Persistence of the Entomopathogenic Fungus, *Beauveria bassiana*, on Phylloplanes of Crested Wheatgrass and Alfalfa

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The influence of three formulations (water, oil, and a 5% oil emulsion) and two canopy positions (top and middle) on the persistence of conidia of *Beauveria bassiana* on phylloplanes of alfalfa (*Medicago sativa*) and crested wheatgrass (*Agropyron cristatum*) was investigated. Penetration into the canopy and coverage of leaves by conidia in oil applied with an ultralow volume (ULV) applicator was equal to that of conidia formulated in water–Tween or the oil emulsion applied at high volumes. Numbers of *B. bassiana* recovered from the top of the canopy immediately following application ranged from 8.3×10^3 to 1.2×10^5 colony-forming units (cfu)/ cm^2 for alfalfa and 5.1×10^3 to 3.5×10^4 cfu/cm^3 for wheatgrass. In alfalfa, but not wheatgrass, the canopy influenced penetration of conidia as evidenced by the significant effect of sampling height. Conidia were relatively short lived on leaves at the top of the canopy in both crops and, by 4 days, conidial populations were reduced by more than 75%. At the middle of the canopy, conidia persisted longer on phylloplanes of both crops. This observation was more pronounced in alfalfa than in wheatgrass; at 16 days populations were reduced by more than 99% on wheatgrass leaves and by 28 to 85% on alfalfa leaves. Formulation had no obvious effect on the persistence of conidia. Mortality of grasshopper nymphs (*Melanoplus sanguinipes*) fed leaves sprayed with conidia from the top of the canopy corresponded to the conidial population data. Immediately after application of conidia, nymph mortality attributed to *B. bassiana* ranged from 31 to 58%, whereas mortality of nymphs fed leaves 2 days postapplication of conidia ranged from 0 to 5.5%. Methods to increase the persistence of conidia are required if plant surfaces are targeted in a strategy to control grasshoppers with *B. bassiana*. © 1993 Academic Press, Inc.

KEY WORDS: *Beauveria bassiana*; persistence; crested wheatgrass; *Agropyron cristatum*; alfalfa; *Medicago sativa*; phylloplanes; grasshopper; *Melanoplus sanguinipes*; formulation oil; ultralow volume.

INTRODUCTION

There are several reports of field infection of acridids by *Beauveria bassiana* (Bals.) Vuill. (MacLeod, 1954;

Humber and Soper, 1986; Li, 1988; Moore and Erlandson, 1988) and pathogenicity of *B. bassiana* toward grasshoppers has been demonstrated under laboratory (Marcandier and Khachatourians, 1987; Johnson *et al.*, 1988; Moore and Erlandson, 1988; Bidochka and Khachatourians, 1990; Goettel and Johnson, 1992) and field (Johnson *et al.*, 1992; Lobo Lima *et al.*, 1992; Johnson and Goettel, 1993) conditions. Penetration through the external integument is the most common route of invasion of nonacridid insects by *B. bassiana* (Ferron, 1978) but infection has also been reported via the respiratory system (Clark *et al.*, 1968), alimentary canal (Gabriel, 1959; Bao and Yendol, 1971; Broome *et al.*, 1976; Yanagita, 1987; Miranpuri and Khachatourians, 1991), and buccal cavity (Siebeneicher *et al.*, 1992). Infection of *per os*-inoculated Colorado potato beetle larvae occurred externally after surface contamination of the integument by conidia in frass (Allee *et al.*, 1990). The mode of infection in grasshoppers is unknown. However, ingestion of wheat, lettuce, and bran inoculated with *B. bassiana* conidia by grasshoppers resulted in significant mortality (Johnson *et al.*, 1988; Goettel and Johnson, 1992; Lobo Lima *et al.*, 1992), suggesting that infection occurred via contaminated mouth parts or the alimentary tract. The application of conidia onto foliage or in baits, rather than directly onto integuments, would have obvious advantages for the efficacious implementation of a biological control strategy for grasshoppers with *B. bassiana*.

Attempts to control a number of insect pests with foliar applications of *B. bassiana* conidia have been reported (Gardner *et al.*, 1977; Ignoffo *et al.*, 1979; Lewis and Cossentine, 1986; Hajek *et al.*, 1987; Johnson *et al.*, 1992; Lobo Lima *et al.*, 1992). Although little is known about the survival of conidia of *B. bassiana* in epigeal habitats, Daoust and Pereira (1986a) suggested that exposure to sunlight was the most important factor limiting survival of conidia on cowpea foliage. Limited information is available on the influence of canopy and crop on penetration, coverage, and persistence of conidial populations in a field environment using conventional application methods. The influence of conidial formulation on these parameters also requires investigation.

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Recently, several entomopathogens have been applied in oil at ultralow volumes (Pan *et al.*, 1988; Delgado *et al.*, 1991; Bateman *et al.*, 1992; Johnson *et al.*, 1992; Lobo Lima *et al.*, 1992) in an attempt to increase their efficacy. Although the application of entomopathogens in oil allows decreased spray volumes, the possibility of increased adhesion of propagules to either plant or insect cuticles and increased resistance of propagules to suboptimal environmental conditions, and the possible predisposition of insects to infection are unknown.

