungi and bacteria were isolated on the basis of colony morphology, allowed to grow on the appropriate medium in slant culture, and stored at 5°C until characterized. Fungi were identified on the basis of conidiogenesis according to standard references. Catalase, oxidase, and gram stain reactions were determined for all bacteria after 24 hr growth on NA. Bacteria were then grouped on the basis of their fatty acid profiles using gas–liquid chromatography (MIDI Inc., Newark, DE). Representative taxa were further characterized using standard physiological tests.

Beauveria Associated with Abdomens

Four ovipositing females per cage per replicate in each experiment were marked with paint on the dorsal surface of their thorax. Marked females were collected within 12 hr of oviposition and killed by freezing (−20°C for 4 to 6 hr); freezing for short periods has no effect on *B. bassiana* conidia (Inglis *et al.*, 1995). Abdomens from the marked females were excised, weighed, individually homogenized in 1 ml of buffer Tween with a micropestle (Kontes, Concept Inc., Clearwater, FL), the homogenate diluted (10-fold), 100 µl of the homogenate for each dilution spread onto oatmeal–dodine agar in duplicate, and numbers of *B. bassiana* CFU were recorded as described for the quantification of conidia in soil.

Statistical Analyses

All experiments were arranged as randomized complete block designs (RBD) with three blocks conducted in sequence. To ensure homogeneity of variance, residuals were plotted against predicted values, and, if necessary, an appropriate transformation was used. The soil texture and soil sterilization experiment was analyzed as a factorial experiment in a RBD with two levels of sterilization and three levels of soil texture. Comparisons of disease progress were analyzed as a split-plot in time (Gomez and Gomez, 1984); a Box correction was used as a conservative test for time and the time–conidial concentration interaction (Milliken and Johnson, 1984). In the absence of a block effect in the conidial concentration–mortality experiment, mortality data was combined across blocks and probit-transformed regressions of maximum mycosis at 14 days by log-concentration were fitted by maximum likelihood using the S108 Multiline Quantal Bioassay program (Agriculture and Agri-Food Canada, Ottawa, ON). Mortality was adjusted relative to the control treatment (Abbott, 1925). The program also tested for goodness of fit and estimated the median lethal concentration (LC_{50}) with 95% fiducial limits. For conidial concentrations where the incidence of mycosis by 14 days exceeded 50%, data were fitted to a Weibull distribution, and median lethal times with upper and lower 95% confidence limits (CL) were estimated using the LIFEREG procedure (SAS Institute, 1988). To obtain an estimate of *B. bassiana* on fecundity, cumulative grasshopper days were calculated and divided by

the total number of eggs produced over the 14-day duration of the experiment.

RESULTS

Conidial Concentration

Populations of *B. bassiana* conidia recovered from the loamy-sand soil averaged 0, 3.2 (SE = 0.13), 4.0 (SE = 0.06), 4.1 (SE = 0.07), 4.6 (SE = 0.03), 5.1 (SE = 0.04), and 5.6 (SE = 0.09) log CFU per gram for the 0, $10^{3.5}$, 10^{4}, $10^{4.5}$, 10^{5}, $10^{5.5}$, and 10^{6} conidial concentration treatments, respectively. Mating activity was reduced after 7 to 10 days and a higher prevalence ($F = 17.0$; $df = 6, 12$; $P < 0.001$) of mortality was observed in ovipositing females as the concentration of conidia in soil increased (Fig. 1). There was no difference ($F = 2.2$; $df = 2, 12$; $P = 0.15$) among blocks, and the mortality data was pooled for probit regression analysis. The median lethal concentration observed for ovipositing females was 10^{4} (95% CL = $10^{3.7}$ to $10^{4.2}$) conidia per gram of soil (Fig. 2A). The relationship between conidial concentration and mortality was fitted to the linear equation, $y = 2.42 + 0.64x$, where y is probit mortality and x is log conidial concentration. There was no effect ($F = 3.0$; $P = 5, 10$; $P = 0.07$) of conidial concentration on cadaver colonization by *B. bassiana;* 26.9 (SE = 10.9) to 49.4 (SE = 13.2) % of the females that died were colonized. Although the rate of death was relatively slow for all of the conidial concentrations tested, it was more rapid ($F = 9.9$; $df = 6, 14$; $P < 0.001$) at the higher conidial concentrations (Fig. 1). For example, the LT_{50} was 10.9 days (95% CL = 10.4 to 11.4) days at the 10^{6} conidial concentration compared to 13.5 days (95% CL = 12.7 to 14.4) at the 10^{4} concentration. Relatively small populations of *B. bassiana* conidia were recovered from female abdomens after oviposition. From the 10^{6} and $10^{5.5}$ conidial concentrations, 2.9 (SE = 0.03) and 3.0 (0.21) log CFU per abdomen, respectively, were recovered. From soil containing 10^{5} conidia per gram or less, few or no conidia (0 to 0.84 log CFU per abdomen) were recovered. During the first oviposition period, there was no effect ($F = 1.6$; $df = 6, 12$; $P = 0.22$) of conidial concentration on egg production; egg density ranged from 768 (SE = 70) to

