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Use of Pathogen Combinations to Overcome the Constraints of Temperature on Entomopathogenic Hyphomycetes against Grasshoppers

G. DOUGLAS INGLIS, DAN L. JOHNSON, K.-J. CHENG, AND MARK S. GOETTEL

Agriculture and Agri-Food Canada Research Centre, P.O. Box 3000, Lethbridge, Alberta, Canada T1J 4B1

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The influences of temperature on the mortality of grasshoppers (*Melanoplus sanguinipes*) inoculated with the entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium flavoviride,* alone and in combination was investigated. Basking by grasshoppers had less influence on the prevalence and timing of mortality in nymphs treated with *M. flavoviride* than with *B. bassiana.* In *B. bassiana*-treated nymphs allowed to bask for only 1 h/day, 44% less mortality was observed, decreasing to 98% less death in nymphs basking for ≥4 h/day. In contrast, only a slight decrease in mortality (13 to 23%) was observed in *M. flavoviride*-treated nymphs permitted to bask for 4 to 6 h/day. For both fungi, more mortality was observed in nymphs exposed to 35 than to 40°C for various durations per day. However, exposure to high temperatures had a greater inhibitory effect on *B. bassiana* than *M. flavoviride.* The efficacy of both entomopathogens, alone and in combination, was determined in simulated cool and hot fluctuating temperature environments and at a constant 25°C. The former two environments were derived from weather data on two different days at Lethbridge in July and were adjusted to simulate grasshopper thermoregulation during daylight periods (0600 to 2200 h). At 25°C, there was no difference between treatments in the prevalence of mortality. In the hot temperature environment, less mortality was observed for *B. bassiana* (3%) than for *M. flavoviride* (52%). Conversely, in the cool temperature environment, less mortality was observed for *M. flavoviride* (46%) than for *B. bassiana* (100%). Application of both pathogens simultaneously resulted in a final prevalence of mortality that was greater than that for *M. flavoviride* in the hot temperature environment and equal to that for *B. bassiana* in the cool temperature environment. The application of *B. bassiana* and *M. flavoviride* in combination may be a way to overcome some of the constraints of temperature on entomopathogenic Hyphomycetes against grasshoppers, especially where temperatures fluctuate or are high for a significant period of time.

KEY WORDS: *Beauveria bassiana, Metarhizium flavoviride,* entomopathogens, grasshoppers, *Melanoplus sanguinipes,* temperature, thermoregulation, basking.

INTRODUCTION

Beauveria bassiana (Balsamo) Vuillemin and *Metarhizium flavoviride* Gams & Rozsypal are being developed as inundative control agents of grasshoppers and locusts. However, both fungi have yielded inconsistent results against field populations of acridids. Acridids elevate their body temperatures higher than ambient by habitat selection and/or orientation to solar radiation (Chappell and Whitman, 1990; Heinrich, 1993), and basking by grasshoppers has been shown to adversely affect disease caused by *B. bassiana* in a controlled setting (Inglis *et al.,* 1996a). The ability of grasshoppers to thermoregulate 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 during hot sunny periods (Johnson *et al.,* 1992; Inglis *et al.,* 1997a). Furthermore, a substantially higher prevalence and more rapid development of disease was observed in grasshoppers placed in shaded field cages than in cages exposed to direct sunlight or protected from the ultraviolet-B radiation (Inglis *et al.,* 1997b), also implicating temperature, and in particular the ability of grasshoppers to elevate their body temperatures as a major constraint on *B. bassiana*. *M. flavoviride* has been selected for development as an inundative control agent of locusts and grasshoppers in Africa (Prior *et al.,* 1992), but the effects of elevated acridid body temperatures on *M. flavoviride* have not been reported. The objectives of this study were to: (1) compare the effects of high temperatures and thermoregulation by *Melanoplus sanguinipes* (Fabricius) nymphs on mortality caused by *B. bassiana* and *M. flavoviride* and (2) compare the efficacy of these two entomopathogens, alone and in combination, against

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grasshoppers in simulated hot and cool field environments.

MATERIALS AND METHODS

Grasshoppers

Nymphs of a non-diapause strain of *M. sanguinipes* (Pickford and Randell, 1969) were reared on a diet of bran and wheat seedlings at 20 to 25°C; a vertical heat gradient was produced in the cage by a 25-W incandescent light bulb (General Electric, Mississauga, ON). Fifteen to 20 days after emergence, nymphs (third instar) were individually collected in sterile 20-ml vials stoppered with a sterile polyurethane foam plug and starved for 12 h before inoculation with conidia.

Preparation of Inocula

Conidia of *B. bassiana* (GHA strain) were produced on a starch substrate by Mycotech Corp. (Butte, MT) (Bradley *et al.*, 1992) and stored dry at −10°C. *M. flavoviride* (LRC No. 112; isolated from *Schistocerca pallens* (Thunberg), Rio Grande do Norte, Brazil), from a lyophilized stock culture, was grown on potato dextrose agar (PDA; Difco) at 25°C. Conidia were harvested from the surface of the colony with a sterile spatula after 15 to 20 days of growth and placed at 5°C for a maximum of 48 h. To determine viability, conidia were suspended in distilled water (2×10^7 conidia/ml), and 100 µl was spread on PDA amended with 0.005% Benlate (DuPont). After 24 h at 25 ± 1°C, conidia were fixed with lactophenol, and a minimum of 500 conidia from each of two replicate cultures were observed. Conidia were considered germinated if germ tubes were greater than 3 µm in length.

Immediately before use, conidia were suspended in sunflower oil (Safflo, Culinar Foods Inc., Toronto, ON). Concentrations of viable conidia were estimated using a hemocytometer and adjusted to 2.0×10^8 viable conidia/ml unless indicated otherwise. Nymphs were inoculated using an oil-bait method as described by Inglis *et al.* (1996b). Briefly, 0.5-µl aliquotes of the conidial suspension were pipetted onto 5-mm-diameter lettuce disks (1.0×10^5 viable conidia/disk), and the disks were pierced with a pin and presented to nymphs. Nymphs were allowed to feed on the disks for approximately 2 h. Nymphs that underwent ecdysis or that did not consume the entire disk during this period were removed from the experiment.

Influence of Basking

Treatment groups consisted of *B. bassiana*- and *M. flavoviride*-inoculated nymphs exposed to heat from a 25-W light bulb for 0, 1, 2, 4, or 6 h/day in 40 × 40 × 30 cm cages (Inglis *et al.*, 1996a). The cages were constructed of aluminum sheeting, fitted with a plexiglass front and top and a perforated metal floor to prevent contact with frass; to facilitate air exchange, vents were placed in the plexiglass (front and top) and in the side walls of each cage. A bulb was mounted 21 cm (bulb center) from the bottom of the cage on the back wall. Adjacent to the bulb, a plastic mesh tube (8 cm diameter × 28 cm high) was erected to permit the movement of nymphs vertically in the heat gradient relative to the light bulb. Cages were situated in a controlled environment chamber (CEC) at 24 to 25°C under a 16/8-h light/dark photoperiod provided by fluorescent bulbs. Conditions of ambient and within-cage temperature and relative humidity were monitored with a CR21X micrologger (Campbell Scientific, Logan, UT). When the light was on, cage temperatures were 46 to 49°C adjacent to the bulb on the climbing mesh, 26 to 27°C at the base of the mesh, and near ambient (25 to 26°C) at all other positions at the bottom of the cage. When the bulb was off, temperatures were 25 ± 1°C throughout the cage. Relative humidities within the CEC ranged from 23 to 48% and increased ≤5% in cages after feeding.