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

MATERIALS AND METHODS

Conidial Formulations

Dry conidia of *Beauveria bassiana* (GHA) were supplied by Mycotech Corp. (Butte, MT) and were maintained at 4°C until used. The number of conidia/g was estimated using a hemocytometer. Immediately prior to use, conidia were suspended in either a paraffinic formulation oil (Mycotech No. 9209), in a 5% formulation oil–water emulsion (v/v), or in water with 0.05% Tween 80 (Sigma). The target concentrations of conidia were 1.0×10^{11} conidia/ml in oil and 5.0×10^8 conidia/ml in water–Tween and the oil emulsion. For both the water–Tween and the oil emulsion formulations, conidial suspensions were homogenized in a Waring blender for 1 min and then passed through a double layer of cheesecloth. Following homogenation, concentrations of conidia in water–Tween were estimated with a hemocytometer. Conidial suspensions for all formulations were diluted five to seven times in 10-fold dilution series and 100 μl from each dilution was spread onto a semiselective oatmeal–dodine medium (consisting of 17.5 g oatmeal agar, 2.5 g agar, 0.45 g Cyprex (dodine), 2.5 mg crystal violet, 0.2 g penicillin G, and 0.5 g streptomycin in 500 ml of deionized water) (Chase *et al.*, 1986). Cultures were maintained at 25°C for 6 to 7 days and the number of colony-forming units (cfu) was enumerated at the dilution yielding 20–200 cfu/dish. Germination percentages of conidia in water–Tween also were determined by placing approximately 20 μl of the conidial suspension in 100 μl of Sabouraud's dextrose broth, incubating the conidia for 12 h and counting the number of conidia forming germ tubes. A minimum of 200 conidia were used. Germination percentages were consistently greater than 80%.

Field Plots

Adjacent fields of alfalfa (lucerne, *Medicago sativa* L.) cv. Beaver and crested wheatgrass (*Agropyron cristatum* L.) were established at the Agriculture Canada Research Station, Lethbridge, Alberta. Fields of alfalfa used for trials one, two, and three were established in 1991, 1989, and 1991, respectively. Fields used in trials one and two had row spacings of 35 cm, whereas in trial three the row spacing was 17.5 cm. Alfalfa plants were cut 20, 37, and 24 days prior to the application of conidia in trials one, two, and three, respectively. The field of wheatgrass was seeded in 1989 in 17.5-cm-wide rows at a rate of 11.1 kg/ha. The wheatgrass was last cut in June, 1991.

Three replicate plots per formulation, each measuring 3.0 by 1.5 m were established in each field. At the time of conidial application, crop height was measured and all foliage within a 0.5-m² quadrat was harvested and leaf areas were measured with a leaf area meter (Model 3100, Li-Cor Inc., Lincoln, NE). Leaf area indices (LAI) were determined by dividing the total leaf areas by the soil surface areas.

Application of Conidia

Conidia in water–Tween and the oil emulsion were applied at a rate of 100 liters/ha (45 ml/plot), using a compressed CO_2 (40 PSI) bicycle sprayer (R&D Sprayers Inc., Opelousas, LA) equipped with three 015-F80 nozzles (Lurmark Ltd., Longstanton, Cambridge, UK). The height of the boom was adjusted according to crop height; for wheatgrass the boom height ranged from 50 to 65 cm and for alfalfa from 25 to 80 cm. Conidia in oil were applied with an ultralow volume (ULV) spinning disk sprayer (Micron Sprayers Ltd., Bromyard, UK) operated at 7000 rpm. Conidia in oil were applied at a rate of 5 liters/ha (2.25 ml/plot). To reduce drift, plots were surrounded with a 1.8-m-high sheet of polyethylene during the spray application. Water- and oil-sensitive papers were randomly placed at the top of the canopy and on the soil surface in selected plots for each crop to evaluate the spray distribution. Target concentrations of conidia in all formulations were 2.25 $\times 10^{10}$ conidia/plot (5.0×10^{13} conidia/ha). Conidia were applied at times of low wind velocity (<4 m/s) on the evenings of July 15, August 2, and August 31, for trials one, two, and three, respectively.

Leaf Collection and Preparation

Ten leaves/leaflets from the top and middle (sampling height was determined at the time of application) of the canopy were randomly collected from the center

of each of the three replicate plots (center row). Times of sampling were immediately after (time 0), and 0.5, 1, 2, 4, 8, and 16 days postapplication. At the 8- and 16-d sample times, care was taken to choose older leaves present at the time of conidial application. Within one hour of collection, leaves were transported to the laboratory in plastic bags and stored at 5°C for a maximum of 12 h. Each of the 10 leaves per sample were aseptically cut across the laminae into pieces of ca. 1 and 0.5 cm long for wheatgrass and alfalfa, respectively.

Microbiological Analysis

Leaf pieces from each sample were placed in 5 ml of 0.01 M phosphate buffer with 0.01% Tween 80 (pH 7.0) in 20-ml scintillation vials and washed at ambient temperature for 2 h on a rotary shaker at 300 rpm. After shaking, the wash solution was diluted 2–3 times in a 10-fold dilution series and 100-μl aliquots for each dilution were spread onto the oatmeal–dodine medium. The cultures were incubated at 25°C for 6–7 days and the number of cfu of $B.$ $bassiana$ was enumerated at the appropriate dilution. Representative colonies were isolated and grown in slide culture and the identity of $B.$ $bassiana$ was confirmed by microscopic examination. Following washing the total area of the leaf pieces was determined with the leaf area meter, and the mean number of cfu/cm^2 of adaxial leaf area was calculated.

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

Grasshopper Bioassay

Nymphs of a laboratory strain of a nondiapause $Melanoplus$ $sanguinipes$ (F.) were reared on a diet of lettuce, wheat bran, and wheat leaves. Third- to fourth-instar nymphs were placed in plastic containers (20 nymphs per replicate) and maintained in a Phytotron at a 16/8 h light/dark photoperiod and 25/20°C day/night

temperature. The protocol required that nymphs be fed either alfalfa leaflets or wheatgrass leaves collected 0 and 2 days postapplication of $B.$ $bassiana.$ However, due to inclement weather, application of conidia was delayed 24 h in trial two. This necessitated that nymphs fed leaves immediately after application of conidia be starved for 12 h, fed ad $libitum$ for 12 h, and then starved for an additional 12 h. Nymphs fed leaves at 2 days postapplication of conidia were starved for 12 h. Following the starvation period, nymphs were fed 100 g (at 12-h intervals) of randomly collected leaves from each crop at each sample time. Nymphs were then maintained on a diet of wheat and lettuce leaves for 11 days. During this period dead nymphs were removed daily and kept at 5°C. At 12 days, surviving nymphs were frozen; all nymphs were then placed on moistened filter paper at 25°C and the occurrence of hyphal growth of $B.$ $bassiana$ recorded after 3–7 days. Freezing was previously shown to have no effect on conidial viability.