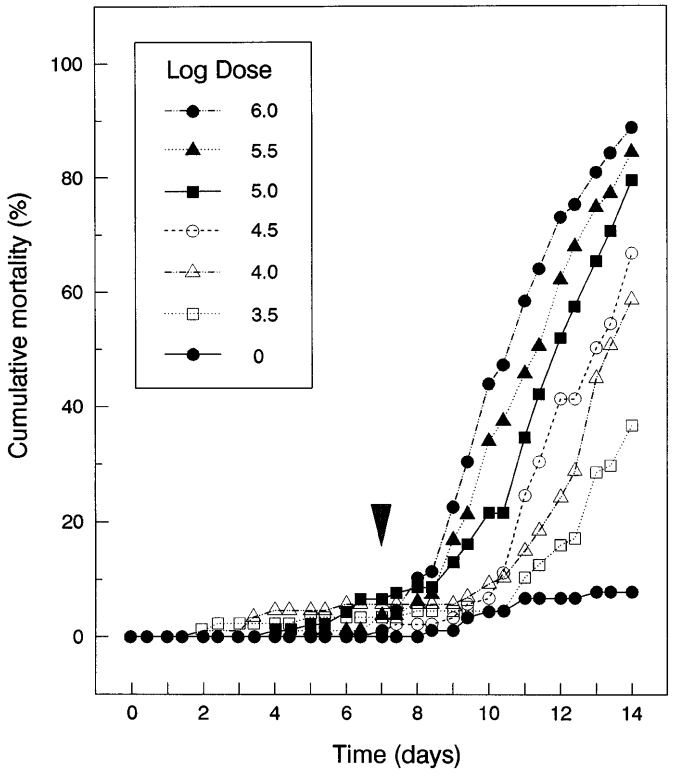

FIG. 1. Progress curves of mean mortality in female grasshoppers (*Melanoplus sanguinipes*) ovipositing into a sterile loamy-sand soil containing various concentrations of *Beauveria bassiana* conidia (log_{10} conidia per gram dry weight; $n = 3$). The arrow indicates the time at which soil was replaced and divides oviposition periods one and two.

