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To cite this article: G. DOUGLAS INGLIS , DAN L. JOHNSON & MARK S. GOETTEL (1996) Effect of Bait Substrate and Formulation on Infection of Grasshopper Nymphs by *Beauveria bassiana*, *Biocontrol Science and Technology*, 6:1, 35-50, DOI: [10.1080/09583159650039511](https://doi.org/10.1080/09583159650039511)

To link to this article: <http://dx.doi.org/10.1080/09583159650039511>



Published online: 28 Jun 2010.



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## Effect of Bait Substrate and Formulation on Infection of Grasshopper Nymphs by *Beauveria bassiana*

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(Received for publication 10 May 1995; revised manuscript accepted 15 October 1995)

The effects of two formulations (oil and water) and two bait substrates (lettuce and bran) on infection of grasshopper nymphs (*Melanoplus sanguinipes*) by *Beauveria bassiana* were investigated. More nymphs died of mycosis after they had ingested substrates inoculated with conidia in oil than in water, but there was no difference between the lettuce and bran substrates. Of the conidia recovered in frass, most (95%) were recovered within 24 h of ingestion of the lettuce and bran substrates by nymphs. Significantly more conidia averaged over time were recovered in frass from the water than from the oil formulation. A higher incidence (33–82%) and more rapid onset of mycosis was observed in nymphs that were surface-sterilized before ingestion, compared with those surface-sterilized after ingestion of lettuce and bran substrates inoculated with *B. bassiana* in both formulations. A similar trend was observed in nymphs receiving the sterilization treatment before, rather than after, ingestion of wheat leaves sprayed with conidia in oil or water. Numerous conidia were observed on the heads, thoraxes and abdomens of nymphs that ingested treated lettuce or bran. Modest numbers of conidia were also recovered from the surfaces of nymphs, but no differences were observed between formulations or substrates. However, when nymphs ingested lettuce disks treated with fluorescent dye in either oil or water, more dye was observed on nymphs with the oil formulation. This laboratory study demonstrates that grasshopper nymphs are highly susceptible to infection by conidia formulated on bait substrates, and that the efficacy of the bait relies on the extent to which nymphs become surface-contaminated during ingestion.

**Keywords:** *Beauveria bassiana*, conidia, grasshopper, *Melanoplus sanguinipes*, nymph, bait formulation, oil, infection

### INTRODUCTION

Grasshoppers are economically important pests in arid agro-systems. During population outbreaks in North America, control measures rely almost exclusively on the use of chemical insecticides. Recognition of the deleterious effects of pesticides has prompted the development of alternative, less obtrusive management strategies, such as the use of microbial control agents. The entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillemin, has shown considerable potential for the management of insects (Feng *et al.*, 1994), and its pathogenicity against

grasshoppers in a field environment has been demonstrated (Johnson & Goettel, 1993). However, the efficacy of *B. bassiana* in field environments has been inconsistent, and improved formulation strategies that enhance the persistence and targeting of *B. bassiana* against grasshoppers is desirable.

Bait formulations reduce the quantity of insecticides required to control grasshoppers by improved targeting (Mukerji *et al.*, 1981; Ewen & Mukerji, 1987; Johnson & Henry, 1987). Formulation of entomopathogens as baits is receiving increasing attention, and bran has been used successfully as a substrate for *Nosema locustae* against grasshoppers in field environments (Henry, 1971; Henry *et al.*, 1973; Onsager *et al.*, 1980; Johnson, 1989). The use of *Steinernema feltiae* (Capinera & Hibbard, 1987) and entomopoxvirus (EPV) (McGuire *et al.*, 1991) in baits against grasshoppers has also been reported, but the efficacy of these pathogens in field environments is unknown. The formulation of entomopathogenic fungi as baits against grasshoppers has not been extensively studied, and if baits are to be developed as a biorational formulation for *B. bassiana*, an understanding of the mechanism of infection of nymphs ingesting baits is of paramount importance. Therefore, the objectives of this study were:

- (1) to determine the efficacy of *B. bassiana* in two formulations (oil and water) and on two bait substrates (lettuce and bran) against grasshopper nymphs;
- (2) to elucidate the mechanism(s) by which *B. bassiana* infects grasshopper nymphs that have come in contact with the bait substrates.

## MATERIALS AND METHODS

### Preparation of Inoculum

Dry conidia of *B. bassiana* (GHA isolate) were supplied by Mycotech Corp. (Butte, MT, USA). Conidia were suspended in distilled water and germination percentages were determined on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) amended with 0.005% benlate (DuPont, Wilmington, DE, USA), 0.04% penicillin G and 0.1% streptomycin. After 24 h at  $25 \pm 1^\circ\text{C}$ , conidia were fixed with lactophenol and germination rates were determined by examination of a minimum of 500 conidia from each of two replicate cultures. Conidia were considered viable if germ tubes were greater than  $3 \mu\text{m}$  in length. Immediately prior to use, conidia were suspended in either sterile distilled water or oil; unless indicated otherwise, the oil used was sunflower oil (Safflo, Culinar Foods Inc., Toronto, ON, Canada). To facilitate suspension, conidia were mixed with a micropestle. Concentrations of conidia were estimated using a hemocytometer and the numbers of viable conidia/ml were calculated as the percentage germination  $\times$  the concentration of conidia, as determined from the hemocytometer counts. Except where indicated otherwise, conidial concentrations were adjusted to  $2 \times 10^8$  viable conidia/ml and 0.5- $\mu\text{l}$  aliquots ( $10^5$  conidia) were pipetted on to the bait substrates.

### Grasshoppers

Nymphs of a non-diapause strain of *Melanoplus sanguinipes* (F.) were reared on a diet of bran and wheat leaves at an ambient temperature of  $20\text{--}25^\circ\text{C}$ ; a vertical heat gradient was produced in the cage ( $40 \times 40 \times 30$  cm) by a 25-W incandescent light-bulb. Third instar nymphs were individually collected in sterile 20-ml glass vials stoppered with a sterile polyurethane foam plug and starved for 12 h before inoculation. During the experimental period, nymphs were maintained on a diet of wheat leaves in a controlled environment chamber under a 16 h/8 h light:dark photoperiod and a  $25/20^\circ\text{C}$  day/night temperature regime. The relative humidity (RH) within the chamber varied from 20 to 55% during the experiments.

### Effect of Substrate and Dose

Aliquots of conidial inoculum in water or oil containing  $10^3$ ,  $10^4$  or  $10^5$  conidia were pipetted on to lettuce disks (5 mm in diameter) or wheat bran flakes (approximately  $3 \times 3$  mm). For control treatments, water or oil alone was placed on the bait. The baits were maintained in the dark for 15–30 min before presentation to nymphs in the vials. To prevent loss of the oil carrier and/or sticking of the bait substrate to the wall of the vial, the inoculated bait was pierced with a pin and then suspended from the foam plug approximately 2 cm into the vial. Nymphs in vials were placed at 25°C under artificial light provided by incandescent and fluorescent bulbs. Although the majority of the nymphs consumed the bait within 30 min, nymphs were kept in the vials for 12 h before transfer to individual 240-ml clear plastic containers. Nymphs that molted during the 12-h period or did not consume the entire bait were removed from the experiment. During the subsequent 14 days, unconsumed diet and frass were removed once daily and dead nymphs were removed twice daily. Cadavers were placed on moistened filter papers at 25°C for 3–5 days in the dark, and those that produced hyphal growth characteristic of *B. bassiana* were recorded as infected. Nymphs that survived the 14-day period of the experiment were killed by freezing, and placed on moistened filter paper and incubated as above. The total number of nymphs/treatment combination ranged from 39 to 46.