Fifty arbitrarily selected nymphs per treatment were placed in each of eight cages in the CEC. Nymphs were maintained on a diet of fresh wheat leaves and bran for 10 days. Cadavers were removed from the cages twice daily and nymphs that survived the 10-day duration of the experiment were killed by freezing. Nymphs were placed on moistened filter paper in petri dishes at 25°C and those that produced hyphal growth of *B. bassiana* or *M. flavoviride* on the cadaver surfaces after 3 to 7 days were recorded. The experiment was arranged as an unbalanced block design with replicates conducted in time. Due to space limitations in the CEC, the control treatment (nymphs not permitted to bask) was replicated four times, whereas the exposure treatments of 1, 2, 4, and 6 h were replicated three times (1520 total nymphs); the order in which the four exposure treatments were run in a given replicate was randomly chosen. Final mortality and colonization of grasshoppers were analyzed as factorial experiments with five levels of exposure time and two levels of fungal treatment. In conjunction with a significant F test, means were compared using the least squares means (lsmeans) function of SAS (SAS Institute Inc., 1988). Comparisons of mortality by day were analyzed as split plots in time (Gomez and Gomez, 1984). For all split-plot analyses, a Box correction was used as a conservative test for time and time–treatment interactions (Milliken and Johnson, 1984); the Box correction reduces the degrees of freedom for time, the time by treatment interactions, and the residual error(time) by time −1. When the final prevalence of mortality exceeded 50%, data were fitted to a Weibull distribution, and median lethal times with upper and lower 95% confidence limits (CL) were estimated.

Exposure to 35 and 40°C

Following inoculation by either *B. bassiana* or *M. flavoviride*, 12 to 15 nymphs per treatment were individually housed in 240-ml plastic cups and exposed to 35 or 40 °C for 0, 1, 2, 4, 6, 8, 12, or 24 h/day for 10 days under a 16/8 h light/dark photoperiod provided by fluorescent lights. The remainder of the day was spent at 25°C. Conditions of relative humidity and temperature within cups were recorded with a CR21 micrologger. Temperatures within cups maintained at constant 25, 35, and 40°C in the CECs were within 1°C of ambient. Relative humidities within cups rose up to 20% higher than ambient after placement of the wheat leaves in the cups and decreased to within 5% of ambient within 3 h at 40°C, 7 h at 35°C, and 12 h at 25°C. Frass was removed from the cups and nymphs were fed fresh wheat leaves once daily, whereas cadavers were removed twice daily. The experiment was arranged as a randomized complete block design (RCBD) with three replicates conducted in time (1200 total nymphs). Final mortality and colonization of grasshoppers were analyzed as factorial experiments with eight levels of exposure time and two levels of fungal treatment or two levels of temperature; lsmeans were used to compare means. Comparisons of mortality by day were analyzed as split plots in time with a Box correction, and LT_{50}s were calculated.

The influence of exposures to 35 or 40°C (same exposure times as above) on fungal colony growth and conidial germination were determined as a RCBD with three replicates conducted in time. For measurements of hyphal growth, 0.5 µl (10^5 conidia) of the conidial the suspension (*B. bassiana* or *M. flavoviride*) was placed centrally on PDA and cultures were incubated in the CEC simultaneously with the grasshoppers. Colony diameters were measured at 24-h intervals for 10 days by taking the mean of two perpendicular measurements for three cultures in each of three replicates. For each fungus–temperature–exposure time combination, cumulative growth of colonies was fit to linear models by day and the slopes obtained from the regression were used as a measure of growth rate in subsequent analyses (r^2 ranged from 0.80 to 0.99). For assessments of conidial germination, germinated conidia on PDA amended with Benlate were recorded after 24 h, as detailed previously. Germination was assessed on two plates for each of three replicates. Both colony growth and conidial germination were analyzed as factorial experiments.

For both fungi, the relationships between mortality, conidial germination, and colony growth rate were fit to linear models in an attempt to elucidate the mechanism by which temperature influences disease. Mortality was logit-transformed and the logit model used, was $[(P + 0.005)/1.005 - P]$, where P is the proportion of final mortality. Negligible mortality was observed in nymphs inoculated with *B. bassiana* and exposed to 40°C for 6 h or more per day and therefore, exposure times of 8, 12, and 24 h/day were not included in the regressions. For *M. flavoviride* at 40°C, the 24-h exposure time was removed from the regression analysis.

Pathogen Combinations

Temperature environments consisted of (1) constant 25°C; (2) a simulated hot day; and (3) a simulated cool day. Hot and cool day treatments were simulated based on temperatures recorded at the Agriculture and Agri-Food Canada Research Centre, Lethbridge on July 26, 1994, and July 13, 1993, respectively; both days were sunny, and net solar radiation was 15.8 and 15.0 MJ/m^2, respectively. An empirical model for *M. sanguinipes* thermoregulation (Kemp, 1986) was used to simulate the natural elevation of grasshopper body temperatures during sunlight hours; between 0600 and 0800 h, and 1900 and 2200 h, the temperature increased/decreased linearly from ambient to or from the elevated condition (Fig. 1). Within-cup and ambient temperatures and ambient relative humidities were recorded with a CR21 micrologger. Ambient temperatures were within 1.5°C of the programmed temperatures in all three CECs, and mean hourly temperatures within cups were within 1°C of ambient. Ambient relative humidities varied among the three temperature environments and ranged from: 22 to 40% in the constant 25°C environment; 9 to 53% in the hot temperature environment; and 27 to 84% in the cool temperature environment.

Treatments consisted of: (1) *B. bassiana* conidia alone (10^5 conidia/nymph); (2) *M. flavoviride* conidia alone (10^5 conidia/nymph); (3) *B. bassiana* (10^5 conidia/nymph) in combination with *M. flavoviride* (10^5 conidia per nymph); and (4) a control (oil alone). To obtain the necessary conidial dose for the combination treatment, initial concentrations of viable conidia were adjusted to 4.0×10^8 viable conidia/ml. After inoculation, 28 to 30 nymphs were individually placed in 240-ml plastic cups. Nymphs were fed, frass was removed, and cadavers were processed as described previously. The experiment was arranged as a RCBD with three replicates conducted in time (1036 total nymphs). Final mortality and colonization of grasshoppers were analyzed as factorial experiments with three levels of environment and three levels of treatment; lsmeans were used to compare means. Comparisons of mortality by day were analyzed as split plots in time with a Box correction, and when the prevalence of mortality exceeded 50%, LT_{50}s were determined. The prevalence of nonsporulating individuals among the mortalities was determined by the index: [(% total mortality − % sporulating cadavers)/% total mortality] × 100.