Scanning Electron Microscopy (SEM)

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

Weather Data

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

Experimental Design and Statistical Analyses

All experiments were arranged as randomized complete block designs with three replicates, and all computations were performed using the ANOVA, GLM, and REG procedures (SAS Institute, 1988). To normalize the conidial population data, $\log_{10}$ transformations were used. Standard errors of the means were calculated from individual treatments and are presented in parentheses. For each trial and crop, experiments were analyzed separately as a split plot in time with three levels of formulation, two levels of canopy position, and seven
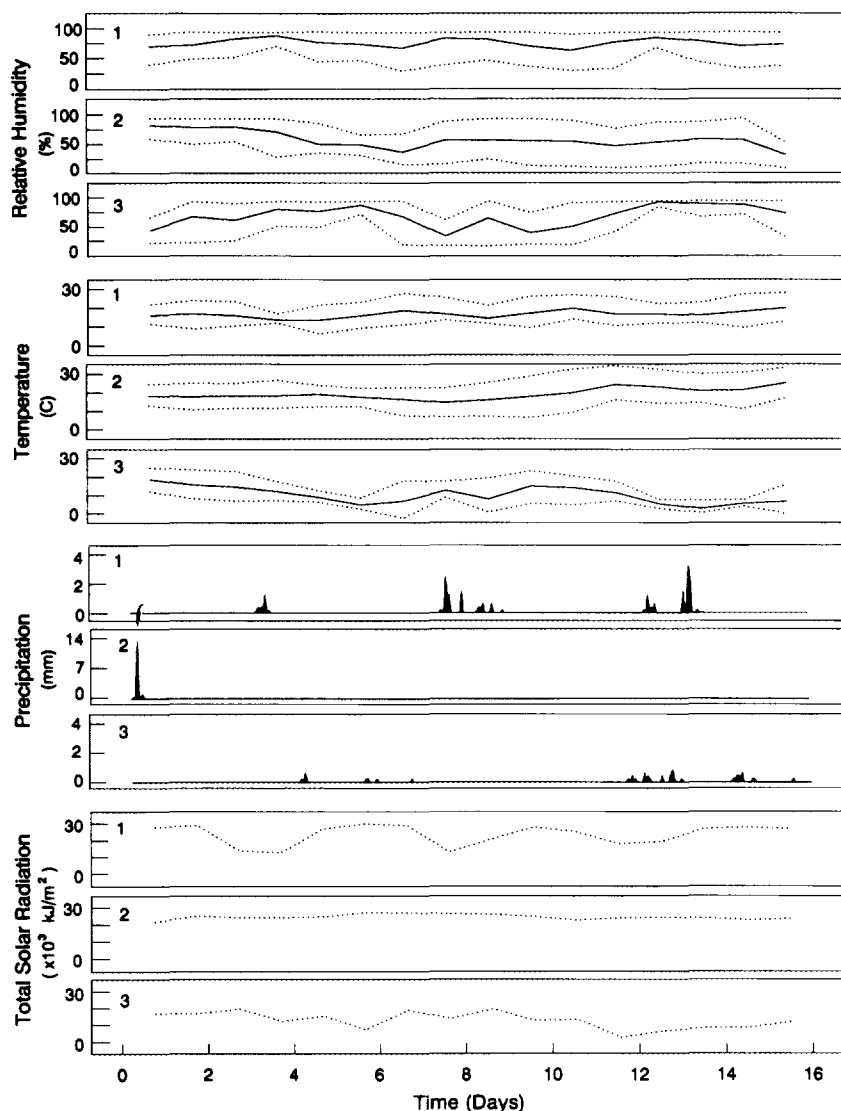
FIG. 1. Daily maximum, minimum, and mean hourly relative humidities (%); daily maximum, minimum, and mean hourly temperatures (°C); hourly precipitation (mm); and daily cumulative hourly incoming light (300–2800 nm) for trials one, two, and three.

sample times. When significant differences ($P \le 0.05$) were observed for a formulation, the data were analyzed at each sample time as a factorial experiment, and Duncan's multiple range test ($\alpha = 0.05$) in conjunction with a significant F test for formulation and a nonsignificant F test for the canopy–formulation interaction was used to separate means. Where indicated by ANOVA, formulation treatments were combined ($n = 9$). Individual formulations or combined formulation data were fitted to linear models within each canopy position. At the top of the wheatgrass canopy, the best fit of the regression lines required $\log_{10}$ transformation of the x-axis (time); for all other regressions the x-axis was not transformed. In addition to time, best fit regressions were conducted against cumulative degree days ($>10°C$) and cumulative

solar radiation. Comparisons of slopes and initial populations (y-intercepts) between trials for each crop were conducted using the GLM procedure. In most instances, no *B. bassiana* was recovered from leaf segments collected from untreated plots and when conidia were recovered, it was at very low levels (<10 cfu/cm^2). Therefore, the control treatment was excluded from the analyses of conidial persistence. Grasshopper mortality data were analyzed as a factorial experiment with three formulations and two crops. Separate analyses were conducted for each of the two sample times. There was no mortality attributed to *B. bassiana* in grasshoppers fed noninoculated leaf segments, and therefore this treatment was excluded from the analysis of *B. bassiana*-incited mortality; for other mortality it was included.

