JOURNAL OF INVERTEBRATE PATHOLOGY **67,** 312–315 (1996)
ARTICLE NO. 0048

# NOTE

# An Oil-Bait Bioassay Method Used to Test the Efficacy of *Beauveria bassiana* against Grasshoppers

*Beauveria bassiana* (Balsamo) Vuillemin is being developed as an inundative microbial control agent against grasshoppers. However, some cases of unsatisfactory field efficacy have necessitated the identification of more virulent genotypes and the elucidation of parameters that limit the activity of the fungus. Bioassays are the most reliable means of testing the efficacy of entomopathogens. Since grasshopper nymphs are usually targeted in inundative applications with *B. bassiana* in North America, a reliable bioassay method using nymphs is necessary. However, 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). Here we describe a bait-inoculation method using conidia in oil to test the efficacy of four isolates of *B. bassiana* against grasshoppers.

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, Alberta, Canada, 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 (Butte, Montana) "GHA," an isolate known to be pathogenic to the migratory grasshopper (*Melanoplus sanguinipes* (Fabricius)). All cultures were lyophilized in skim milk. Cultures were established on potato dextrose agar (PDA; Difco) and grown in the dark at 25°C for 7 to 10 days. The germinability of conidia was measured on PDA amended with 0.005% Benlate (DuPont), 0.04% penicillin G, and 0.1% streptomycin after 24 hr incubation at 25°C (Inglis *et al.,* 1996). Within 24 hr of the germination assessments, conidia were collected from the surfaces of the PDA cultures and suspended in sunflower oil (Safflo, Culinar Foods, Inc.). Concentrations of viable conidia in oil were then estimated using a hemocytometer and conidial densities were adjusted so that 0.5$\mu$l contained $1 \times 10^5$, $3.2 \times 10^4$, $1 \times 10^4$, $3.2 \times 10^3$ or $1 \times 10^3$ viable conidia (5.0, 4.5, 4.0, 3.5, or 3.0 $\log_{10}$ conidia).

Field-collected *M. sanguinipes* adults collected in the fall of 1993 and 1994 were placed in cages in the laboratory and permitted to copulate and oviposit into a sand substrate. Egg pods were sifted from the sand, placed in clean moist sand 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 break diapause. After the cold treatment, eggs were allowed to hatch in a sand–vermiculite substrate, and nymphs were reared on a diet of bran and wheat leaves. After 15 to 20 days, third-instar nymphs were individually collected in sterile 20-ml vials stoppered with a sterile polyurethane foam plug and starved for 12 hr before inoculation. Conidial suspensions from each dose were pipetted (0.5 $\mu$l) onto 5-mm-diameter 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 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 hr 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 × 28 × 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 controlled environment chamber (CEC) under a 16L/8D 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 (Campbell Scientific). Relative humidities within the CEC varied from 20 to 55%. Although humidities



0022-2011/96 $18.00

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-hr 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 randomized complete block design 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 died and subsequently produced hyphal growth of *B. bassiana* on moistened filter paper were considered to have died from mycosis; those not producing hyphal growth were classed as "other mortality." No ($P = 0.33$) interaction was observed between isolate and dose in the incidence 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 (Gomez and Gomez, 1984). A Box correction was used as a conservative test for time and time–treatment interactions (Milliken and Johnson, 1984). Subsequent to a significant $F$ test for the time–treatment interaction, means were compared using the