The influence of the three temperature environments on hyphal growth of *B. bassiana* and *M. flavoviride* was

measured on PDA as detailed previously, with the exception that five plates were measured per replicate. Percent reductions in colony growth in the high and low temperature environments (T_x) relative to the constant 25°C environment (T_{25}) were calculated as: [(growth rate at T_{25} − growth rate at T_x)/growth rate at T_{25}] × 100. Percent increases in colony growth were calculated as: [(growth rate at T_{25} − growth rate at T_x)/ growth rate at T_x] × 100. Conidial germination of *B. bassiana* and *M. flavoviride*, alone and in combination, was measured on PDA with Benlate after 24 h, as detailed previously. For the combination treatment, conidia were combined immediately before use and the final concentration was 1×10^7 conidia/ml for each fungus.

RESULTS

Influence of Basking

We observed that basking affected the rate of death ($F = 7.5, 46.2$; $df = 4, 11$; $P \leq 0.004$) in grasshoppers treated with both *B. bassiana* and *M. flavoviride*. Median lethal times for nymphs inoculated with *B. bassiana* and allowed to bask for 1 h/day was 9.4 days (CL = 8.8 to 10.1) compared to 5.0 days (CL = 4.8 to 5.2) in control nymphs. In nymphs treated with *M. flavoviride*, LT_{50}s were 5.3 (CL = 5.1 to 5.4), 5.7 (5.5 to 5.9), 6.2 (CL = 5.9 to 6.5), 7.2 (CL = 6.9 to 7.6), and 7.9 (CL = 7.5 to 8.3) days for grasshoppers allowed to bask for 0, 1, 2, 4, and 6 h/day, respectively. Final mortality was also reduced ($F = 7.3, 283.3$; $df = 4, 8$; $P \leq 0.009$) as the duration of the basking period increased (Fig. 2) but the effects of basking were more ($F = 34.8$; $df = 4, 19$; $P < 0.001$) inhibitory to *B. bassiana* than to *M. flavoviride*. In *B. bassiana*-treated nymphs allowed to bask for only 1 h/day, 44% less ($P < 0.001$) mortality was observed relative to nonbasking nymphs, decreasing to 93% less ($P < 0.001$) mortality in nymphs permitted to bask for 4 to 6 h/day. Mortality was only reduced ($P \leq 0.04$) by 13 to 23% in nymphs treated with *M. flavoviride* that basked for 4 or 6 h/day. A higher ($F = 16.7$; $df = 1, 19$; $P < 0.001$) prevalence of nonsporulating cadavers was observed for *M. flavoviride*-treated nymphs (Fig. 2).

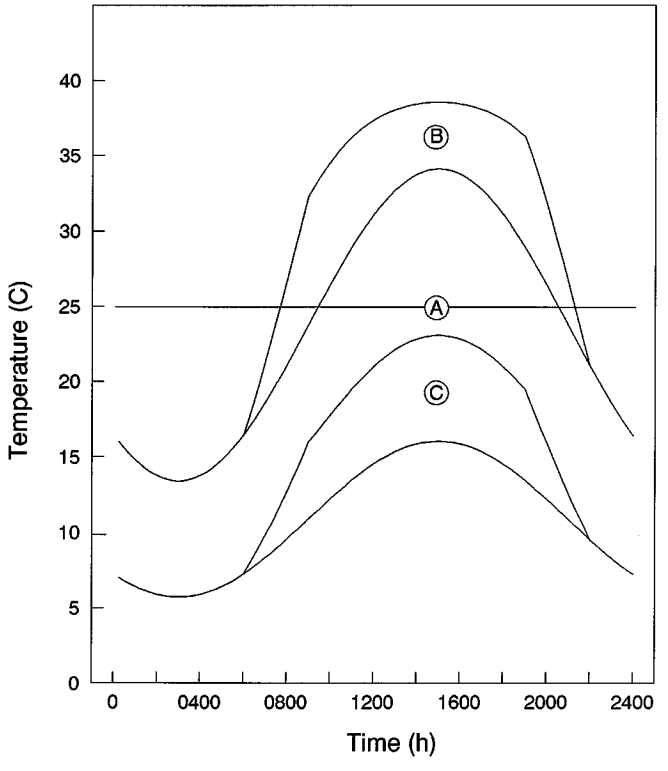

FIG. 1. Temperature profiles within the three test environments. (A) Constant (25°C) temperature environment. (B) Hot temperature environment. The lower curve is the temperature (min) recorded at the Agriculture and Agri-Food Canada (AAFC) Research Centre, Lethbridge, Alberta on July 26, 1994. The upper curve was derived from an empirical model for *Melanoplus sanguinipes* thermoregulation (Kemp, 1986) during daylight hours (0600 to 2200 h); between 0600 and 0800, and 1900 and 2200, the temperature increased/decreased linearly. (C) Cool temperature environment. The lower curve is the temperature recorded at the AAFC Research Centre, Lethbridge on July 13, 1993, whereas the upper curve was derived from Kemp, 1986).

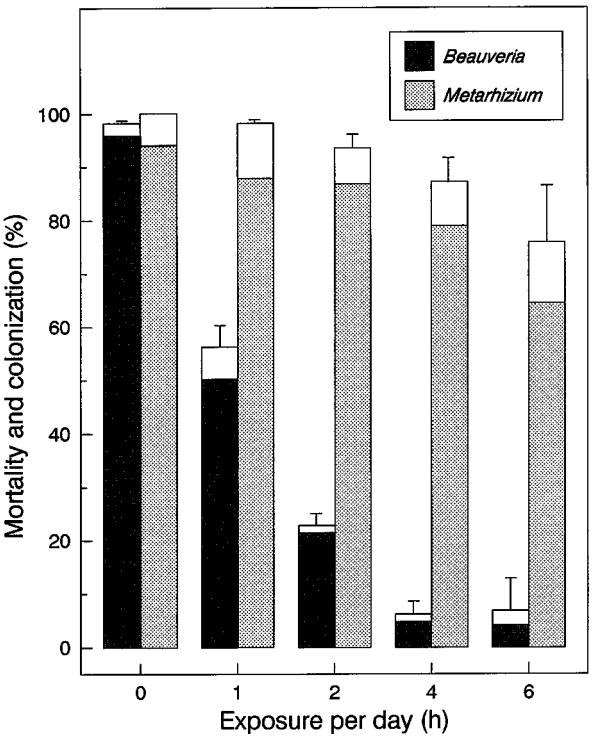

FIG. 2. Mortality (10 days) in *M. sanguinipes* nymphs treated with *Beauveria bassiana* and *Metarhizium flavoviride* and permitted to bask (25-W incandescent light bulb) for various times per day. Open and shaded bars together indicate total mortality (%), shaded bars indicate cadavers colonized by the two Hyphomycetes (%), and the open bars indicate noncolonized nymphs (%). Vertical lines represent standard errors of means for total mortality ($n = 3$ to 4).