RESULTS

Efficacy of the Wash Method for Recovery of Conidia

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

Field Application of Conidia onto Leaves of Crested Wheatgrass and Alfalfa

Only concentrations of conidia suspended in water–Tween could be accurately verified with a hemocytometer. In both the oil and the 5% oil emulsion formulations, the oil obscured the conidia; therefore, conidial concentrations in these formulations were verified using the dilution-spread plate technique. In general, numbers of conidia recovered on the oatmeal–dodine medium for the oil emulsion formulation were slightly less (<10%) than those for the other two formulations. This was attributed to aggregation of conidia.

Immediately following application of conidia, there were no interactions between formulation and canopy position, and with the exception of crested wheatgrass in trial one, there were no significant differences between the formulation treatments in either crop (Figs. 2–4). Populations of *B. bassiana* recovered from the top of the canopy immediately following application ranged from log 3.71 (0.48) to log 4.54 (0.12) cfu/cm^2 and from log 3.92 (0.13) to log 5.09 (0.15) cfu/cm^2 for wheatgrass and alfalfa, respectively. Conidia applied in the oil emulsion in trial one were less numerous ($P < 0.05$) than conidia applied in either water–Tween or oil on wheatgrass phylloplanes (Fig. 2A). Although winds were light (1.5 to 3.8 m/s), ULV droplets were observed to drift during application in all three trials.

In general, fewer droplets/cm^2 were observed on oil and water-sensitive papers at the bottom (soil level) of the canopy relative to the top of the canopy in alfalfa but not wheatgrass. There were no differences in numbers of cfu recovered from either the top or the middle of the crested wheatgrass canopy; LAI were 0.55, 0.24, and 0.35 in trials one, two, and three, respectively (Figs. 2A, 3, 4A). Fewer cfu ($P = 0.007$ and $P \leq 0.0001$) were recovered from the middle than the top of the alfalfa canopy in trials one and three; populations recovered ranged from log 3.52 (0.25) to log 4.34 (0.07) cfu/cm^2 (Figs. 2B, 4B). For these trials, LAI were 1.8 and 1.9, respectively.

However, in trial three, when the LAI of alfalfa was greater than 2, there was no effect of canopy position on the recovery of *B. bassiana* conidia. Microscopic observations of leaf surfaces showed a degree of conidial aggregation of all formulations (Figs. 5A–5D). In several instances, aggregation of conidia in circles around the point of droplet impact on wheatgrass leaves were observed for conidia applied in water and the oil emulsion (Figs. 5E, 5F).

Weather Conditions

Conditions of precipitation, temperature, and incoming solar radiation differed substantially among the three trials (Fig. 1). Cumulative light was similar between trials one (3.7 × 10^5 kJ/m^2) and two (3.9 × 10^5 kJ/m^2) but substantially less in trial three (2.2 × 10^5 kJ/m^2). In trials one, two, and three, mean hourly temperatures at 1 m were 16.6, 19.2, and 9.7°C, respectively. Total precipitation was 22, 15, and 8.4 mm for trials one, two, and three, respectively. Immediately following application of conidia in trial two, an intense storm lasting less than 1 h occurred; 14 mm of rain accompanied by hail fell and winds gusted from 60 to 80 km/h. Relative humidities at 1 m averaged 75, 58, and 69% for the three trials, respectively. Both temperature and relative humidity at canopy midheight for crested wheatgrass were similar to ambient conditions over a 24-h period. In the alfalfa canopy, temperatures were similar but relative humidities were slightly higher by 3 to 5% on average. During daylight hours over a 12-h period (800 AM to 800 PM), there were minimal differences in temperature due to canopy position in both wheatgrass and alfalfa. However, relative humidities were higher by 6 to 9% in alfalfa; there were no conspicuous differences in relative humidities in the wheatgrass canopy.