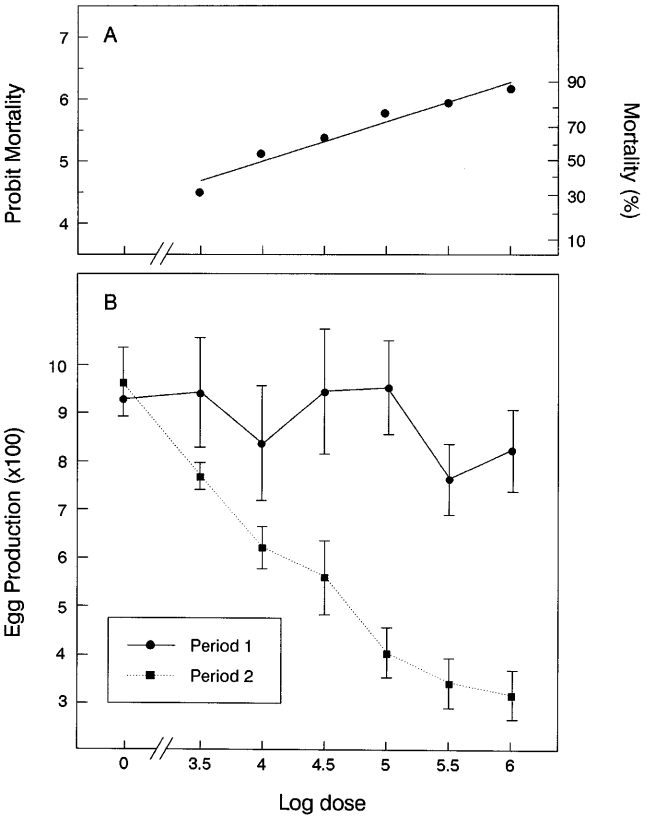

FIG. 2. Mortality in grasshoppers (*Melanoplus sanguinipes*) ovipositing into a sterile loamy-sand soil containing various concentrations of *Beauveria bassiana* conidia (log_{10} conidia per gram dry weight). (A) Probit regressions of mortality at 14 days (Abbott's adjusted). (B) Mean egg production per container during the two oviposition periods. Vertical bars represent standard errors of the means ($n = 3$).

956 (SE = 97) eggs per oviposition container. At the end of the second oviposition period, there was a strong effect of conidial concentration ($F = 27.2$; $df = 6, 12$; $P < 0.001$) on egg production, and relative to the control treatment, fewer ($P \leq 0.011$) eggs were recovered from all of the containers amended with *B. bassiana* conidia (Fig. 2B). There was no effect of conidial concentration on female fecundity ($F = 1.8$; $df = 6, 12$; $P = 0.18$); estimates of grasshopper fecundity ranged from 0.22 (SE = 0.007) to 0.29 (0.046) grasshopper days per total eggs produced.

At the end of the 14-day experimental period, male mortality for all treatments ranged from 13.3 (SE = 4.4) to 29.3 (SE = 12.2) %. Conidial concentration in the soil had no effect ($F = 1.2$; $df = 6, 12$; $P = 0.38$) on mortality of male grasshoppers. Only two of the males that died ($n = 92$) were subsequently colonized by *B. bassiana*.

Texture and Sterilization

The three soils tested had diverse characteristics (Table 1). After autoclaving, small populations of bacteria (≤2.0 log CFU per gram) but no fungi were recovered from the soils (Fig. 3). From unsterilized soils, fungal populations ranged from 4.0 (SE = 0.64) to 5.0 (SE = 0.05) log CFU per gram, and bacterial populations ranged from 6.8 (SE = 0.14) to 7.3 (SE = 0.09) log CFU per gram. The fungal taxa most frequently isolated from unsterilized soils included species of *Fusarium, Gliocladium, Penicillium,* and *Trichoderma* (Table 2). The most commonly isolated bacteria from unsterilized soils were species of *Bacillus, Paenibacillus,* and *Pseudomonas.* At the end of the 7-day oviposition period, bacterial populations from sterilized soil ranged from 7.2 (SE = 0.13) to 8.2 (SE = 0.29) log CFU per gram (Fig. 3); *Bacillus* and *Paenibacillus* species were most frequently isolated. Relatively small (≤2.2, SE = 1.3 log CFU per gram) numbers of fungi were also isolated from sterilized soils after 7 days, with the exception of the sandy-loam and clay-loam soils during the first oviposition period (4.3, SE = 0.23, and 4.5, SE = 0.28 log CFU per gram, respectively). The fungi recovered from sterilized soil at the end of the oviposition period were primarily *Chrysosporium pannorum* (Link) Hughes, *Penicillium* spp., *Trichoderma harzianum* Rifai, and *Trichothecium roseum* Link.