Exposure to 35 and 40°C

For both *B. bassiana* and *M. flavoviride*, exposures to 35°C had less ($F = 20.8, 80.3$; $df = 1, 26$; $P < 0.001$)

impact on final mortality than did exposures to 40°C (Fig. 3A–B). Exposures to 35°C affected final mortality in nymphs treated with *B. bassiana* ($F = 21.7$; $df = 7, 14$; $P = 0.001$) but not *M. flavoviride* ($F = 1.8$; $df = 7, 14$; $P = 0.17$); a minimum exposure of 4 h or more per day influenced ($P = 0.05$) mortality in *B. bassiana*-treated nymphs. The timing of mortality was also increased by exposures to 35°C for *B. bassiana* ($F = 13.9$; $df = 7, 16$; $P < 0.001$) but not *M. flavoviride* ($F = 1.9$; $df = 7, 16$; $P = 0.14$). Exposures to 40°C affected ($F = 31.0, 48.5$; $df = 7, 14$; $P < 0.001$) nymphal mortality after 10 days for both entomopathogens. However, *B. bassiana* was more affected ($F = 148.2$; $df = 1, 30$; $P < 0.001$) by 40°C than was *M. flavoviride;* exposures of ≥2 h/day (P = 0.001) and 4 h/day (P = 0.05) reduced mortality for the *B. bassiana* and *M. flavoviride* treatments, respectively. The timing of death was also lengthened ($F = 21.3, 24.0$; $df = 7, 16$; $P < 0.01$) by exposures to 40°C. For example, the median lethal times to death for nymphs treated with *M. flavoviride* and placed at 40°C for 2, 4, 6, and 8 h/day were 5.9 (CL = 5.2 to 6.6), 6.3 (CL = 5.4 to 7.3), 7.3 (CL = 6.5 to 8.3), and 8.4 (CL = 7.3 to 9.6) days, respectively compared to 4.8 days (CL = 4.6 to 5.1) for nymphs at constant

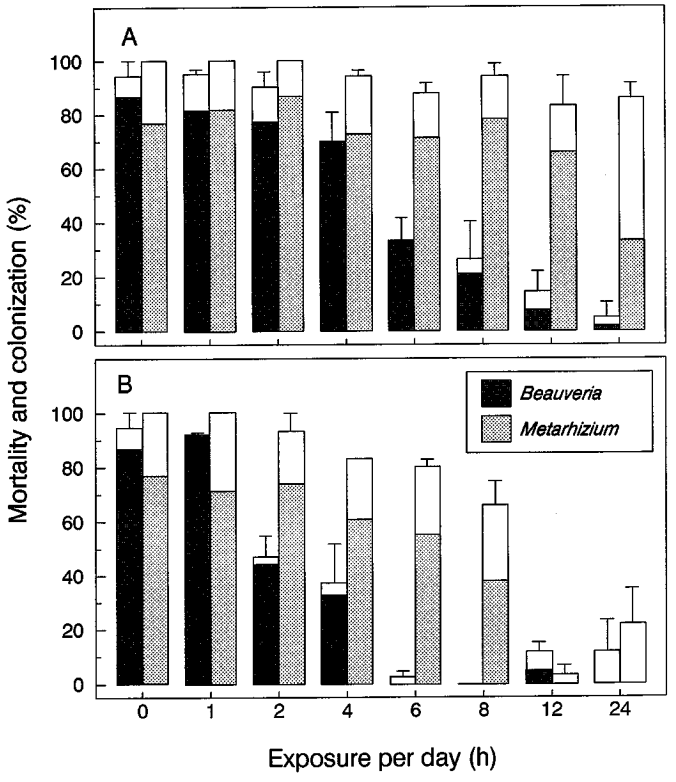

FIG. 3. Mortality (10 days) in *M. sanguinipes* nymphs treated with *B. bassiana* and *M. flavoviride* and exposed to high temperatures for various times per day. (A) 35°C; (B) 40°C. The open and shaded bars together indicate total mortality (%), the shaded bars indicate cadavers colonized by the two Hyphomycetes (%), and the open bars indicate noncolonized nymphs (%). Vertical lines represent standard errors of means for total mortality ($n = 3$).

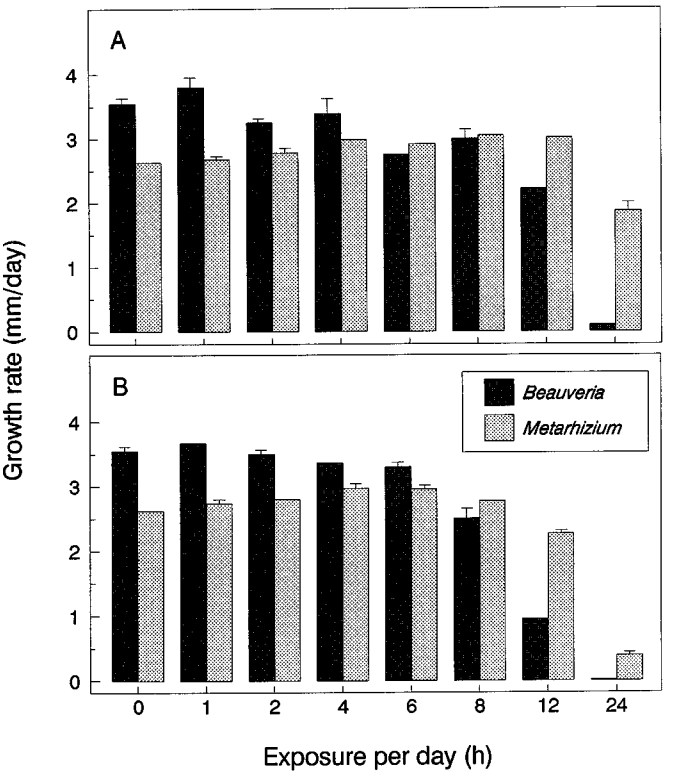

FIG. 4. Growth rates (mm/day) of *B. bassiana* and *M. flavoviride* colonies on potato dextrose agar exposed to high temperatures for various times per day. (A) 35°C; (B) 40°C. Vertical lines represent standard errors of means ($n = 3$).

25°C. A higher ($F = 50.6$; $df = 1, 62$; $P < 0.001$) percentage of cadavers was colonized by *B. bassiana* than by *M. flavoviride.*

Colony development was affected ($F = 42.9, 59.2$; $df = 7, 29$; $P < 0.001$) by exposures to 35 and 40°C for *B. bassiana* and *M. flavoviride* (Fig. 4). Exposures to 40°C were more ($F = 14.4, 63.4$; $df = 7, 28$; $P < 0.001$) inhibitory than 35°C for both fungi; differences in growth rate were observed for exposure times exceeding 6 to 8 h/day. Growth of *M. flavoviride* was less affected than *B. bassiana* by increasing exposures to 35° ($F = 54.3$; $df = 7, 29$; $P < 0.001$) and 40°C ($F = 78.3$; $df = 7, 29$; $P < 0.001$). While *M. flavoviride* grew under all conditions, growth of *B. bassiana* hyphae was severely restricted at constant 35°C and completely suppressed at constant 40°C. The growth rate of *M. flavoviride* hyphae was increased ($P < 0.05$) by exposures to 35°C for 4 to 12 h/day and to 40°C for 2 to 8 h/day.