Persistence of Conidia on Phylloplanes of Crested Wheatgrass

The only interaction that consistently influenced persistence of *B. bassiana* conidia was between canopy position and time ($F = 5.8$–10.9, $df = 6$, $P \leq 0.05$). There were no interactions among canopy, formulation, and sampling time, thus justifying the analysis of the data as a split plot over time. Interactions between formulation and canopy also were nonsignificant. Time was highly significant ($F = 80.5$–97.4, $df = 6$, $P \leq 0.0001$) and with the exception of trial one ($F = 2.2$, $df = 12$, $P = 0.022$), there were no interactions between formulation and time; the positive interaction in trial one was attributed to the oil emulsion formulation at the midcanopy position (Fig. 2A). When populations of conidia recovered at Time 0 and 0.5 days were compared, time was not significant in trials one and three but was in trial two ($P = 0.0002$). The influence of canopy position alone was highly significant ($F = 8.9$–22.5, $df = 1$, $P \leq 0.0001$); a
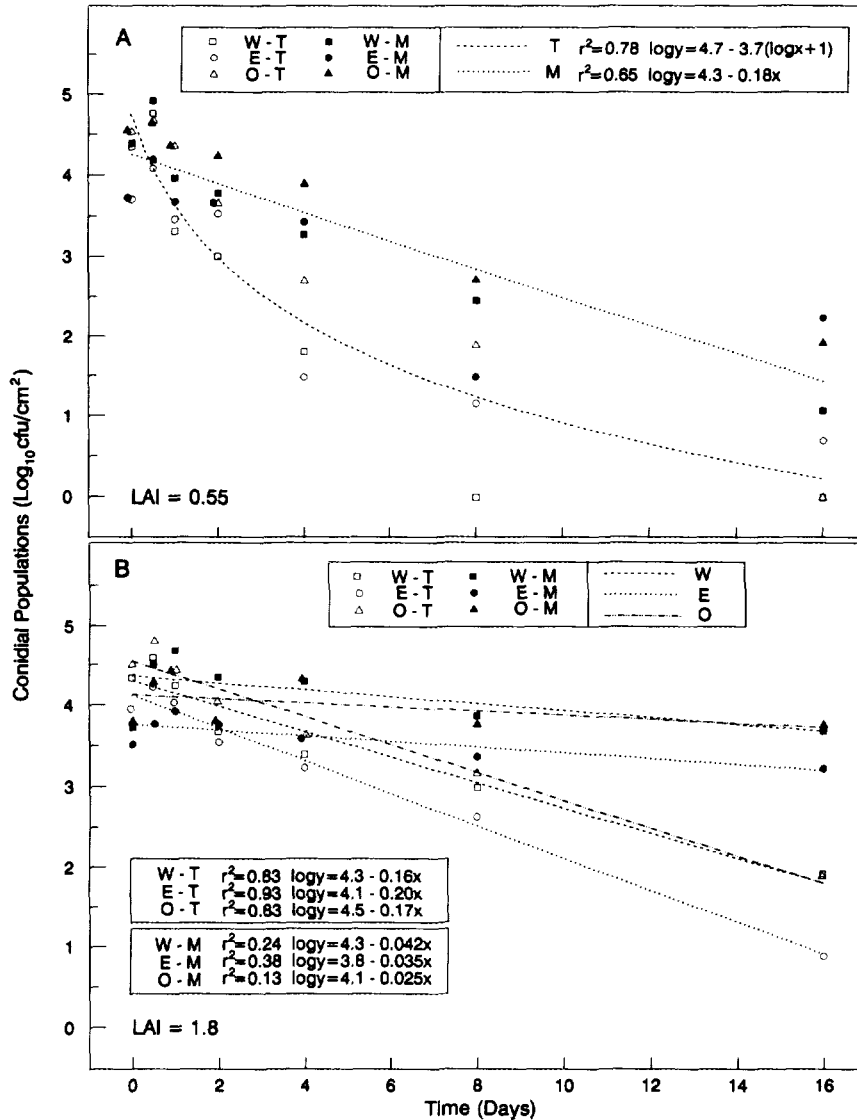
FIG. 2. Persistence of *Beauveria bassiana* conidia on the phylloplanes of crested wheatgrass and alfalfa from July 15 to 31. (A) Conidial populations ($\log_{10}$ colony-forming units (cfu)/cm^2 of leaf area) for conidia applied on phylloplanes of crested wheatgrass in water amended with 0.5% Tween 80 (W), in a 5% oil emulsion (E) and in formulation oil (O) at the top (T) and middle (M) of the canopy; and (B) populations of conidia (log cfu/cm^2) applied on phylloplanes of alfalfa. Leaf area indexes (LAI) for both crops were measured at the time of application.

canopy effect was first observed at 1 day postapplication of conidia and was subsequently maintained throughout the 16-day duration of the experiment in trials one and three but not in trial two. In trial two, there was no influence of canopy on persistence of conidia at 16 days postapplication. Formulation had no effect on persistence of conidia; therefore, data for formulations were pooled at each canopy position and a single regression equation was used to model the response of conidial survival to time. Substantial aggregation of conidia was observed by SEM on leaves 1 to 4 days postapplication for all formulations. However, in contrast to conidia on leaves collected immediately after application, conidia were found to be congregated in the intracellular depressions of wheatgrass leaves (Fig. 6A). At the middle of the wheatgrass canopy, conidial persistence declined logarithmically over time (Figs. 2A, 3, 4A). In contrast, at the top of the canopy, data were fitted to linear regressions following log-transformation of both cfu and time data. Formulations were combined within each canopy position to calculate the coefficient of determination (r^2); these ranged over the three trials from 0.66 to 0.78 at the top and from 0.65 to 0.72 from the middle of the canopy. Slopes ranged from −2.9 to −3.7 for the top and from −0.17 to −0.18 for the middle canopy position. There were no differences among
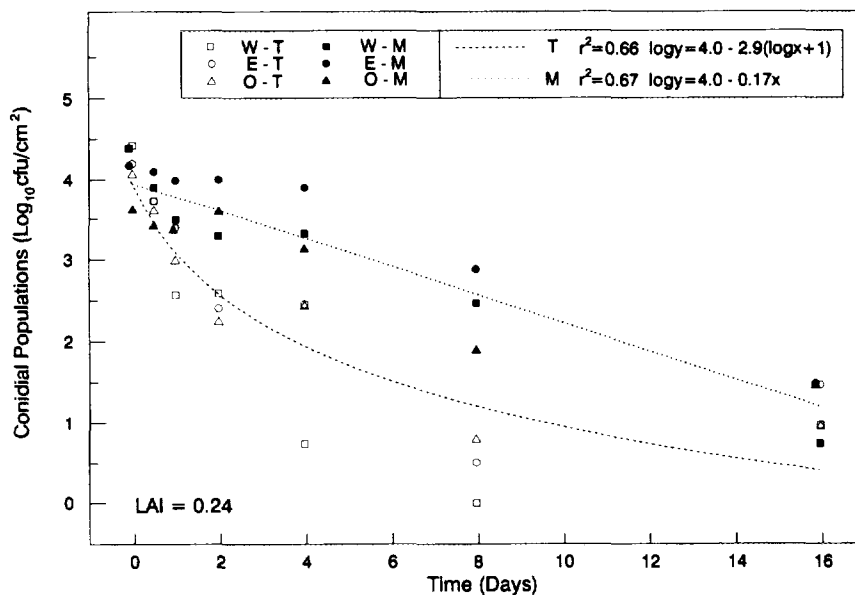
FIG. 3. Persistence of *Beauveria bassiana* conidia on phylloplanes of crested wheatgrass from August 2 to 18. Conidial populations (log$_{10}$ cfu/cm² of leaf area) for conidia applied in water amended with 0.5% Tween 80 (W), in a 5% oil emulsion (E), and in formulation oil (O) at the top (T) and middle (M) of the canopy. LAI was estimated at the time of application.

slopes of conidial persistence between trials for either the top or middle canopy positions. Slopes for each canopy position differed ($P \leqslant 0.0001$) from zero. However, the y-intercepts (average initial populations) differed among trials for both the top ($P \leqslant 0.0001$) and the middle ($P \leqslant 0.0001$) canopy positions. Accumulated degree days and cumulative light were less effective as predictors of conidial persistence than was time.