After incorporation of conidia, populations of *B. bassiana* in soil ranged from 4.7 (SE = 0.10) to 4.9 (SE = 0.08) log CFU per gram and were similar among textures ($F = 0.96$; $df = 2, 22$; $P = 0.40$), sterilization

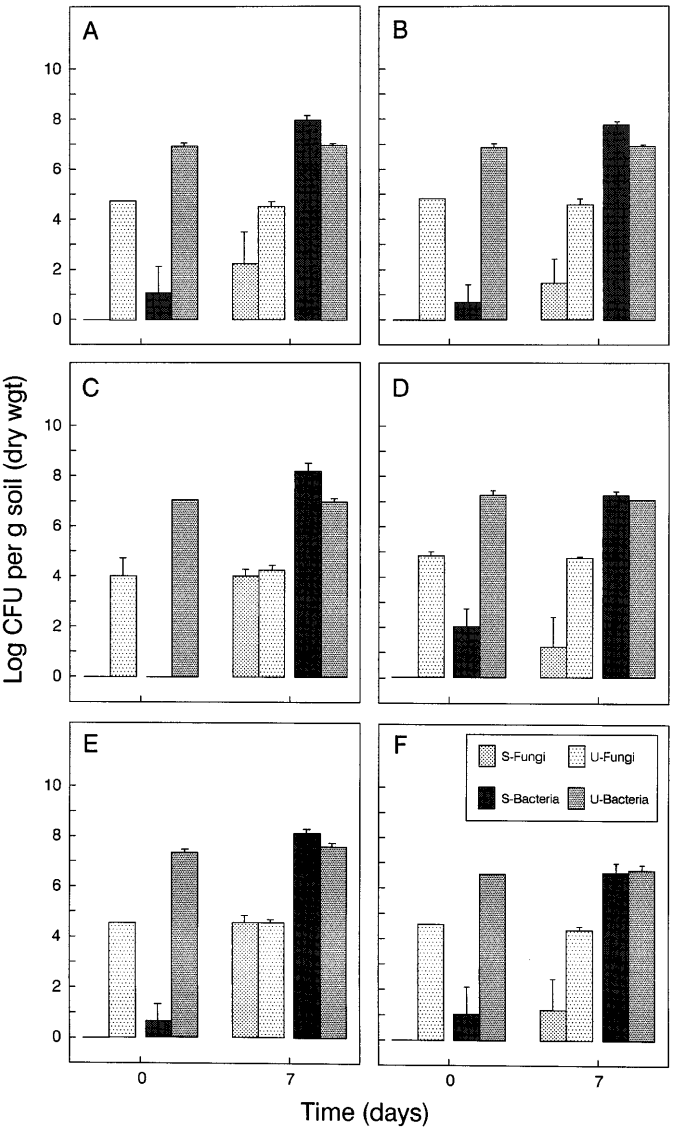

FIG. 3. Mean populations of bacteria and fungi in sterilized (S) and unsterilized (U) soils at the start and end of oviposition period one (0 to 7 days) and two (7 to 14 days). (A) loamy-sand during oviposition period one; (B) loamy-sand soil during oviposition period two; (C) sandy-loam soil during oviposition period one; (D) sandy-loam soil during oviposition period two; (E) clay-loam soil during oviposition period one; and (F) clay-loam soil during oviposition period two. Vertical bars represent standard errors of the mean ($n = 3$).

TABLE 1

Physical Characteristics of the Brown Chernozemic Soils Used to Test the Efficacy of *Beauveria bassiana* Against Ovipositing Grasshoppers (*Melanoplus sanguinipes*)

		%					
Texture[a]	Location	Sand	Clay	Silt	FC[b]	OM[c]	pH
Loamy-sand	Purple Springs, AB	85.6	9.2	5.2	17.2	1.5	7.1
Sandy-loam	Lethbridge, AB	53.6	17.7	28.7	19.4	2.2	7.7
Clay-loam	Magrath, AB	26.1	29.4	44.5	35.1	3.0	7.8

[a] Soil textures were determined using the sedimentation method.
[b] Field capacity (FC) of soils were approximated using a wetting front method.
[c] Organic matter content (OM) was estimated from organic carbon (1.72 × organic carbon).

treatments ($F = 0.01$; $df = 1, 22$; $P = 0.97$) and oviposition periods ($F = 0.81$; $df = 1, 22$; $P = 0.38$).