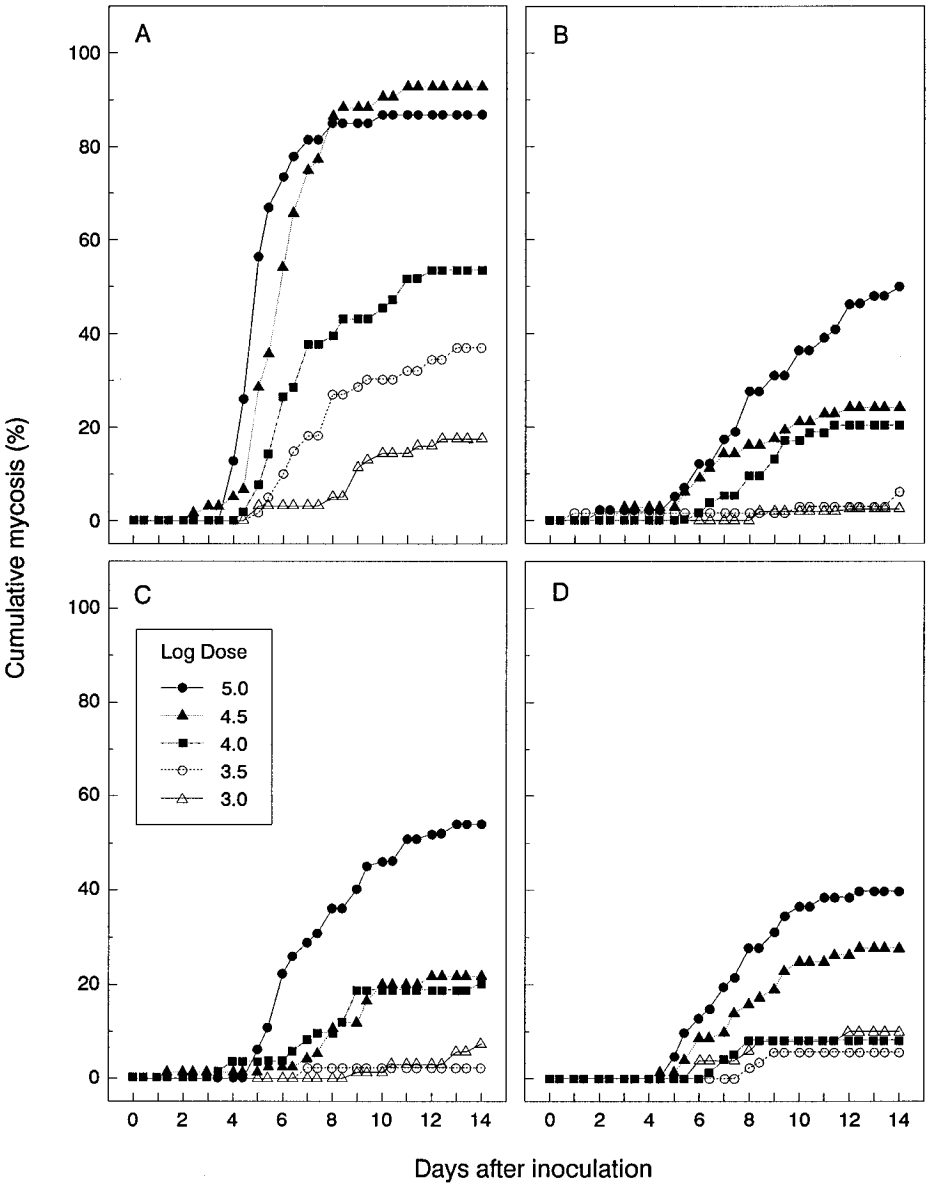


**FIG. 1.** (A–D) Disease progress curves for grasshopper (*Melanoplus sanguinipes*) nymphs ingesting lettuce disks inoculated with *Beauveria bassiana* conidia in oil containing 3.0, 3.5, 4.0, 4.5, or 5.0 $\log_{10}$ conidia per nymph: (A) Mycotech Corp. "GHA" isolate; (B) soil isolate from Canada; (C) soil isolate from Burkina Faso, Africa; and (D) alligator isolate.

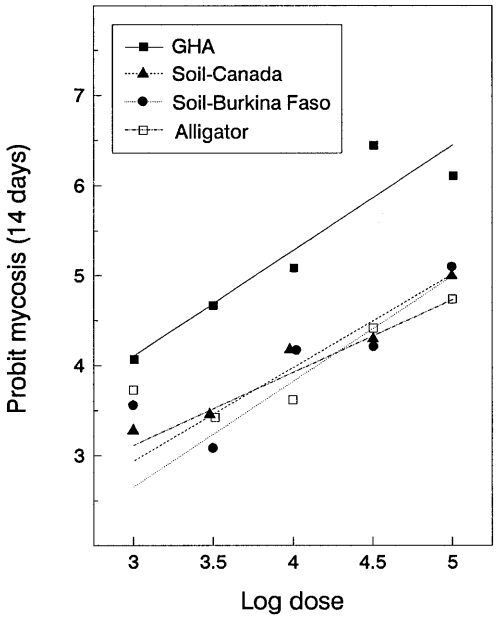


**FIG. 2.** Probit regressions of final mycosis (14 days) by isolate and dose.

least squares means function of SAS (SAS Institute, Inc., 1988). The incidence of final mycosis (14 days) was analyzed with four levels of isolate and five levels of dose; means were also compared using least square means test. 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, Ontario, K1A OC5). The program also tested for goodness of fit, estimated lethal dose ($LD_{50}$) with 95% fiducial limits, and tested for parallelism and for common $y$-intercepts. For isolate–dose combinations where the incidence 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.