### Effect of Sterilization and Freezing on Conidial Viability

Conidial suspensions (100  $\mu$ l) in oil or water were applied with an airbrush (Artek, Rockford, IL, USA) at 103 kPa to nymphs in a 10.8-cm diameter by 35.5-cm high Plexiglass cylinder. Nymphs were killed by freezing, thawed, weighed and placed on their ventral surface before spray application. Droplet size, density and distribution patterns were monitored using water- and oil-sensitive papers. To quantify the deposition of conidia, three sterile round glass coverslips (13 mm in diameter) were placed equidistant at the bottom of the spray cylinder. Following the application of conidia, the coverslips were aseptically placed in 5 ml of 0.01 M-sodium phosphate buffer (pH 7.0) amended with 0.05% Tween 80 (buffer–Tween) in 20-ml vials with approximately 0.5 g of sterile sand and washed at ambient temperature for 2 h on a rotary shaker at 300 rpm. After shaking, the wash solution was diluted two to three times in a 10-fold dilution series and 100- $\mu$ l aliquots for each dilution were spread on to semi-selective oatmeal–dodine agar (consisting of 17.5 g of oatmeal agar, 2.5 g of agar, 0.45 g of cyprex, 2.5 mg of crystal violet, 0.2 g of penicillin G and 0.5 g of streptomycin in 500 ml of deionized water; Chase *et al.*, 1986). Cultures were incubated at 25°C for 6–7 days and the numbers of colony-forming units (CFU) were enumerated at the dilution yielding 30–300 *B. bassiana* CFU/dish. The mean number of CFU  $\text{cm}^{-2}$  of coverslip area was then calculated.

Following inoculation, nymphs were maintained in the dark at ambient temperature for 15–20 min. Subsequent treatments were:

- (1) nymphs submerged in 1.0% sodium hypochlorite with 0.05% Tween 80 for 2 min and then rinsed twice in sterile water;
- (2) nymphs submerged in 1.0% sodium hypochlorite without Tween 80 for 2 min and then rinsed twice in sterile water;
- (3) nymphs frozen at  $-20^\circ\text{C}$  for 6 h;
- (4) nymphs maintained at ambient temperature in the dark.

Five nymphs for each treatment were individually macerated in buffer–Tween and CFU were enumerated on oatmeal–dodine agar. Data were calculated as the number of CFU/standardized nymph (adjusted for differential body weight). Reductions in conidial number/replicate ( $x_i$ ) were determined relative to the mean value of the control treatment ( $\bar{X}_c$ ) and were calculated as:

$$\sigma(((\text{CFU/nymph } \bar{X}_c - \text{CFU/nymph } x_i)/\text{CFU/nymph } \bar{X}_c) \times 100)/n$$

If the number of conidia recovered for  $x_i$  was larger than for  $\bar{X}_c$ , the percentage increase in number was calculated as:

$$100((CFU/nymph \bar{X}_c - CFU/nymph x_i)/CFU/nymph x_i) \times 100/n$$

The experiment was repeated once.

### Effect of Surface Sterilization of Grasshopper on Mortality

To measure the relative importance of external versus *per os* infection, the mortality of nymphs surface-sterilized before or after feeding on lettuce or bran substrates treated with *B. bassiana* conidia in oil or water was measured. Nymphs were surface-sterilized at 10°C by submersion in 1.0% sodium hypochlorite for 2 min followed by two rinses in sterile distilled water. Nymphs were surface-sterilized either before placement in the glass vial with the bait substrate or after they had ingested the bait in the vial. Nymphs were then transferred to 21 × 28 × 12 cm Plexiglass cages fitted with a perforated metal floor to minimize contact with frass. There were no temperature differences between the ambient and the cage environment in the controlled environment chamber during the dark period, but the temperature within the cages was 2.5–3.1°C higher than ambient during the light period. Although RH values within the cages were similar to ambient for most of the day and ranged from 24 to 34%, they rose 4–8% higher than ambient levels for a 2–3 h period after placement of the wheat leaves in cages. Cadavers were collected twice daily and were incubated on moistened filter paper to detect internal *B. bassiana*. The total number of nymphs/treatment combination ranged from 48 to 69.

Conidia suspended in an emulsifiable paraffinic-based oil (Mycotech Corp. #9209) or water ( $1.8 \times 10^8$  conidia ml<sup>-1</sup>) were applied to wheat leaves (100 μl) with the Artek airbrush. At the time of application, the numbers of conidia cm<sup>-2</sup> on randomly selected leaf segments/replicate were quantified using a wash/dilution-spread plate technique (Inglis *et al.*, 1993). Nymphs were placed in plastic cages (21 × 6.5 × 8.5 cm) and one-half of the nymphs were surface-sterilized as described previously. Sterilized and non-sterilized nymphs were allowed to feed *ad libitum* on 1.5 g (fresh weight) of the treated wheat within 5 min of conidial application. After 6 h, unconsumed wheat was removed, air dried and weighed and the remaining nymphs were surface-sterilized. Nymphs were then placed in the controlled environment chamber for 16 days, and dead nymphs were removed daily and checked for internal *B. bassiana*. The total number of nymphs/treatment combination ranged from 42 to 49.

### Passage of Conidia Through the Alimentary Canal

Nymphs were fed lettuce or bran bait inoculated with conidia in oil or water and maintained in the controlled environment chamber in 240-ml clear plastic containers as described previously. Control treatments consisted of lettuce disks or bran flakes inoculated with either water or oil. Frass was collected from individual nymphs at 24-h intervals for 5 days, weighed and stored at 5°C for a maximum of 48 h. Frass from nymphs which had molted in the previous 24 h was excluded, and the mean quantity of frass was calculated only for the non-molting nymphs in each replicate. Frass was macerated in 1 ml of buffer-Tween, diluted and spread on oatmeal-dodine agar to recover conidia. Nymphs which survived for 5 days were killed by freezing at -20°C for approximately 6 h, surface-sterilized in 70% ethanol for 1 min and rinsed twice in sterile deionized water. The alimentary canal was then removed aseptically. It was then macerated and the number of CFU in the macerate enumerated on oatmeal-dodine agar.

To determine if conidia passed in frass could act as a source of inoculum, frass collected from 13–20 *B. bassiana*-treated and -untreated nymphs/replicate was placed daily in plastic cages containing 12–20 nymphs not previously exposed to *B. bassiana*. All nymphs were maintained in the controlled environment chamber for 14 days, and dead nymphs were removed daily and checked for internal *B. bassiana*. Introduced frass was not weighed, but the amount of frass produced by nymphs inoculated with conidia decreased noticeably after 3–4 days, due to the effects of mycosis. The total number of nymphs exposed to frass ranged from 45 to 48 nymphs/treatment.