Soil texture did not affect either the rate of death ($F = 1.2–1.3$; $df = 2, 6$; $P \geq 0.36$) or the prevalence of final mortality after 14 days ($F = 0.14$; $df = 2, 10$; $P = 0.87$) (Fig. 4). In contrast, a higher prevalence of final mortality ($F = 41.8$; $df = 1, 10$; $P < 0.001$) was observed in females ovipositing into sterilized than into unsterilized soils. More mortality was observed after 14 days in females ovipositing into the sterilized clay-loam ($P < 0.001$) and sandy-loam ($P = 0.008$) soils; a weak effect ($P = 0.066$) was also observed for grasshoppers ovipositing into the sterilized loamy-sand soil. A more rapid development of mortality ($F = 9.3$; $df = 1, 4$; $P = 0.038$) in the sterilized relative to the unsterilized soil treatment was only observed for the clay-loam soil. Neither the texture ($F = 0.24$; $df = 2, 10$; $P = 0.79$) nor sterilization ($F = 0.09$; $df = 1, 10$; $P = 0.77$) treatments had an influence on colonization of cadavers by *B. bassiana*. Of the females that died during the course of the experiment, 52.0 (SE = 19.0) to 81.8 (SE = 10.2) % were subsequently colonized. Small numbers of conidia (0.48, SE = 0.48, to 1.5, SE = 0.05 log CFU per abdomen) were recovered from the abdomens of females collected within 12 hr of oviposition.

Egg densities ranged from 411 (SE = 205) to 643 (SE = 279) eggs per container per oviposition period. Neither soil texture ($F = 1.8–2.4$; $df = 2, 10$; $P \geq 0.14$) nor sterilization of soil ($F = 1.0–2.6$; $df = 1, 10$;

TABLE 2
Filamentous Fungi Isolated from Unsterilized Soils[a]

Taxa	Loamy-sand	Sandy-loam	Clay-loam
Absidia corymbifera (Cohn) Sacc. & Trotter			+
Acremonium sp.	+		+
Aspergillus sp.	+		
Chrysosporium pannorum (Link) Hughes	+		+
Cladosporium cladosporioides (Fres.) de Vries	+		
Cladosporium herbarum (Pers.) Link ex Gray	+		
Fusarium merismoides Corda		++	+
Fusarium spp.[b]	++		+
Gliocladium sp.	+++		++
Mucor sp.		+	
Penicillium aurantiogriseum Dierckx			+
Penicillium chrysogenum Thom		++	
Penicillium citreonigrum Dierckx		+	
Penicillium decumbens Thom		+	
Penicillium expansum Link	+		
Penicillium janthinellum Biourge[c]	+		
Penicillium miczynskii Zaleski	++		
Penicillium restrictum Gilman & Abbott	+		
Penicillium viridicatum Westling			+++
Penicillium sp.		+	
Rhizopus sp.			++
Spicaria violacaea Abbott		++	
Stachybotrys chartarum (Ehrenb. ex Link) Hughes		+	
Trichoderma harzianum Rifai	+	+	+
Trichoderma viride Pers. ex Gray	+++		
Trichothecium roseum Link	+		
Verticillium sp.	+		
Mycelia sterilia	+	+	

[a] Fungi were isolated using a dilution, spread-plate method, where + is 0 to 10%, ++ is 10–25%, and +++ is >25% of the colony-forming units isolated.

[b] On the basis of colony and morphological characteristics, a minimum of five *Fusarium* taxa was isolated.

[c] Identification indefinite.