Persistence of Conidia on Phylloplanes of Alfalfa

The severe storm encountered immediately after application of conidia in trial two disrupted the canopy structure of the alfalfa. Therefore, only data for Time 0 at both canopy positions, and for the Times 0 to 4 days for the top of the canopy were used in this trial. Substantial numbers of cfu were isolated from the top of the canopy following the rain storm. Populations recovered from the top of the canopy 12 h after the application of conidia were log 3.22 (0.33), log 3.91 (0.19), and log 3.79 (0.39) cfu/cm² for the water–Tween, the oil emulsion and oil formulations, respectively. As was the case with wheatgrass, populations of *B. bassiana* in all three formulations were reduced ($P \leqslant 0.0001$) at 0.5 days relative to Time 0 at the top of the alfalfa canopy in trial two.

In trials one and three there were no interactions among canopy, formulation, and sampling time. The interaction between canopy position and time was highly significant ($F = 27.6$ and 40.7, $df = 6$, $P \leqslant 0.0001$) in both these trials. There was no interaction between canopy position and formulation in trial one, whereas a weak

interaction ($F = 5.8$, $df = 2$, $P = 0.020$) was observed in trial three. In trial three ($F = 4.6$, $df = 12$, $P \leqslant 0.0001$) but not one, formulation influenced the rate of decline over time. Similar to the case of wheatgrass, both time ($F = 59.7$ and 89.8, $df = 6$, $P \leqslant 0.0001$) and canopy ($F = 39.3$ and 9.2, $df = 1$, $P \leqslant 0.0001$) were highly significant. In both trials, the effect of formulation on the persistence of conidia was highly significant ($F = 25.1$–10.8, $df = 2$, $P \leqslant 0.001$). Therefore, data for the three formulations were not pooled as in the case of wheatgrass, and individual regressions were calculated for each formulation (Figs. 2B, 4B). A comparison of slopes between trials for each formulation and canopy position indicated that for conidia applied in both water–Tween and the oil emulsion at the top of the alfalfa canopy, slopes differed ($P = 0.0002$ and $P = 0.009$) between trials one and three. For all other formulation–canopy combinations there were no significant differences among slopes. With the exception of conidia applied in oil and sampled at the middle of the canopy, initial populations (y-intercepts) differed between trials for all formulation–canopy combinations. Densities of conidia at the middle of the canopy were less at Time 0 ($P = 0.0070$ and $P \leqslant 0.0001$) and 0.5 days ($P = 0.0012$ and $P \leqslant 0.0001$) but not at 1 and 2 days postapplication. Between 2 and 4 days, conidia at the middle of the canopy became more numerous ($P \leqslant 0.0001$) than those at the top of the canopy, and this effect was maintained throughout the experiment. Although the surface topography of alfalfa leaflets differed substantially from wheatgrass leaves, similarly to wheatgrass, conidial aggregations in intra-
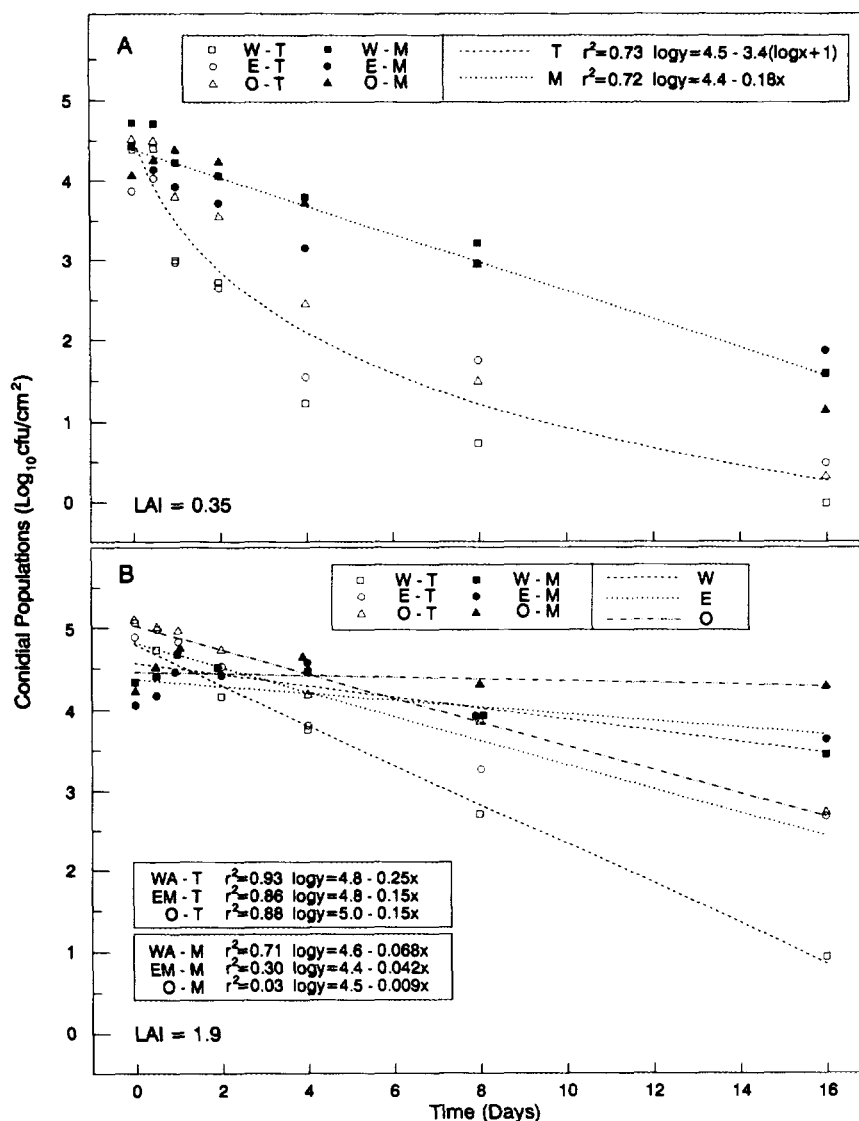
FIG. 4. Persistence of *Beauveria bassiana* conidia on the phylloplanes of crested wheatgrass and alfalfa from August 31 to September 16. (A) conidial populations ($\log_{10}$ cfu/cm^2 of leaf area) for conidia applied on phylloplanes of crested wheatgrass in water amended with 0.5% Tween 80 (W), in a 5% oil emulsion (E), and in formulation oil (O) at the top (T) and middle (M) of the canopy, and (B) populations of conidia (log cfu/cm^2) applied on phylloplanes of alfalfa. LAI for both crops were measured at the time of application.