### Deposition of Conidia and Dye on Nymphs

Nymphs were fed bran or lettuce inoculated with conidia suspended in water or oil. Control treatments consisted of nymphs allowed to ingest lettuce treated with just water or oil. Within 1 h of ingestion of the substrates, nymphs were killed by freezing. After freezing, heads and the last abdominal segment were submerged in sterile molten paraffin to prevent possible contamination by conidia from frass or regurgitation. Nymphs were then placed individually in 5 ml of buffer–Tween with sterile sand and washed for 2 h on a rotary shaker. CFUs were enumerated on oatmeal–dodine agar; 12–15 nymphs/bait formulation treatment were used.

Ten additional nymphs fed bran or lettuce inoculated with conidia in water or oil were killed by freezing, fixed in 2% glutaraldehyde, dehydrated in ethanol, critical-point dried in liquid CO<sub>2</sub> sputter-coated with gold and examined with a Hitachi S-570 scanning electron microscope (Nissei Sangyo Canada Inc., Rexdale, Ont., Canada). Twenty-five nymphs were also fed bran flakes or lettuce disks treated with 0.1% (w/w) fluorescent dye (Day-Glo Color Corp., Cleveland, OH, USA) in water or oil. Following ingestion, nymphs were immediately killed by freezing, thawed and photographed under long-wave UV light.

### Statistical Analyses

All computations were performed using the GLM, REG and LIFEREG procedures of SAS (SAS Institute Inc., 1988). Nymphs that died and subsequently produced hyphal growth of *B. bassiana* following incubation on moistened filter paper were considered to have died from mycosis; those not producing hyphal growth were recorded as ‘other mortality’. With the exception of the influence of sterilization procedure and deposition of conidia and dye experiments which were repeated, all experiments were replicated in time (minimum of three replicates) and analyzed as randomized complete block designs. The influence of sterilization and freezing on conidial viability and the effect of sterilization and formulation on ingestion of wheat leaves were analyzed using one-way analysis of variance (ANOVA). In conjunction with a significant *F*-test ( $P \leq 0.05$ ), Tukey’s studentized range test ( $\alpha = 0.05$ ) was used to separate treatment means. When disease progress or passage of conidia over time was included in the model, the experiments were analyzed as split-plots in time (Gomez & Gomez, 1984). A Box correction was used as a conservative test for time (*t*) and time–treatment interactions (Milliken & Johnson, 1984); the Box correction reduces the degrees of freedom for time (*t*), the time–treatment interactions and the residual error (time) by  $t - 1$ . In all instances, residuals were plotted against predicted values, and, where necessary, the appropriate transformations were used to normalize the variance. The conidial population data were always log<sub>10</sub> transformed, and log values for the means and standard errors of the mean (SE) are presented in parentheses throughout the text. In several instances, an arcsine transformation was applied to the incidence of mycosis data, but untransformed means and SE in parentheses are presented. For each treatment–dose combination (influence of substrate and dose experiment), the mycosis data were fitted to a Weibull function, and median lethal times with upper and lower 95% confidence limits (CL) were estimated.

## RESULTS

### Effect of Substrate and Dose

The grasshopper mortality from mycosis increased with conidial dose, and this effect was greater for nymphs consuming baits inoculated with conidia suspended in oil than those treated with conidia in water ( $F = 11.0$ ; degrees of freedom (df) = 2, 21;  $P = 0.0006$ ) (Figure 1). Disease development was more rapid among nymphs fed bait substrates inoculated with conidia in oil on lettuce ( $F = 18.2$ ; df = 1, 4;  $P < 0.01$ ) and bran ( $F = 28.5$ , df = 1, 4;  $P < 0.01$ ). For the oil formulation, LT<sub>50</sub> values at the 10<sup>5</sup> dose were 4.9 (95% CL = 4.6–5.2) and 6.0 (95% CL = 5.3–6.7) days for the lettuce and bran substrates respectively. For the water formulation, LT<sub>50</sub> values at the same dose were 9.8 (95% CL = 8.2–11.8) and 11.0 (95% CL = 9.2–13.2) days on lettuce and bran respectively. In contrast to the conspicuous differences between formulations, there was no difference between substrates ( $F = 1.8$ ;

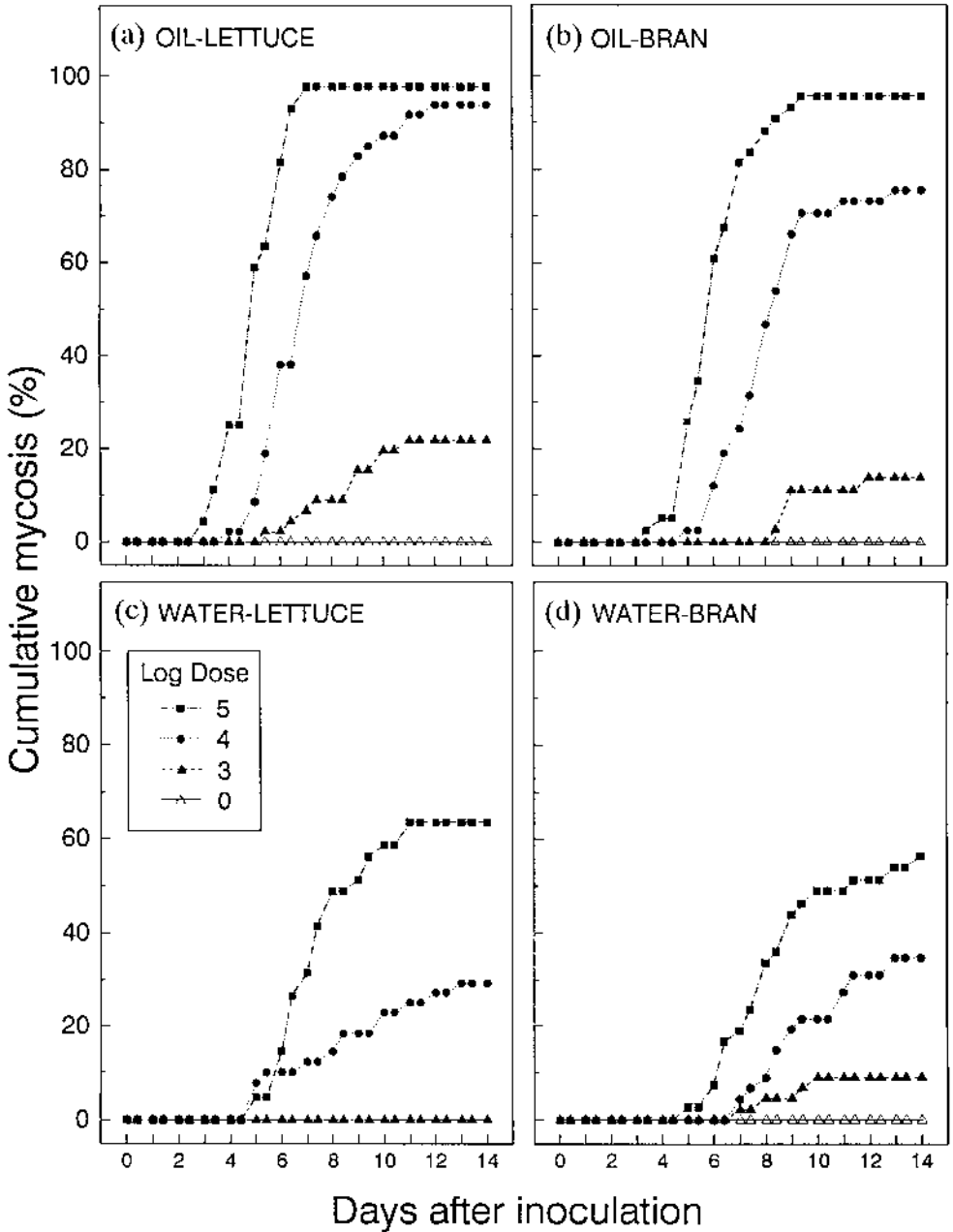


FIGURE 1. Cumulative mortality due to mycosis (%) in grasshopper (*M. sanguinipes*) nymphs that fed on lettuce and bran substrates inoculated with *B. bassiana* conidia in oil or water.