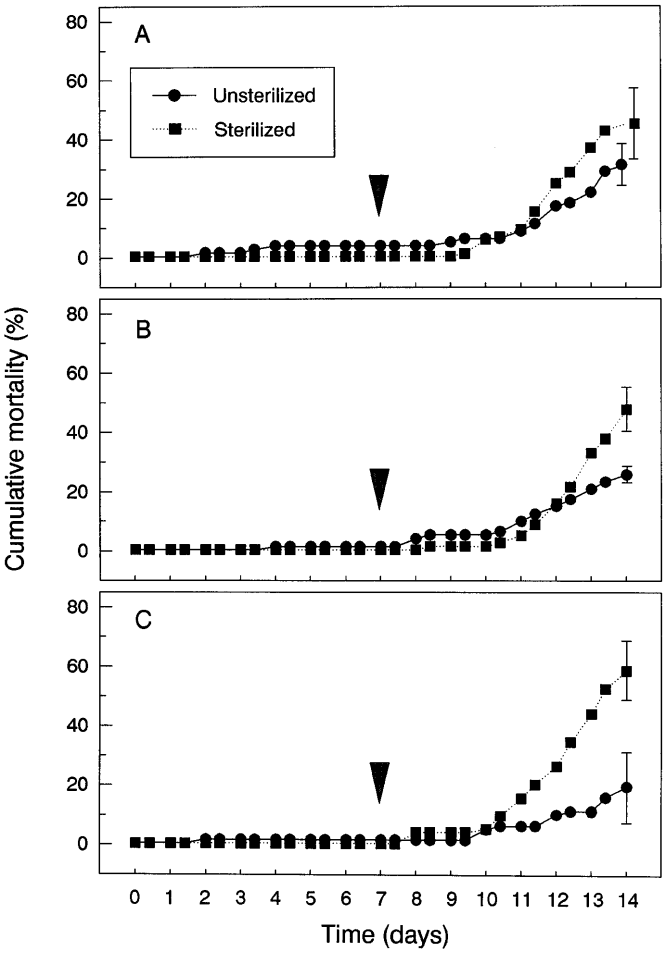

FIG. 4. Progress curves of mean mortality in grasshoppers (*Melanoplus sanguinipes*) ovipositing into sterilized or unsterilized soil containing 10^5 *Beauveria bassiana* conidia per gram dry weight. (A) loamy-sand soil, (B) sandy-loam soil, and (C) clay-loam soil are shown. Vertical bars represent standard errors of the mean ($n = 3$). The arrows indicate the time at which soil was replaced and divides the two oviposition periods.

$P \geq 0.14$) affected the number of eggs deposited by females during the first or second oviposition periods. Additionally, neither texture ($F = 2.38$; $df = 2, 10$; $P = 0.14$) nor sterilization ($F = 0.08$; $df = 1, 10$; $P = 0.78$) had an influence on female fecundity which ranged from 0.39 (SE = 0.10) to 0.62 (0.24) grasshopper days per total eggs produced. Texture ($F = 1.8$; $df = 2, 22$; $P = 0.19$), sterilization ($F = 1.8$; $df = 1, 22$; $P = 0.20$) or the oviposition period ($F = 0.40$; $df = 1, 22$; $P = 0.53$) also had no effect on the depth to which eggs were deposited; mean egg depth ranged from 19.0 (SE = 3.7) to 28.7 (SE = 8.7) mm.

Both the texture ($F = 5.4$; $df = 2, 10$; $P = 0.026$) and sterilization ($F = 33.9$; $df = 1, 10$; $P < 0.001$) treatments affected male mortality after 14 days. A higher prevalence of mortality ($P \leq 0.05$) occurred in males placed in cages containing the sterilized clay-loam (57.9%, SE = 13.9) and loamy-sand (41.9%, SE = 4.1) soils compared to those placed in cages with the sterilized sandy-loam (23.0%, SE = 6.7) and unsterilized (12.9%, SE = 7.2 to 20.5%, SE = 8.0) soils. Of the males that died during the course of the experiment ($n = 100$), only 17 cadavers were colonized by *B. bassiana*.