Conidial viability was equally high ($P = 0.44$) for *B. bassiana* (95.0%, SE = 0.32) and *M. flavoviride* (88.9%, SE = 1.3) after 24 h at 25°C. As the time of exposure to 35° ($F = 6.4$; $df = 7, 30$; $P < 0.001$) and 40°C ($F = 3.0$; $df = 7, 30$; $P < 0.02$) increased, conidial germination of *B. bassiana* was more inhibited than *M. flavoviride* (Figs. 5A and 5B). Only 43% of *B. bassiana* conidia maintained at continuous 35°C germinated after 24 h, while 35°C had no effect ($P = 0.69$) on germination of

M. flavoviride conidia. Exposures to 40°C for 12 h or more inhibited ($P < 0.001$) conidial germination for both fungi.

Time ($r^2 = 0.83$ and 0.95 at 35 and 40°C, respectively) was a better predictor of mortality in nymphs treated with *B. bassiana* than was either colony growth ($r^2 = 0.69$ and 0.79) or conidial germination ($r^2 = 0.35$ and 0.30). While the same trend was observed for *M. flavoviride*, in general, the relationships between mortality and time ($r^2 = 0.64$ and 0.95) and mortality and colony growth ($r^2 = 0.002$ and 0.20) were less distinct than those for *B. bassiana*. In contrast, the relationship between mortality and germination of *M. flavoviride* conidia ($r^2 = 0.63$ and 0.71) was stronger than that for *B. bassiana*.

Pathogen Combinations

The efficacy of *B. bassiana* and *M. flavoviride*, alone and in combination, was determined against grasshoppers in cool and hot temperature environments. Mortality in nymphs treated with oil ranged from 1.1% (SE = 1.1) to 8.9% (SE = 5.6) and this treatment was excluded from subsequent analyses. Mortality (14 days) in nymphs treated with *B. bassiana* and/or *M. flavoviride* differed ($F = 54.3$; $df = 4, 16$; $P \leq 0.001$) across environments (Fig. 6). There was no difference in final mortality of nymphs between treatments (100%) in the 25°C environment. Although there was no significant difference ($F = 3.1$; $df = 2, 6$; $P = 0.12$) in the rate of death at 25°C, LT_{50}s were 4.7 days (CL = 4.4 to 4.9) for the combination treatment compared to 5.5 (CL = 5.1 to 5.8) and 5.3 (CL = 5.2 to 5.4) for *B. bassiana* and *M. flavoviride* alone (Fig. 7). In the hot temperature environment, significantly less mortality ($P < 0.001$) was observed for *B. bassiana* (3.4%, SE = 1.9) than for *M. flavoviride* (51.8%, SE = 2.9). Conversely, in the cool temperature environment, less mortality ($P < 0.01$) was observed for *M. flavoviride* (45.5%, SE = 7.3) than for *B. bassiana* (100%); the rate of mortality was also slower ($F = 18.0$; $df = 1, 4$; $P = 0.013$) for the *M. flavoviride* treatment (Fig. 7). Application of *B. bassiana* in combination with *M. flavoviride* resulted in greater ($P = 0.007$) mortality than *M. flavoviride* in the hot temperature environment and the same ($P = 0.84$) level of mortality to *B. bassiana* in the cool temperature environment (Figs. 6 and 7). The time to death was similar ($F = 0.6$; $df = 1, 4$; $P = 0.50$) between the combination ($LT_{50} = 9.2$ days, CL = 8.8 to 9.6) and *B. bassiana* ($LT_{50} = 9.7$ days, CL = 9.3 to 10.1) treatments in the cool temperature environment. In the hot environment, the rate of death was slightly slower ($F = 6.1$; $df = 1, 4$; $P = 0.069$) for the *M. flavoviride* than for the combination treatment. The median time to death for the combination treatment was 10.1 days (CL = 8.8 to 11.6) compared to 13.1 days (CL = 11.3 to 15.1) for *M. flavoviride*. The prevalence of colonization relative to total mortality was similar ($F = 0.51$; $df = 2, 16$; $P = 0.61$) between environments but differed ($F = 6.5$; $df = 2, 16$; $P = 0.009$) between treatments;

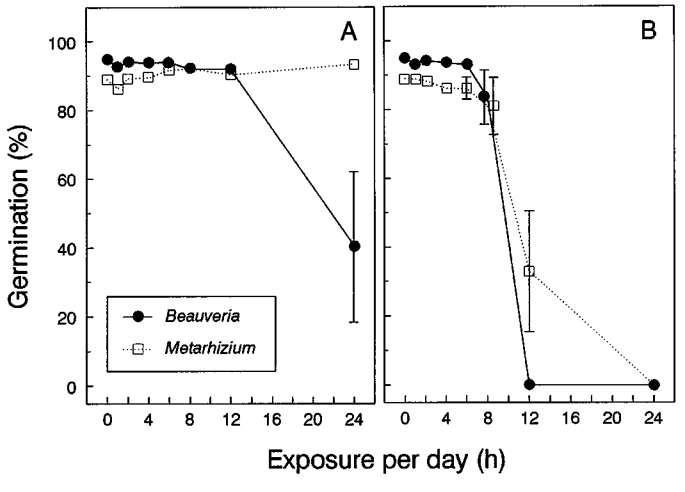

FIG. 5. Germination of *B. bassiana* and *M. flavoviride* conidia on potato dextrose agar (24 h) exposed to high temperatures for various times. (A) 35°C; (B) 40°C. Vertical lines represent standard errors of means ($n = 3$).