df = 1, 21;  $P = 0.20$ ) in the incidence of total mycosis. Also the rate of disease development did not differ between substrates for either the oil ( $F = 5.45$ ; df = 1, 4;  $P > 0.05$ ) or water ( $F = 1.23$ ; df = 1, 4;  $P > 0.05$ ) formulation. Mortality not attributed to *B. bassiana* ranged from 2.4 (2.4) to

TABLE 1. Effect of freezing or exposure to sodium hypochlorite on recovery of viable *B. bassiana* conidia from the surface of *M. sanguinipes* nymphs sprayed with conidia in oil or water

Treatment	Oil		Water	
	log <sub>10</sub> CFU/nymph <sup>a</sup>	Reduction (%)	log <sub>10</sub> CFU/nymph <sup>a</sup>	Reduction (%)
Untreated (post-spray)	3.92 (0.06) <sup>b</sup>	8.5 (10.8)	3.96 (0.13) <sup>b</sup>	10.5 (14.5)
Frozen (-20°C for 6 h)	3.91 (0.05)a	10.3 (9.5)	4.13 (0.05)a	-8.8 (9.4)
NaOCl (1%)	2.05 (0.25)b	97.6 (0.6)	1.75 (0.33)b	98.2 (0.7)
NaOCl + Tween (0.05%)	1.91 (0.28)b	97.4 (0.9)	1.45 (0.18)b	99.6 (0.1)

<sup>a</sup> Conidia (log<sub>10</sub> CFU/nymph) recovered from nymphs (standardized by weight) sprayed with conidia in oil or water. Values in parentheses following means represent standard errors of the means.

<sup>b</sup> Means not followed by the same letter within each formulation-substrate group are significantly different ( $\alpha = 0.05$ ) according to Tukey's studentized range test. The experiment was conducted twice.

20.6% (10.7), and was similar between formulations ( $F = 0.11$ ;  $df = 1, 29$ ;  $P = 0.74$ ), substrates ( $F = 0.03$ ;  $df = 1, 29$ ;  $P = 0.87$ ) and doses ( $F = 2.2$ ;  $df = 3, 29$ ;  $P = 0.10$ ).

### Effect of Sterilization and Freezing on Conidial Viability

Surface-sterilization of nymphs by submersion in 1% sodium hypochlorite for 2 min reduced ( $\alpha = 0.05$ ) the number of viable conidia on nymphs by more than 97% (Table 1). Nevertheless, considerable numbers of conidia (46–240 CFU/nymph) were recoverable from treated nymphs. Amendment of sodium hypochlorite with Tween 80 (0.05%) did not significantly increase ( $\alpha = 0.05$ ) the efficacy of sterilization. Freezing nymphs (-20°C for 6 h) had no effect ( $\alpha = 0.05$ ) on conidial survival (Table 1). Whether conidia were applied to nymphs in oil or water also had no effect ( $F = 0.86$ ;  $df = 1, 63$ ;  $P = 0.36$ ).

### Effect of Surface Sterilization of Grasshoppers on Mortality

The incidence of total mycosis (14 days) in nymphs that were surface-sterilized before ingesting lettuce or bran substrates infested with conidia in oil or water was 33–82% greater ( $F = 47.0$ ;  $df = 1, 21$ ;  $P \leq 0.0001$ ) than that observed in nymphs sterilized after ingesting the substrates (Figure 2). For all formulation-substrate combinations except oil on lettuce ( $F = 4.2$ ,  $df = 1, 6$ ;  $P > 0.05$ ), the rate of mycosis development was slower ( $F = 8.0$  to  $21.2$ ;  $df = 1, 6$ ;  $P < 0.05$ ) for the post-sterilization treatment. With both sterilization treatments, the cumulative incidence of mycosis ( $F = 54.6$ ;  $df = 1, 21$ ;  $P \leq 0.0001$ ) was higher among nymphs ingesting conidia applied in oil on lettuce than in nymphs ingesting conidia in oil on bran or water applied to either substrate; a weak interaction ( $F = 5.8$ ;  $df = 1, 21$ ;  $P = 0.025$ ) was observed between formulation and substrate. Total mortality due to mycosis was higher ( $F = 27.1$ ;  $df = 1, 21$ ;  $P \leq 0.0001$ ) in nymphs that ingested conidia on lettuce than bran for both the oil and water formulations. Mortality not attributed to *B. bassiana* ranged from 18.4 (11.2) to 33.5% (11.8), and was not influenced by sterilization treatment ( $F = 0.06$ ;  $df = 1, 21$ ;  $P = 0.80$ ), formulation ( $F = 2.1$ ;  $df = 1, 21$ ;  $P = 0.17$ ) or substrate ( $F = 0.01$ ;  $df = 1, 21$ ;  $P = 0.93$ ).

To obtain a distribution of conidia on foliage that would be more representative of a field test, conidia in oil or water were applied to wheat leaves with an airbrush. Conspicuously less free oil was evident on the wheat leaves than on the lettuce substrate of the preceding experiment. Both oil and water droplets appeared to be uniform in distribution and size on water- and oil-sensitive papers, and similar numbers of conidia applied in oil (4.72, standard error (SE) = 0.039 log CFU cm<sup>-2</sup>) and water (4.58, SE = 0.034 log CFU cm<sup>-2</sup>) were recovered from wheat leaves. For the oil formulation, 44.4% (5.0) mycosis was observed over 16 days in nymphs sterilized prior to ingestion of inoculated wheat as compared with 10.0% (5.8) mycosis in