DISCUSSION

Effective deployment of *B. bassiana* against grasshoppers just before or during the egg-laying period may be possible since many of the economically important grasshopper species, including *M. sanguinipes*, oviposit in field margins (Criddle, 1918, 1933; Johnson, 1989). Although ovipositing grasshoppers were observed to be highly susceptible to *B. bassiana* conidia in a sand substrate treated with $\approx 10^7$ conidia per gram, the effect of conidial concentration on mortality was not measured (Inglis *et al.*, 1995). In the present study, we observed a significant but relatively gradual log concentration response probit curve (slope = 0.64) in grasshoppers ovipositing into a sterile sandy-loam soil with a median lethal concentration of 10^4 *B. bassiana* conidia per gram of soil. Conidial populations of approximately 10^4 conidia per gram of soil can be economically obtained by spraying *B. bassiana* with conventional application equipment. For example, conidia formulated in water and applied over crested wheatgrass at a rate of 2.5×10^{13} conidia per hectare penetrated the canopy and were subsequently recovered from soil at densities reaching 10^4 CFU per gram of soil (Inglis *et al.*, 1997a). While the rate of death was relatively slow (commencing at 11 to 14 days), reductions in eggs laid were observed by 14 days for all of the conidial concentrations tested ($\geq$3.5 log conidia per gram). Evidence suggested that reductions in egg production were the result of female mortality and not due to reductions in fecundity. Therefore, the lower prevalence of mortality (19 to 59%) observed in grasshoppers ovipositing into sterile and nonsterile soils of different textures may explain, in part, why there was no significant reduction in egg production during the second oviposition period of this experiment.

Mycosis in males copulating with females ovipositing into a sterile sand substrate containing *B. bassiana* conidia reached 100% in one trial and 50% in a second trial (Inglis *et al.*, 1995). The delay in mycosis in males relative to females suggested that males either came in contact with conidia brought to the sand surface through oviposition activity or they were infested with conidia after direct contact with females. In the present study, we observed no effect of conidial concentration on male mortality (13 to 29%) after 14 days and only 2% of the males that died were colonized by *B. bassiana*. With the exception of the sterilized clay-loam (58%) and loamy-sand (42%) treatments, the prevalence of male mortality was also relatively low (13 to 23%) in the texture–sterilization experiment. Reasons for the slightly higher male mortality in the two sterilized soil treatments are unknown, but 14 of the 57 males were colonized by *B. bassiana*, suggesting that at least a portion of these males were infected. Despite the slightly higher mortality in males placed in cages containing sterilized clay-loam and loamy-sand soils, there was no effect on egg production by females relative to other treatments. Sexually active male *M. sanguinipes* can copulate several times per day and under cage conditions averaged 0.9 copulations per day over a 12-week period (Pickford and Gillott, 1972). Therefore, a reduction in egg production would not necessarily result from the death of a portion of males.

Whether inoculation of oviposition sites will be an efficacious method of applying *B. bassiana* to manage grasshoppers will depend on the degree to which a number of potentially detrimental abiotic and biotic factors influence the pathogen and the host–pathogen interaction. Water availability and soil texture have been shown to impact on the efficacy of *B. bassiana* (e.g., Studdert and Kaya, 1990). Oviposition depth tends to vary according to soil texture and moisture, and grasshoppers will readily oviposit into soil at or near field capacity but not into dry soils (Uvarov, 1966). To study the impact of soil texture independent of soil moisture, we used a central watering tube that allowed the addition of water to the bottom of the soil profile in an attempt to simulate a field soil; grasshoppers place their eggs near the top of the soil profile if water is added to the soil surface. We observed that texture had no significant effect on the positioning of eggs, in numbers of eggs laid, or in the efficacy of *B. bassiana* against ovipositing grasshoppers. Acridids can extend their abdomen 10-fold by stretching the soft cuticle and muscles between abdominal segments (Vincent and Wood, 1972; Vincent, 1975) and after completion of egg laying, the abdomen is retracted. We recovered relatively small numbers of *B. bassiana* conidia from

abdomens of ovipositing females, but Inglis *et al.* (1995) observed many conidia aggregated within intersegmental folds and suggested that the cuticle between abdominal sclerites is the primary site of infection in ovipositing grasshoppers. Furthermore, they suggested that since conidia are trapped within folds during abdominal retraction, the influence of soil texture and other abiotic and biotic characteristics of soil may have less impact on *B. bassiana* infection in grasshoppers than on soil-dwelling insects.