The oil-bait method facilitated the rapid inoculation of grasshopper nymphs; within 1 hr, we easily inoculated 350 nymphs. In a subsequent study using the same method, we were able to inoculate 1650 nymphs within a 4-hr period (unpublished data). The oil-bait method unquestionably expedites the inoculation of nymphs relative to direct topical application of conidia. We also observed very low levels of nymphal mortality within 48 hr 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 to nymphs. 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 ($F_{(4,15)} = 5.0$ to 6.0, $P \leq 0.01$) dose response of mycosis was observed (Figs. 1 and 2). Between isolates, substantial differences ($F_{(3,57)} = 27.0$, $P \leq 0.0001$) were observed in the incidence of final mycosis averaged over dose (Fig. 1). With the exception of the 3.0 $\log_{10}$ dose, a higher ($P \leq 0.01$) incidence of final mycosis was observed for the GHA than the alligator and soil isolates. Since there was no ($P = 0.12$) differences among replications, the mycosis data were pooled for probit regression analysis. The median lethal dose observed for the GHA isolate was $5.8 \times 10^3$ conidia per nymph compared to $\geq 9.5 \times 10^4$ conidia per nymph for the soil isolates (Table 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 ($\chi^2 = 2.5$, $df = 3$, $P \geq 0.05$) among isolates (Table 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 ($F_{(3,12)} = 5.7$

**TABLE 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] | $\chi^2$ heterogeneity |
|---|---|---|---|---|
| GHA | $5.8 \times 10^3$ ($3.9 \times 10^3$–$8.3 \times 10^3$) | 0.74a[e] | 1.13 (0.14) | 1.77 |
| Soil, Canada (LRC 14) | $9.5 \times 10^4$ ($5.8 \times 10^4$–$2.3 \times 10^5$) | –0.32b | 1.07 (0.19) | 1.47 |
| Soil, Africa (LRC 30) | $1.0 \times 10^5$ ($6.5 \times 10^4$–$2.2 \times 10^5$) | –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%.

to 18.1, $P \leq 0.05$) between isolates (Fig. 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 5.0 $\log_{10}$ conidial 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.,* 1996). Although infection via 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.,* 1996). 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.

KEY WORDS: *Beauveria bassiana; Melanoplus sanguinipes;* microbial control; grasshopper; fungus; conidia.

We thank Ron Feniuk, Eva Pavlik, and Craig Andrews, Agriculture and Agri-Food Canada (AAFC), Lethbridge, for technical assistance. We also thank Lynne Sigler, University of Alberta Microfungus Collection and Herbarium, for providing the isolate of *B. bassiana* from the alligator, and Jay Yanke, Dicky Yu and Helen McMenamin (AAFC) for critically reviewing the manuscript. Research was funded in part by a grant from Farming for the Future, Alberta Agricultural Research Institute. This manuscript is LRC contribution No. 3879540.

## REFERENCES

Bateman, R. P., Carey, M., Moore, D., and Prior, C. 1993. *Ann. Appl. Biol.* **122,** 145–152.

Ferron, P. 1978. *Ann. Rev. Entomol.* **23,** 409–442.

Goettel, M. S., and Johnson, D. L. 1992. 25th Annual Meeting, Soc. Invertebr. Pathol., Heidelberg, Germany, August 16–21, p. 79.

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

Inglis, G. D., Johnson, D. L., and Goettel, M. S. 1996. *Biocontrol Sci. Technol.,* in press.

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

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

G. DOUGLAS INGLIS*,†
DAN L. JOHNSON*
MARK S. GOETTEL*

** Research Centre*
*Agriculture and Agri-Food Canada*
*P.O. Box 3000*
*Lethbridge, Alberta*
*Canada T1J4B1*

*† Centre for Pest Management*
*Simon Fraser University*
*Burnaby, British Columbia*
*Canada V5A 1S6*

*Received September 25, 1995; accepted January 19, 1996*