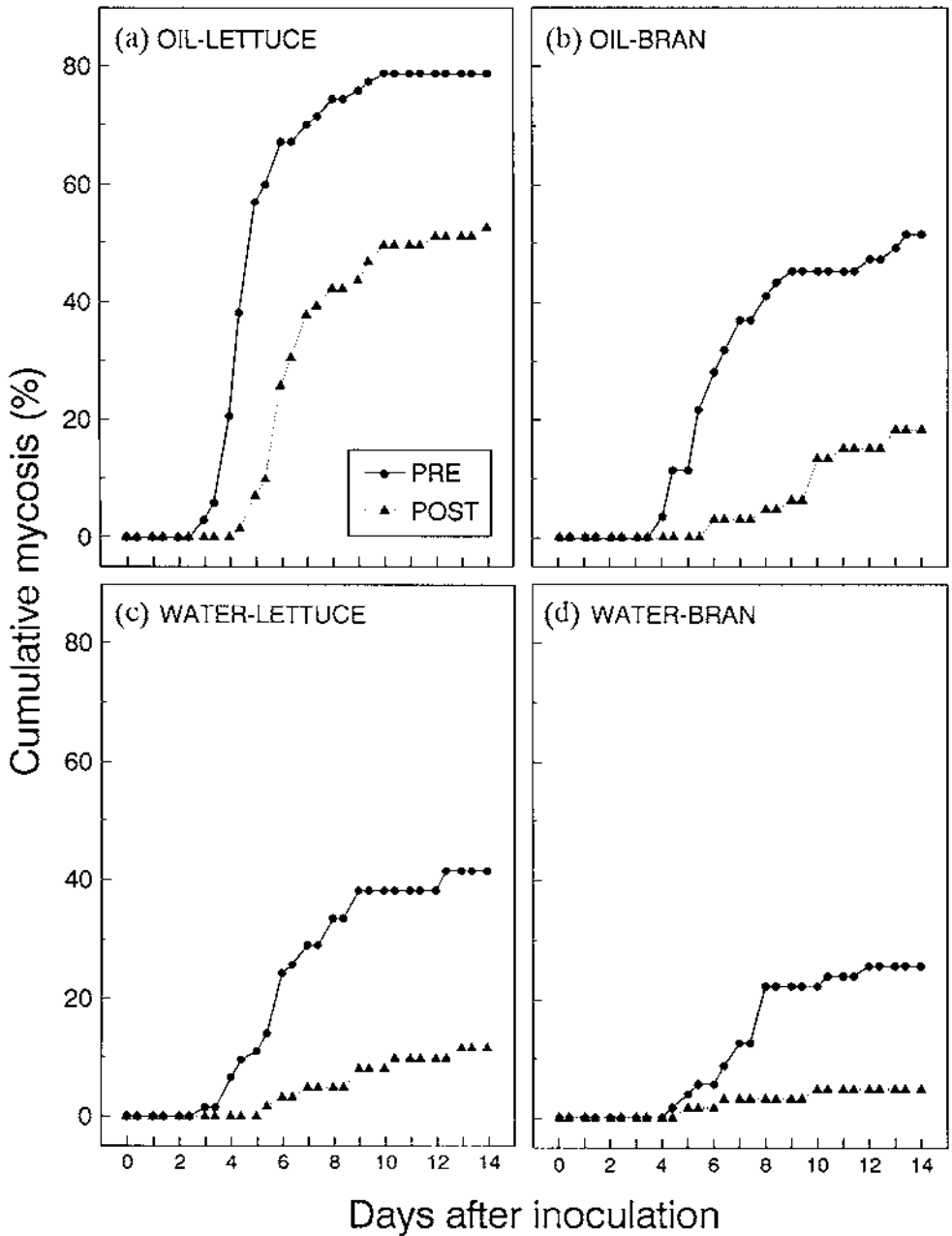


FIGURE 2. Cumulative mortality due to mycosis (%) in grasshopper (*M. sanguinipes*) nymphs surface-sterilized before (pre-) and after (post-) ingestion of lettuce and bran substrates inoculated with  $10^5$  *B. bassiana* conidia in oil or water.

those sterilized after ingestion (Figure 3). For the water formulation, the incidence of mycosis was 34.2% (11.1) and 13.2% (9.4) nymphs sterilized before and after ingestion of inoculated wheat respectively. The sterilization treatment ( $F = 10.9$ ;  $df = 1, 6$ ;  $P = 0.016$ ) but not formu-

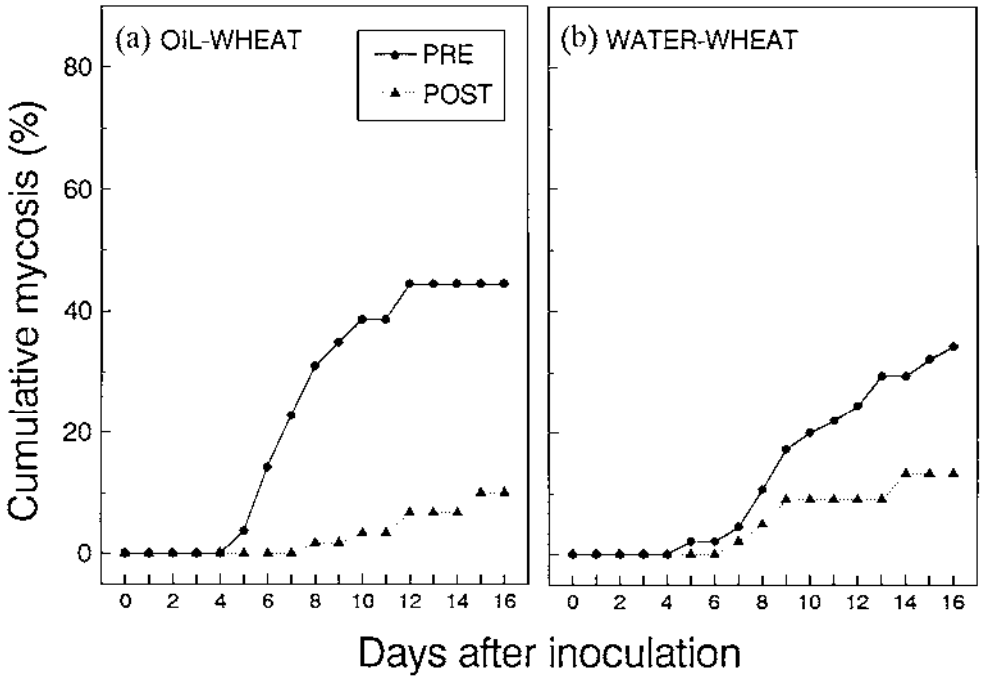


FIGURE 3. Cumulative mortality due to mycosis (%) in grasshopper (*M. sanguinipes*) nymphs surface-sterilized before (pre-) and after (post-) ingestion of wheat leaves sprayed with *B. bassiana* conidia in oil or water.

lation ( $F=0.17$ ;  $df=1, 6$ ;  $P=0.69$ ) nor the interaction between formulation and treatment ( $F=0.63$ ;  $df=1, 6$ ;  $P=0.46$ ) influenced the amount of mycosis observed after 16 days. For the oil ( $F=15.9$ ;  $df=1, 4$ ;  $P<0.05$ ) but not the water ( $F=1.7$ ;  $df=1, 4$ ;  $P>0.05$ ) formulation, the development of mycosis was more rapid in nymphs sterilized before than after ingestion of conidia. The incidence of non-*Beauveria* mortality was relatively high, ranging from 20.9 (12.1) to 53.7% (6.7), but was not influenced by formulation ( $F=0.07$ ;  $df=2, 10$ ;  $F=0.94$ ) or sterilization treatment ( $F=0.13$ ;  $df=1, 10$ ;  $P=0.72$ ).

To determine if the oil formulation on wheat leaves or treatment of nymphs with sodium hypochlorite affected consumption, uningested leaf material was collected after 6 h. Weights of unconsumed leaves from cages with nymphs sterilized before feeding on treated foliage were 92.8 (7.1), 131.4 (10.0) and 56.8 (19.2) mg for the water, oil and control (untreated) treatments respectively. For nymphs sterilized after feeding on treated foliage, the weights of uningested foliage were 91.3 (3.4), 111.1 (11.9) and 61.8 (8.0) for the water, oil and control treatments respectively. The timing of the sterilization treatment (before or after food presentation) had no effect ( $F=0.32$ ;  $df=1, 10$ ;  $P=0.58$ ) on the amount of foliage consumed. However, formulation influenced the amount of leaves ingested ( $F=13.1$ ;  $df=2, 10$ ;  $P=0.0016$ ) independent of the sterilization treatment ( $F=0.59$ ;  $df=2, 10$ ;  $P=0.57$ ). Less ( $\alpha=0.05$ ) foliage treated with conidia in oil was consumed by grasshoppers relative to untreated leaves, whereas similar amounts ( $\alpha=0.05$ ) of water and untreated leaves were consumed.