Soil microorganisms may decrease the persistence (e.g., Lingg and Donaldson, 1981) and efficacy of *B. bassiana* against insects (e.g., Groden and Lockwood, 1991; Pereira *et al.*, 1993). We observed less mortality in grasshoppers that oviposited into unsterilized than sterilized soils for two of the three soils tested, suggesting a detrimental impact of the soil microflora on *B. bassiana*. Although the media and techniques we used select for certain types of microorganisms, large populations of bacteria ($\approx 10^7$ CFU per gram) and fungi ($\approx 10^4$ to 10^5 CFU per gram) were isolated from unsterilized soils. Fungal populations were two to three orders of magnitude smaller than bacterial populations but the dilution spread-plate technique does not provide an accurate measure of fungal biomass. Furthermore, the spread-plate method selects for fungi that are prolific sporulators and the vast majority of the fungi that we isolated are Hyphomycetes that produce abundant conidia. Nevertheless, many of the microorganisms that we isolated are well documented antagonists of fungi in soil (e.g., species of *Bacillus, Gliocladium, Penicillium, Trichoderma*). We detected no effect of unsterilized soils on conidial survival over the 7-day duration of each oviposition period, suggesting that soil microorganisms inhibited conidial viability, conidial attachment, conidial germination, or penetration of the host. Lingg and Donaldson (1981) observed that *Penicillium griseofulvum* Dierckx (=*Penicillium urticae*) was frequently isolated from soil and produced a water-soluble inhibitor of *B. bassiana* in culture. However, antagonism *in vitro* and *in vivo* rarely correspond, and despite reports of mycoparasitism and antibiosis by many of the fungi and bacteria that we isolated, the mechanism by which these microorganisms may inhibit *B. bassiana* in soils is unknown. Further studies using soils amended with microorganisms of specific taxa may elucidate the mechanisms by which the efficacy of *B. bassiana* against grasshoppers is affected.

While autoclaving eliminated or substantially reduced numbers of viable microorganisms in soil, the use of sterilization methods to study the influence of the indigenous microflora on the efficacy of entomopathogens has some limitations. All sterilization methods cause chemical alterations to soil, and heat sterilization causes hydrolyzation of carbohydrates, coagulation of proteins, and influences a variety of other soil properties (Wolf and Skipper, 1994). Therefore, the increased susceptibility of ovipositing grasshoppers to *B. bassiana* in sterilized soils may also have been influenced by chemical alterations or by an increase in available nutrients to germinating conidia. Another problem with the use of sterilization to study the impact of the soil microflora on *B. bassiana* is the reintroduction of microorganisms to the soils from air and grasshoppers. We observed substantial populations ($>10^7$ CFU per gram) of bacteria and in some instances fungi ($>10^4$ CFU per gram) in sterilized soils at the end of each oviposition period. However, the diversity of culturable bacteria and fungi was substantially reduced relative to unsterilized soils, and the recovered microorganisms likely proliferated as pioneer colonizers in the absence of a viable microflora, possibly aided by the release of nutrients upon autoclaving. The rapid proliferation of microorganisms emphasizes the importance of monitoring microbial populations in similar experiments using sterilized soil.

This study shows that ovipositing females are susceptible to *B. bassiana* conidia in soil at densities that can be attained using conventional application technology. However, relatively moderate levels of mortality ($<60\%$) were observed in females by 14 days in the texture–sterilization experiment. While there was no influence of soil texture, mortality was increased in females ovipositing into two of three soils that were heat sterilized, suggesting that the presence of a viable microflora may inhibit the efficacy of *B. bassiana* in field soils. The normal reproductive period (43 to 142 days) for *M. sanguinipes* (Pickford and Gillott, 1972) is substantially longer than the 14-day duration of our experiments, and higher levels of mortality, irregardless of the presence of an antagonistic soil microflora, may occur over this period. Additional studies are required to measure the impact of the microflora of field soils and other factors (e.g., effects of grasshopper thermoregulation) on mycosis and to elucidate possible mechanisms by which antagonistic microorganisms affect the efficacy of *B. bassiana* against ovipositing grasshoppers. Larval integuments (vermiform sheath or provisional cuticles) did not protect hatchling nymphs from soil-borne conidia (Inglis *et al.*, 1995) and information on the influence of the biotic and abiotic properties of field soils on infection of emerging nymphs is also necessary before *B. bassiana* can be effectively deployed against grasshoppers in oviposition sites.

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