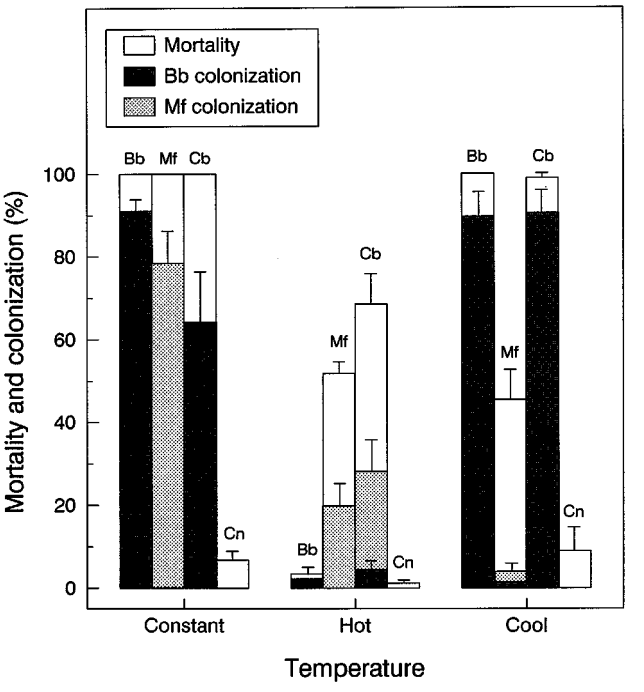

FIG. 6. Mortality (14 days) in *M. sanguinipes* nymphs in three temperature environments (constant 25°C, hot, and cool). Treatments consisted of: (Bb) *B. bassiana* (10^5 conidia/nymph); (Mf) *M. flavoviride* (10^5 conidia/nymph); (Cb) *B. bassiana* and *M. flavoviride* in combination (10^5 conidia/nymph/fungus); and (Cn) control (oil alone). Open and shaded bars together indicate total mortality (%), shaded bars indicate cadavers colonized by the two Hyphomycetes (%), and open bars indicate noncolonized nymphs (%). Vertical bars represent standard errors of means ($n = 3$).

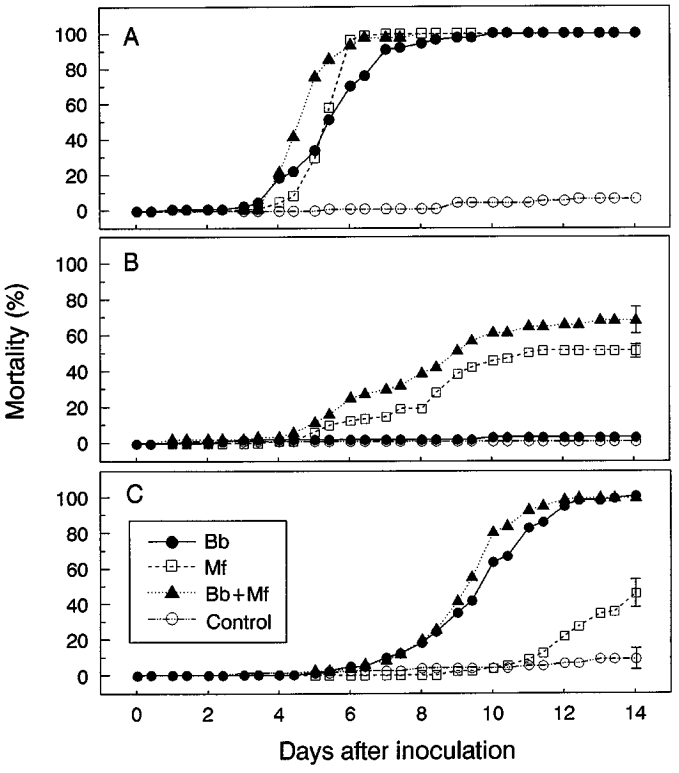

FIG. 7. Mortality rates for *M. sanguinipes* nymphs in the three temperature environments. (A) Constant 25°C; (B) Hot temperature environment; and (C) Cool temperature environment. Treatments consisted of: (Bb) *B. bassiana* (Bb; 10^5 conidia/nymph); (Mf) *M. flavoviride* (Mf; 10^5 conidia/nymph); (Bb + Mf) *B. bassiana* and *M. flavoviride* in combination (10^5 conidia/nymph/fungus); and control (oil alone). Vertical bars represent standard errors of means for total mortality at 14 days ($n = 3$).

the prevalence of noncolonized nymphs was lower ($P = 0.05$) for the *B. bassiana* treatment across environments. In only one instance were both fungi observed externally on a nymph that was coinoculated with *B. bassiana* and *M. flavoviride.*

Growth of *B. bassiana* and *M. flavoviride* colonies by day were linear ($r^2 = 0.91$ to 0.99), and the rate of growth differed ($F = 73.6$; $df = 2, 10$; $P < 0.001$) between the two fungi in the three temperature environments. Although colony development was inhibited ($P < 0.001$) for both fungi in the hot and cool temperature environments, *B. bassiana* was more inhibited ($P < 0.001$) in the hot environment and *M. flavoviride* was more inhibited ($P = 0.002$) in the cool environment. *Beauveria bassiana* colony development (relative to the constant 25°C environment) was reduced by 82% (SE = 0.5) and 67% (SE = 1.7) in the hot and cool temperature environments, respectively. Conversely, *M. flavoviride* colony growth was reduced by 58% (SE = 1.0) and 81% (SE = 0.4) in the hot and cool temperature environments, respectively.

Conidial germination differed ($F = 9.4$; $df = 4, 16$; $P < 0.001$) among the three treatments in the three temperature environments. Germination was equally ($P \geq 0.69$) high for *B. bassiana* alone (96.0%, SE = 1.4), *M. flavoviride* (92.1%, SE = 0.3) alone, and for both fungi in combination (94.7%, SE = 0.18) at constant 25°C. In the hot temperature environment, there were also no differences ($P \geq 0.31$) among treatments but conidial germination was reduced ($P = 0.003$ to 0.060) relative to constant 25°C; germination by 24 h was 63% (SE = 9.3), 73% (SE = 15.1), and 73% (SE = 5.1) for *B. bassiana* alone, *M. flavoviride* alone, and the combination treatment, respectively. In the cool temperature environment, no *M. flavoviride* conidia had germinated by 24 h compared to 67% (SE = 5.9) and 43% (SE = 8.9) germination for *B. bassiana* and the combination treatment, respectively. While more ($P = 0.02$) conidia had germinated for *B. bassiana* than the combination treatment by 24 h in the cool temperature environment, germination for both treatments was reduced ($P \leq .007$) relative to constant 25°C.

DISCUSSION

The optimal temperature for feeding and development of many grasshoppers, including *M. sanguinipes,* is 38 to 40°C (Hilbert and Logan, 1983; Lactin and Johnson, 1995), and nymphs that are permitted to bask adjacent to an incandescent light bulb optimize their body temperatures (Inglis *et al.,* 1996a; Lactin and Johnson, 1996). We observed that basking adversely affected mortality in *M. sanguinipes* nymphs inoculated with *B. bassiana* or *M. flavoviride,* but basking was substantially more inhibitory to *B. bassiana.* For example, basking for 6 h/day reduced mortality in nymphs inoculated with *B. bassiana* by 98% compared to only a 23% reduction in nymphs treated with *M. flavoviride.* Inoculated nymphs were also exposed to 35 or 40°C for various periods each day to determine the effects of suboptimal as well as optimal body temperatures on mortality. At continuous 35°C, mortality was reduced in nymphs inoculated with *B. bassiana* but not *M. flavoviride;* a minimum exposure of 4 h affected mortality caused by *B. bassiana.* In instances where grasshoppers are able to bask but are unable to elevate their body temperatures to optimum levels, as is frequently the case in temperate prairie agroecosystems (Kemp, 1986; Carruthers *et al.,* 1992), our results suggest that suboptimal body temperatures (e.g., 35°C) would impact on *B. bassiana* but not on *M. flavoviride.* Milner (1997) observed that mortality of *Locusta migratoria* (L.) adults inoculated with *M. flavoviride* was significantly reduced at continuous 40°C but not at 25, 30, or 35°C. We observed equally low mortality in *M. sanguinipes* nymphs inoculated with *B. bassiana* (11%) and *M. flavoviride* (22%) and maintained at continuous 40°C. However, acridids primarily elevate their body temperatures during periods of sunlight, and we at-

tempted to mimic a situation in which grasshoppers were capable of optimizing their body temperatures for various times each day. In nymphs exposed to 40°C for various periods, mortality levels were consistent with those observed in the basking experiment and this would be expected given our supposition that grasshoppers optimize their body temperatures by basking. Our results clearly indicate that while *M. flavoviride* is not immune to the effects of high temperature, it is less affected by elevated body temperatures in acridids than is *B. bassiana*.