cellular depressions were observed on leaves 1 to 4 days postapplication (Fig. 6B).

Grasshopper Bioassay

Average total mortality of grasshopper nymphs fed crested wheatgrass and alfalfa leaves immediately after the application of *B. bassiana* conidia ranged from 51 to 82% for the three formulations (Table 1). There was no difference between either crops ($P = 0.89$) or formulations ($P = 0.49$) in the incidence of mortality attributed to infection by *B. bassiana;* such mortality ranged from 31 to 58%. In nymphs fed untreated leaves, there was no

mortality that could be attributed to *B. bassiana.* Non-*Beauveria bassiana* mortality ranged from 17 to 34%, and there was no difference between crops or formulation, including the control treatment.

Both *B. bassiana*-incited and other mortality in nymphs fed crested wheatgrass and alfalfa leaves 2 days postapplication of *B. bassiana* were considerably less than those fed leaves immediately after application (Table 1). Mortality attributed to *B. bassiana* ranged from 0 to 5.5%, and the incidence of other mortality ranged from 0 to 17%. A difference ($P = 0.018$) in other mortality due to crop was observed in nymphs fed leaves 2 days postapplication of conidia. For both sample times, the
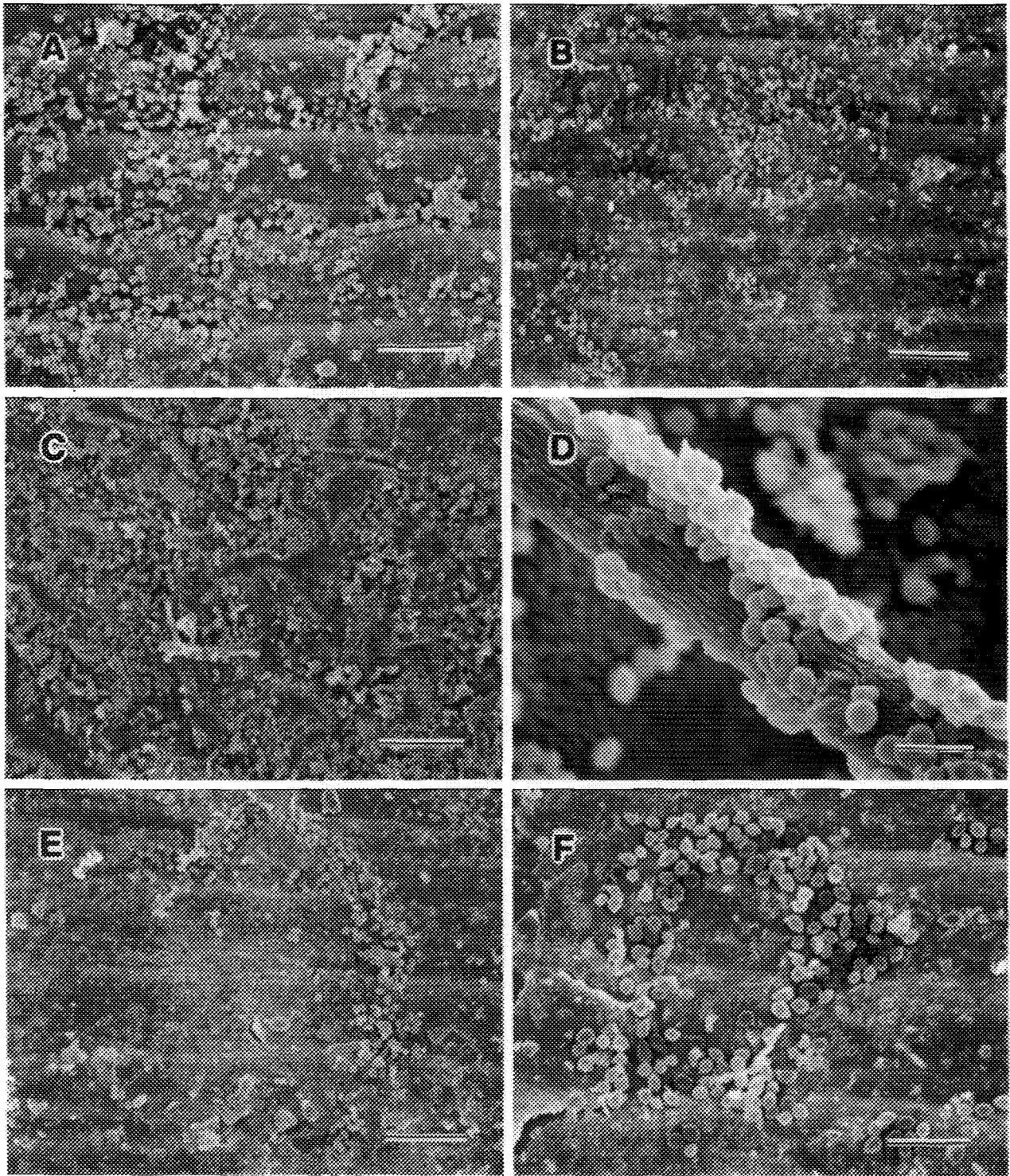
FIG. 5.   Scanning electron micrographs of *Beauveria bassiana* conidia on phylloplanes of crested wheatgrass and alfalfa immediately following application. (A–C) Bars, 20 μm. (A) Conidia applied onto a wheatgrass leaf in water amended with 0.05% Tween 80; (B) conidia applied onto a wheatgrass leaf in a 5% oil emulsion; (C) conidia applied onto a alfalfa leaflet in oil; (D) conidia adhering to an alfalfa cuticular hair. Bar, 5 μm. (E–F) aggregation of conidia in circles around the site of droplet impact. (E) conidia applied in a 5% oil emulsion. Bar, 20 μm; (F) Conidia applied in water–Tween. Bar, 10 μm.
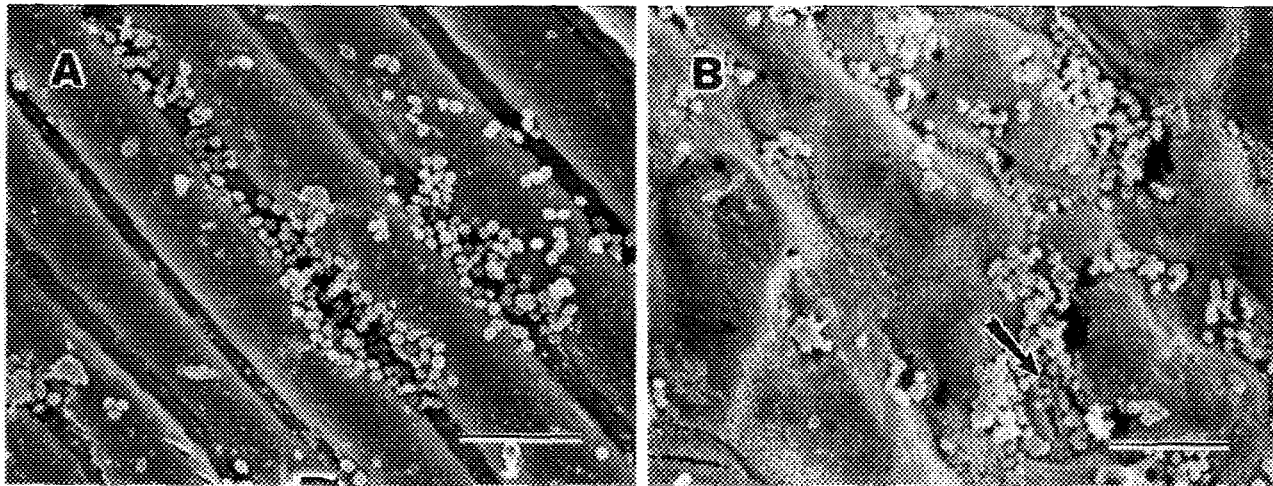
FIG. 6. Scanning electron micrographs of *Beauveria bassiana* conidia on phylloplanes of crested wheatgrass and alfalfa 4 days postapplication. (A) Aggregation of conidia in intracellular depressions of a wheatgrass leaf; (B) conidia aggregated in a depression of an alfalfa leaflet. Arrow points to conidia in a stoma. Bars, 20 µm.