#### Passage of Conidia Through the Alimentary Canal

Most of the conidia recovered from the frass were recovered within 24 h of ingestion, but small numbers of conidia were recovered for up to 5 days after ingestion (Figure 4). Only 1.3–2.5% and 7.7–11.7% of the conidia inoculated on to baits in oil or water respectively were recovered

in frass. For both formulations, the number of conidia recovered in frass decreased over time ( $F = 83.7$ ;  $df = 1, 10$ ;  $P < 0.01$ ). There was no interaction between formulation and time ( $F = 2.5$ ;  $df = 1, 10$ ;  $P > 0.05$ ) but averaged over time, fewer conidia ( $F = 9.3$ ;  $df = 1, 7$ ;  $P = 0.019$ ) were recovered from the frass of nymphs fed baits treated with the oil formulation. The substrate had no effect ( $F = 0.47$ ;  $df = 1, 7$ ;  $P = 0.52$ ) on conidial passage. After 5 days, conidia were recovered from the alimentary canals of only 7/47 nymphs (14.9%). There was no influence of formulation ( $F = 4.5$ ;  $df = 1, 10$ ;  $P = 0.060$ ) or substrate ( $F = 0.46$ ;  $df = 1, 10$ ;  $P = 0.51$ ) on the presence of conidia in guts. Mean numbers of conidia recovered from alimentary canals ranged from 0.13 (0.13) to 0.79 (0.16) log CFU/nymph.

Most nymphs molted during the 5-day incubation period, causing considerable variability in frass production despite the removal of individuals that molted during each 24-h collection period. No differences ( $F = 1.1$ – $2.0$ ;  $df = 1, 5$ ;  $P > 0.05$ ) were observed in the amount of frass produced by nymphs between the control and *B. bassiana* treatments over time within each of the formulation–substrate combinations.

No mycosis was observed in test nymphs exposed to frass from the nymphs fed conidia in oil on lettuce, even though the incidence of mycosis in the nymphs from which the frass was collected was 93.8% (3.5) after 10 days. The density of conidia in this frass was measured in only one of the three replicates and only in frass produced during the first 24-h period after inoculation; the conidial populations averaged 2.41 (0.23) log CFU mg<sup>-1</sup> of frass. No conidia were recovered from the frass of control nymphs. The incidence of other mortality was similar ( $F = 0.27$ ,  $df = 3, 6$ ;  $P = 0.84$ ) between treatments and ranged from 8.6 (1.7) to 13.9% (4.9).

### Deposition of Conidia and Dye

Relatively small numbers of conidia were recovered from the surfaces of nymphs ingesting lettuce or bran inoculated with conidia in either oil or water. Conidial populations ranged from 1.7 (0.30) to 2.1 (0.26) log CFU/nymph. There was no effect due to formulation ( $F = 0.03$ ;  $df = 1, 34$ ;  $P = 0.87$ ), substrate ( $F = 0.06$ ;  $df = 1, 34$ ;  $P = 0.80$ ) or the interaction between them ( $F = 1.6$ ;  $df = 1, 34$ ;  $P = 0.22$ ). Conidia were unevenly distributed on the integument, and were observed on the thorax, abdomen and head. In most instances, conidia were clumped (Figure 5). Most of the nymphs examined had regurgitated, and conidia observed near their mouthparts were frequently observed to be regurgitate-coated (Figure 5(a)). When nymphs were fed bait treated with fluorescent dye in either oil or water, considerably more dye was observed on nymphs ingesting baits treated with oil (Figure 6). Most of the dye was observed on the head (including antennae and mouthparts), legs (in particular the forelegs) and thorax (Figure 6(a)).

## DISCUSSION

Grasshopper nymphs ingesting bait substrates (lettuce or wheat bran) inoculated with conidia in oil or water are susceptible to infection under laboratory conditions. The formulation of conidia in oil was consistently more efficacious than in water, but the superiority of the lettuce substrate relative to bran was less evident. Although *B. bassiana* formulated as baits has been used against a variety of non-acridid insect pests (Feng *et al.*, 1994), bait formulations of entomopathogenic fungi for the control of grasshoppers have been little studied, despite the common use of baits for the application of insecticides and *N. locustae*. Moore and Erlandson (1988) reported that *B. bassiana* (10<sup>5</sup> conidia) applied in water to lettuce disks caused significant mortality in nymphs, and the incidence of disease that they observed (53%) was similar to that observed in the present study. Johnson *et al.* (1988) also found that the application of *Verticillium lecanii* in bran caused significant mortality in caged grasshoppers. Although *B. bassiana* conidia formulated as a bran bait caused significant reductions of field populations of grasshoppers (Johnson & Goettel, 1993), it was speculated that infection occurred from conidia liberated from the bran substrate during application. Furthermore, Johnson and Goettel (1993) observed that mycosis was low in nymphs