The decreased susceptibility of *M. flavoviride* to high temperatures would be expected given that its optimal and upper cardinal temperatures are generally higher than those of *B. bassiana* (Roberts and Campbell, 1977; Fargues *et al.*, 1992, 1997). We observed that the cardinal temperature for hyphal growth was approximately 35°C for *B. bassiana* (GHA), whereas some growth of *M. flavoviride* (LRC No. 112) hyphae was observed at a constant 40°C. Although the optimal temperature for growth of *M. flavoviride* hyphae is approximately 27 to 28°C, exposures to 35 and 40°C for 2 to 8 h/day increased the rate of growth relative to 25°C. The reason for the increased rate of growth of *M. flavoviride* hyphae exposed to high temperatures is uncertain but recently the induction of heat shock proteins (HSPs) was observed in *B. brongniartii* blastospores exposed to 45°C (Xavier and Khachatourians, 1996) and the formation of HSPs in hyphae may explain the observed increase in vegetative growth. Relatively poor relationships were observed between mortality and hyphal growth or conidial germination. Although the mechanism by which temperature impacts on entomopathogenic fungi is uncertain, the results of this study suggest that factors other than the direct effects of high temperatures on fungal growth (e.g., indirect effects of temperature on the immune response of the host) affect disease development, and this possibility warrants additional study.

While *M. flavoviride* is less influenced by high temperatures than *B. bassiana,* the impact of low temperatures on disease of acridids has largely been ignored. In temperate prairie agroecosystems, summer temperatures can be relatively cool (<15°C), particularly at night. To test the effects of cool and hot temperatures on *B. bassiana* and *M. flavoviride,* we maintained inoculated nymphs in simulated cool and hot temperature environments derived from actual weather data at Lethbridge but modified to simulate grasshopper thermoregulation (Kemp, 1986). Relative to a constant 25°C environment, mortality caused by *B. bassiana* and *M. flavoviride* was affected in both the hot and cool temperature environments. However, in the hot environment less mortality was observed in nymphs treated with *B. bassiana* (4%) than with *M. flavoviride* (52%). In the cool environment, *B. bassiana* (100%) was superior to *M. flavoviride* (45%). Our results indicate that while *M. flavoviride* is superior to *B. bassiana* in its ability to withstand high temperatures (e.g., elevated body temperatures in behaviorally thermoregulating grasshoppers), its efficacy may be severely inhibited in temperate agroecosystems due to low temperatures. The relative ability of *M. flavoviride* to incite mortality at high but not at low temperatures may also explain its prevalence in tropical and subtropical but not in temperate climates (Goettel *et al.,* 1995; Milner, 1997).

Relatively few studies have reported the combined application of entomopathogens with the aim of increasing efficacy (e.g., Lecuona and Alves, 1988; Barbercheck and Kaya, 1990; Choo *et al.,* 1996; Glare, 1994; Kaya *et al.,* 1995; Thurston *et al.,* 1994). We tested the hypothesis that the coapplication of *B. bassiana* and *M. flavoviride* could be used to increase the temperature range over which the individual fungi alone would incite mortality in acridids. We observed that the final mortality was greater for the combination treatment than for *M. flavoviride* alone in the hot temperature environment and equal to that of *B. bassiana* alone in the cool temperature environment. While this was a relatively simple simulation, it demonstrates the potential value of utilizing fungal "cocktails" to overcome the constraints of temperature on entomopathogenic hyphomycetous fungi against acridids.

The degree to which temperature influences the competitive infection and colonization of the host by *B. bassiana* and *M. flavoviride* may affect the efficacy of control. We observed a consistently higher prevalence of nonsporulating cadavers for *M. flavoviride* than for *B. bassiana* in all three environments and this may represent differences in mechanisms of pathogenesis and/or in saprophytic colonization of the host. In general, conidial germination on PDA appeared to be unaffected by antagonism but whether antagonism limits conidial germination *in vivo* remains to be determined. Barbercheck and Kaya (1990) observed that when *B. bassiana,* and the nematodes *Steinernema carpocapsae* (Weiser) (=*S. feltiae*) or *Heterorhabditis bacteriophora* Poinar (=*H. heliothidis*) co-infected greater wax moth (*Galleria mellonella*) larvae, *B. bassiana* was more likely to develop in larvae below 20°C but the reverse was true at 22 and 30°C. We obtained some evidence to suggest that temperature also reduced interspecies antagonism by favoring growth of one of the pathogens. Hyphal growth rates for *B. bassiana* were reduced by 82% in the hot and 67% in the cool temperature environments, whereas growth of *M. flavoviride* hyphae was reduced by 58 and 81% in the hot and cool temperature environments, respectively. Furthermore, only 10% of the grasshopper cadavers that were colonized by one of the fungi were colonized by *B. bassiana* in the hot temperature environment compared to 100% of cadavers in the cool temperature

environment. At 25°C, the final prevalence and rate of mortality was the same for all three pathogen treatments, but none of the nymphs that were co-inoculated were colonized by *M. flavoviride* after death. This would suggest that while *M. flavoviride* is able to out compete *B. bassiana* at hot temperatures, *B. bassiana* is able to competitively exclude *M. flavoviride* from cadavers at temperatures at or below 25°C.

This study demonstrates that by elevating their body temperature, grasshoppers can inhibit and/or prevent disease caused by *B. bassiana* and *M. flavoviride.* However, the effects of thermoregulation are considerably more inhibitory to *B. bassiana* than to *M. flavoviride.* While *M. flavoviride* was superior to *B. bassiana* in its ability to cause disease in thermoregulating grasshoppers, cool temperatures limited its efficacy. By co-inoculating grasshoppers with *B. bassiana* and *M. flavoviride,* high levels of mortality were achieved in both cool and hot temperature environments. This study indicates that the combined application of *B. bassiana* and *M. flavoviride* may be a means to overcome some of the constraints of high and low temperatures on entomopathogenic Hyphomycetes against grasshoppers. This possibility is presently under investigation at Lethbridge.