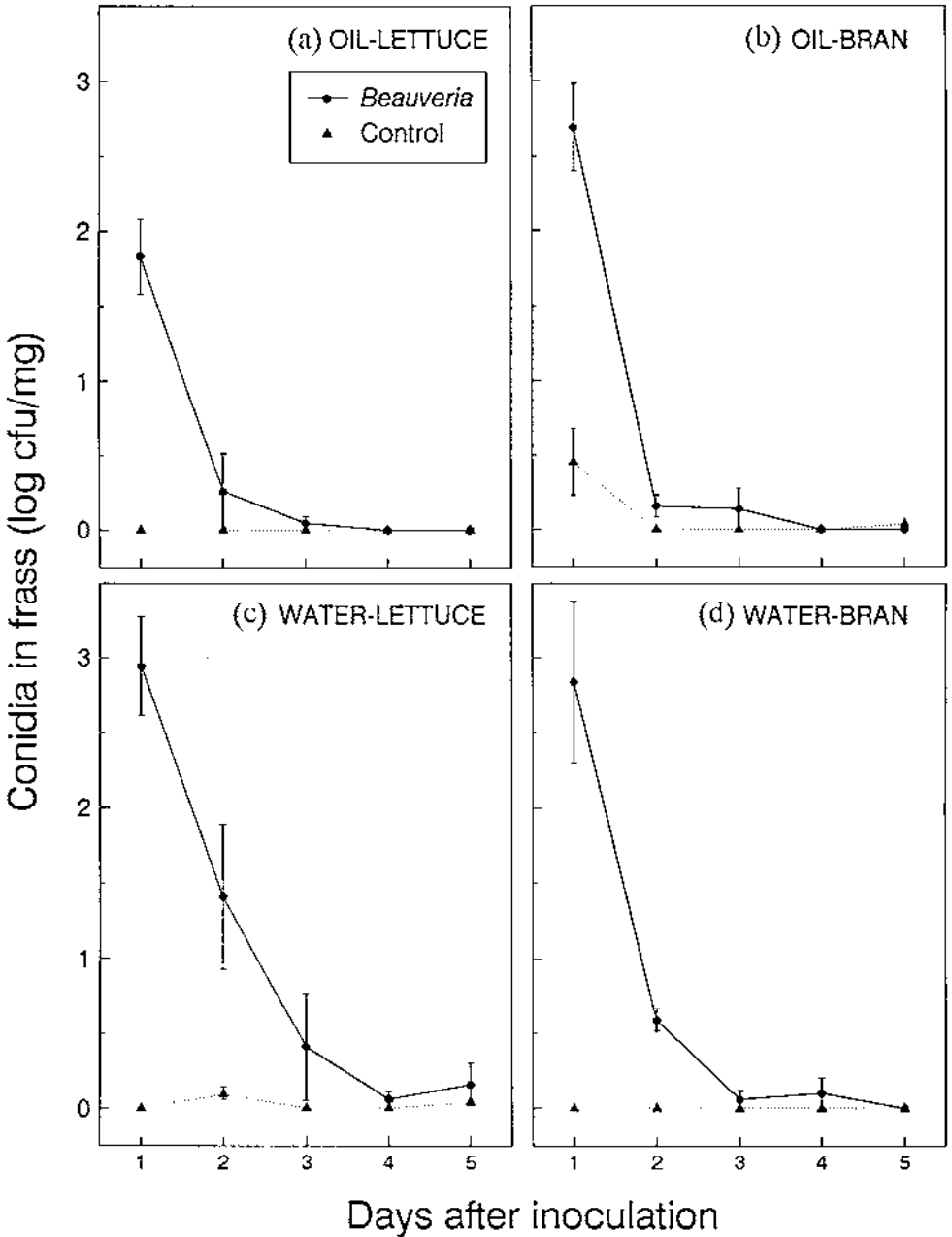


FIGURE 4. Recovery of ingested *B. bassiana* conidia in frass (log<sub>10</sub> CFU mg<sup>-1</sup> fresh weight) from grasshopper (*M. sanguinipes*) nymphs that were fed lettuce and bran substrates inoculated with 10<sup>5</sup> *B. bassiana* conidia in oil or water or with oil or water alone (control).

ingesting the bran in the laboratory, raising a question as to the efficacy of *B. bassiana* applied as a bran bait. Despite anecdotal accounts of successful use of bait formulations of entomopathogenic fungi, very little information is available on the mode of action of these fungi in/on baits.

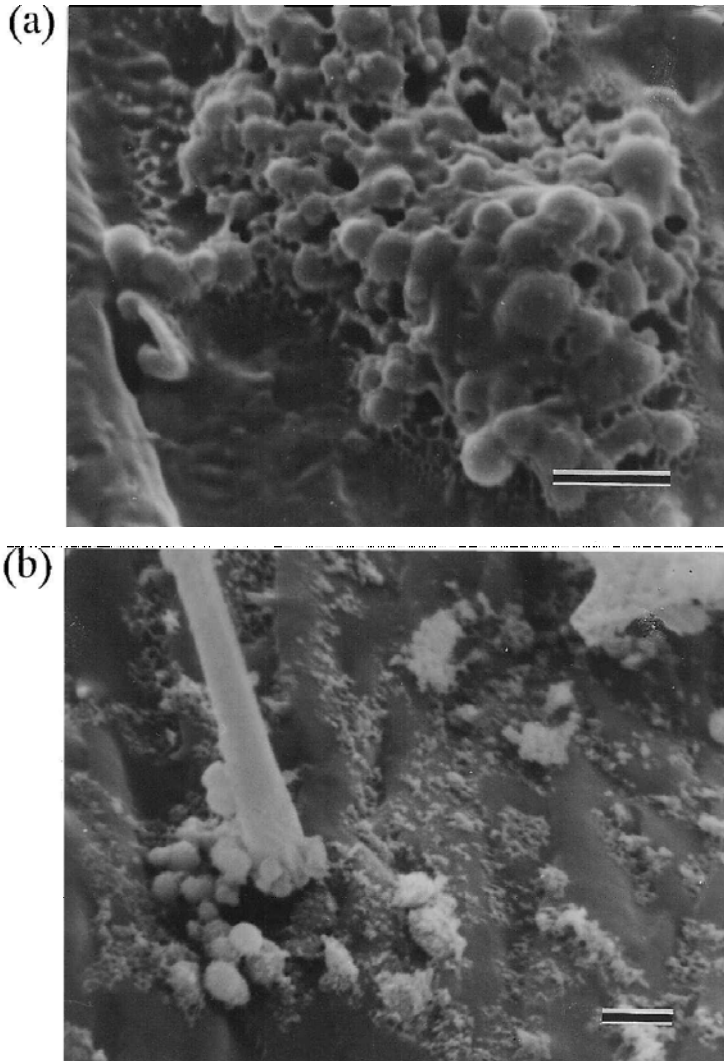


FIGURE 5. Scanning electron micrographs of *B. bassiana* conidia on grasshopper (*M. sanguinipes*) nymphs that ingested lettuce treated with conidia in oil (bars = 5  $\mu$ m): (a) regurgitate-coated conidia on head (labrum); (b) conidia on abdominal tergum.

An understanding of the mechanism(s) of action is necessary if entomopathogenic fungi are to be effectively deployed in bait formulations.

This study indicates that grasshopper nymphs become surface-contaminated with conidia during ingestion of the bait substrate, and that infection then occurs through the external integument. Numerous conidia were observed on the surfaces of nymphs that ingested baits, and conidia were also recovered from nymphs that had been surface-washed (< 130 conidia/nymph on average), even though the heads and posterior abdominal segments had been coated with paraffin. Furthermore, when nymphs were fed baits treated with fluorescent dye, substantial quantities of dye were observed on the nymphs, mostly on body parts involved in the detection and handling of food (mouthparts, antennae and forelegs) or on the parts of the body that would most likely come into contact with the bait substrate (head sclerites and the ventral surface of the

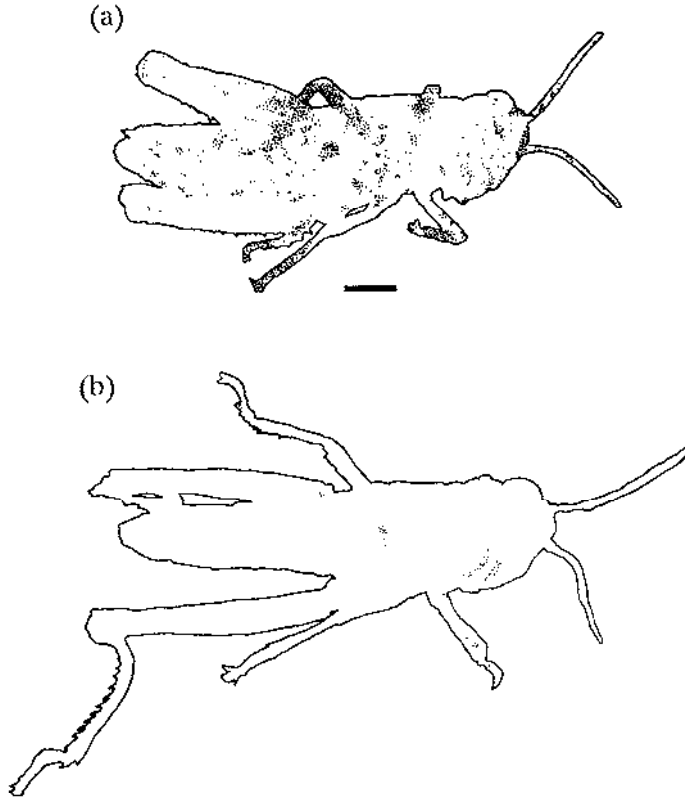


FIGURE 6. Deposition of dye (dots) on *M. sanguinipes* nymphs (bar = 1 mm). Nymphs were fed lettuce disks treated with fluorescent dye in oil (a) and water (b), and representative drawings were traced from color photographs taken under UV light.