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REFERENCES

Barbercheck, M. E., and Kaya, H. K. 1990. Interactions between *Beauveria bassiana* and the entomogenous nematodes, *Steinernema feltiae* and *Heterorhabditis heliothidis. J. Invertebr. Pathol.* **55,** 225–234.

Bradley, C. A., Black, W. E., Kearns, R., and Wood, P. 1992. Role of production technology in mycoinsecticide development. *In* "Frontiers in Industrial Mycology" (G. F. Leatham, Ed.), pp. 160–173. Chapman & Hall, New York.

Carruthers, R. I., Larkin, T. S., and Firstencel, H. 1992. Influence of thermal ecology on the mycosis of a rangeland grasshopper. *Ecology* **73,** 190–204.

Chappell, M. A., and Whitman, D. W. 1990. Grasshopper thermoregulation. In "Biology of Grasshoppers" (R. Chapman, Ed.), pp. 143–172. Wiley Interscience, New York.

Choo, H. Y., Koppenhofer, A. M., and Kaya, H. K. 1996. Combination of two entomopathogenic nematode species for suppression of an insect pest. *J. Econ. Entomol.* **89,** 97–103.

Fargues, J., Goettel, M. S., Smits, N., Ouedraogo, A., and Rougier, M. 1997. Effect of temperature on vegetative growth of *Beauveria bassiana* isolates from different origins. *Mycologia* (in press).

Fargues, J., Maniania, N. K., Delmas, J. C., and Smits, N. 1992. Influence de la temperature sur la croissance *in vitro* d'hyphomycetes entomopathogenes. *Agronomie* **12,** 557–564.

Glare, T. R. 1994. Stage-dependent synergism using *Metarhizium anisopliae* and *Serratia entomophila* against *Costelytra zealandica. Biocontrol. Sci. Technol.* **4,** 321–329.

Goettel, M. S., Johnson, D. L., and Inglis, G. D. 1995. The role of fungi in the control of grasshoppers. *Can. J. Bot.* **73**(Suppl. 1), S71–S75.

Gomez, K. A., and Gomez, A. A. 1984. "Statistical Procedures for Agricultural Research," p. 680. Wiley, New York.

Heinrich, B. 1993. "The Hot-Blooded Insects," p. 601. Harvard Univ. Press, Cambridge, Massachusetts.

Hilbert, D. A., and Logan, J. A. 1983. Empirical model of nymphal development for the migratory grasshopper, *Melanoplus sanguinipes* (Orthoptera: Acrididae). *Environ. Entomol.* **12,** 1–5.

Inglis, G. D., Johnson, D. L., and Goettel, M. S. 1997a. Field and laboratory evaluation of two conidial batches of *Beauveria bassiana* against grasshoppers. *Can. Entomol.* (in press).

Inglis, G. D., Johnson, D. L., and Goettel, M. S. 1997b. Effects of temperature and sunlight on mycosis (*Beauveria bassiana*) of grasshoppers under field conditions. *Environ. Entomol.* (in press).

Inglis, G. D., Johnson, D. L., and Goettel, M. S. 1996a. Effects of temperature and thermoregulation on mycosis by *Beauveria bassiana* in grasshoppers. *Biol. Control.* **7,** 131–139.

Inglis, G. D., Johnson, D. L., and Goettel, M. S. 1996b. An oil-bait bioassay method used to test the efficacy of *Beauveria bassiana* against grasshoppers. *J. Invertebr. Pathol.* **67,** 312–315.

Johnson, D. L., and Goettel, M. S. 1993. Reduction of grasshopper populations following field application of the fungus *Beauveria bassiana. Biocontrol. Sci. Technol.* **3,** 165–175.

Johnson, D. L., Goettel, M. S., Bradley, C., van der Paauw, H., and Maiga, B. 1992. Field trials with the entomopathogenic fungus *Beauveria bassiana* against grasshoppers in Mali, West Africa, July, 1990. *In* "Biological Control of Locusts and Grasshoppers" (C. J. Lomer, and C. Prior, Eds.), pp. 296–310. CAB Int., Wallingford, UK.

Kaya, H. K., Choo, H. Y., Burlando, T. M., and Thurston, G. S. 1995. Integration of entomopathogenic nematodes with *Bacillus thuringiensis* or pesticidal soap for control of insect pests. *Biol. Control.* **5,** 432–441.

Kemp, W. 1986. Thermoregulation in three rangeland grasshopper species. *Can. Entomol.* **118,** 335–343.

Lactin, D. J., and Johnson, D. L. 1995. Temperature-dependent feeding rates of *Melanoplus sanguinipes* nymphs (Orthoptera: Acrididae) in laboratory trials. *Environ. Entomol.* **24,** 1291–1296.

Lactin, D. J., and Johnson, D. L. 1996. Behavioural optimization of body temperature by nymphal grasshoppers (*Melanoplus sanguinipes*, Orthoptera: Acrididae) in temperature gradients established using incandescent bulbs. *J. Therm. Biol.* **21,** 231–238.

Lecuona, R. E., and Alves, S. B. 1988. Efficiency of *Beauveria bassiana* (Bals.) Vuill., *B. brongniartii* (Sacc.) Petch. and granulose virus on *Diatraea saccharalis* (F., 1794) at different temperatures. *J. Appl. Entomol.* **105,** 223–228.

Milliken, G. A., and Johnson, D. E. 1984. "Analysis of Messy Data. Vol. 1: Designed Experiments," p. 473. Van Nostrand–Reinhold, New York.

Milner, R. J. 1997. *Metarhizium flavoviride* (F1985) as a mycoinsecticide for Australian acridids. *In* "Microbial Control of Grasshoppers and Locusts" (M. S. Goettel and D. L. Johnson, Eds.). *Mem. Entomol. Soc. Can.* (in press).

Pickford, R., and Randell, R. L. 1969. A non-diapause strain of the

migratory grasshopper *Melanoplus sanguinipes* (Orthoptera: Acrididae). *Can. Entomol.* **101,** 894–896.

Prior, C., Lomer, C. J., Herren, H., Paraiso, A., Kooyman, C., and Smit, J. J. 1992. The IIBC/IITA/DFPV collaborative research programme on the biological control of locusts and grasshoppers. *In* "Biological Control of Locusts and Grasshoppers" (C. J. Lomer, and C. Prior, Eds.), pp. 8–18. CAB Int., Wallingford, UK.

Roberts, D., and Campbell, A. 1977. Stability of entomopathogenic fungi. *Misc. Publ. Entomol. Soc. Am.* **10,** 19–76.

SAS Institute Inc. 1988. "SAS/Stat User's Guide." SAS Institute, Cary, NC.

Thurston, G. S., Kaya, H. K., and Gaugler, R. 1994. Characterizing the enhanced susceptibility of milky disease-infected scarabaeid grubs to entomopathogenic nematodes. *Biol. Control.* **4,** 67–73.

Xavier, I. J., and Khachatourians, G. G. 1996. Heat-shock response of the entomopathogenic fungus *Beauveria brongniartii*. *Can. J. Microbiol.* **42,** 577–585.