thorax) (Figure 6). Infection of insects by *B. bassiana* occurs most frequently through the external integument (Ferron, 1978). *Per os* infection of insects by entomopathogenic fungi has also been demonstrated using both histopathological and indirect methods (Dillon & Charnley, 1991), and Veen (1966) observed hyphae in the maxillary palps and heads of locust nymphs sterilized after they ingested *M. anisopliae* conidia on leaves, thereby suggesting an infection site in the mouth region. In this study, a surface-sterilization technique was employed in an attempt to show quantitatively the importance of external versus *per os* infection in grasshopper nymphs feeding on baits. Conidia of *B. bassiana* are rapidly killed by low concentrations of sodium hypochlorite (Ignoffo & Dutky, 1963), and sodium hypochlorite was confirmed to kill the majority (>97%) of conidia on grasshopper nymphs. In nymphs sterilized after the ingestion of substrates inoculated with *B. bassiana*, 33–82% less mycosis was observed than in nymphs sterilized before ingestion of the baits. This suggests that infection occurs largely through the external integument. However, some mycosis also occurred in grasshopper nymphs sterilized after ingestion, and although infection may have occurred *per os*, additional possibilities include infection following the surface contamination of nymphs with *B. bassiana* conidia excreted in feces, as described previously for Colorado potato beetles (Allee *et al.*, 1990) and/or external infection due to the incomplete sterilization of nymphs. Inoculation of nymphs from conidia in

frass seems unlikely in grasshopper nymphs, as frass pellets are encapsulated by peritrophic membrane (Dillon & Charnley, 1991). Further, we observed no mycosis in nymphs exposed to frass that contained conidia. A threshold of inoculum is required to cause disease in insects, and although the quantities of viable conidia present on nymphs after sterilization would not normally be sufficient to cause mycosis, it is possible the submersion of nymphs in sodium hypochlorite predisposed them to infection. Histological studies will be necessary to ascertain if *per os* infection by *B. bassiana* occurs in acridids.

The alimentary canal of insects is considered to be a relatively inhospitable environment for fungi (Dillon & Charnley, 1991). However, many microorganisms can survive passage through the alimentary canal of acridids, including bacteria (Mead *et al.*, 1988; Chapco & Kelln, 1994) and filamentous fungi (Dillon & Charnley, 1991). It was observed that some ingested *B. bassiana* conidia passed through the alimentary canal of *M. sanguinipes* nymphs and that viable conidia could be recovered from frass. Acridids have a rapid alimentary transit time of 2–6 h (Ueckert & Hansen, 1972; Dillon & Charnley, 1986; Mead *et al.*, 1988). The majority of the *B. bassiana* conidia that passed through the alimentary canal of grasshopper nymphs did so within 24 h of ingestion, as has been reported for other fungal propagules (Hasan, 1982; Huang & Harper, 1985; Dillon & Charnley, 1986). However, very small proportions of the conidia placed on the bait substrates were subsequently recovered in frass (1.3–11.7%). Loss of conidial viability may have occurred during transit through the alimentary canal. In addition, the maceration spread-plate technique measures CFU and not necessarily individual conidia, and therefore populations may have been underestimated due to the clumping of conidia. For successful infection via the gut, propagules of a fungus must remain in the alimentary canal for a sufficient time to permit germination and penetration, as has been observed for *M. anisopliae* conidia in the hindgut of the desert locust (Dillon & Charnley, 1986). However, in *M. sanguinipes* nymphs, the rapid excretion of conidia in frass along with the low incidence of conidial retention in alimentary canals (viable conidia were found in the guts of only 15% of the nymphs 5 days after bait ingestion) is consistent with the apparent importance of an external mechanism of infection in grasshoppers ingesting bait formulations of *B. bassiana*.

When oil was used as a carrier for *B. bassiana*, more mycosis was observed in nymphs than when water was used, regardless of the bait substrate ingested. This observation is in agreement with the findings from a preliminary study (Goettel & Johnson, 1992). Possible advantages of the use of oil, rather than water, as a carrier of fungal propagules include:

- (1) infection at lower humidities (Bateman *et al.*, 1993);
- (2) stimulated germination (Winder & Van Dyke, 1990);
- (3) longer duration of viability (Prior *et al.*, 1988);
- (4) decreased sensitivity to high temperature in storage (McClatchie *et al.*, 1994);
- (5) decreased sensitivity to UV radiation (Moore *et al.*, 1993; Inglis *et al.*, 1995);
- (6) enhanced attachment to the hydrophobic surfaces of insect integument.

In addition, the formulation of *B. bassiana* in oil may improve the adhesion of the conidia to the bait, and thereby reduce the dislodgement of inoculum from the bait substrate during application (Johnson & Goettel, 1993). Despite the superiority of formulation in oil relative to water in the present study, little difference was observed in mycosis when conidia in oil or water were applied to wheat leaves with an airbrush. Inglis *et al.* (1993) also observed no difference in infection of nymphs fed wheatgrass or alfalfa leaves sprayed with *B. bassiana* conidia in oil or water in the field, and that the oil was rapidly absorbed into leaf tissues following application at ultra-low volumes (ULV). In this study, in contrast to ULV application, a considerable volume of the oil pipetted onto the lettuce, and to a lesser degree on to the bran substrate, remained unabsorbed; in the surface-sterilization experiment, absorption of oil into bran may explain the decreased efficacy observed relative to the lettuce substrate. Conspicuously more fluorescent dye was also observed on nymphs ingesting bait substrates treated with dye in oil than in water, suggesting the efficacy of the oil relative to the water formulation was due to the increased coverage of nymphs with the oil carrier.

This study demonstrates that grasshopper nymphs are highly susceptible to infection by *B. bassiana* formulated on bait substrates, and that the efficacy of bait formulation appears to be dependent on the degree to which nymphs become surface-contaminated during handling and ingestion of the bait. Therefore, future development of bait formulations should focus on baits that promote tactile handling by the grasshoppers, thereby enhancing transfer of conidia to the integument surface.

#### ACKNOWLEDGEMENTS

The authors thank the following people at the Agriculture and Agri-Food Canada (AAFC) Lethbridge Research Centre (LRC): Ron Feniuk for his technical assistance, Eva Pavlik for providing grasshopper eggs, Ken Sinclair for photographing the nymphs and Toby Entz for his statistical advice. The authors also wish to thank Dr Martin Erlandson (AAFC, Saskatoon) and Dr Kevin Floate, Dr Bob Byers and Ms Helen McMenamin (AAFC, Lethbridge) 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 #3879504.

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