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

DISCUSSION

Conidia of *B. bassiana* applied on phylloplanes of crested wheatgrass and alfalfa in a field environment were found to be relatively short-lived at the top of the canopy for both crops. Persistence of conidia appeared to be somewhat enhanced at the top of the alfalfa canopy relative to wheatgrass; by 4 days populations were reduced by over 99% on wheatgrass and by 75–90% on alfalfa leaves. Daoust and Pereira (1986a) observed reduced germination of *B. bassiana* conidia recovered from cowpea foliage in a field setting; conidial half-lives were 1–2 days and no conidia were viable after 1 week. We found that conidia within the canopy of both wheatgrass and alfalfa survived substantially longer than those at the top. Conidia on leaves within the canopy of alfalfa survived much longer than those in the wheatgrass canopy. At the middle of the wheatgrass canopy, conidial populations were reduced by 79 to 81% after 4 days. On alfalfa leaves at the midcanopy position, populations were only reduced by 8.0 to 47% after 4 days, and, by 16 days, substantial numbers of viable conidia were still recovered (populations reduced by 29 to 85%). Feeding by grasshoppers is not limited to the top of the canopy and the persistence of conidia within canopies may have positive consequences for implementation of a biological control program with *B. bassiana*.

In the present study, conditions of temperature, relative humidity, and precipitation fluctuated considerably within and between trials. In trial two, substantial rainfall and hail accompanied by driving winds occurred im-

mediately following application of conidia, yet only slight population decreases were observed for both crops. When the slopes of conidial persistence between trials were compared, no significant differences at either the top or the middle of the canopy of the wheat-

TABLE 1

Mortality of Grasshopper (*Melanoplus sanguinipes*) Nymphs Fed Leaf Segments of Alfalfa and Crested Wheatgrass Collected from the Top of the Canopy at 0 and 2 Days Postapplication of Conidia of *Beauveria bassiana*

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

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

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

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

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

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

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

grass were observed. Similarly, slopes of conidial persistence between trials at the middle of the alfalfa canopy did not differ. These observations suggest that temperature, relative humidity, and rainfall do not have an overriding impact on conidial survival.

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

An endophytic existence would buffer *B. bassiana* from the suboptimal environmental conditions encountered on phylloplanes and it has been reported to exist as an endophyte in corn (Bing and Lewis, 1991). Although there are numerous reports of direct penetration of insect cuticles by *B. bassiana* (Ferron, 1978), direct penetration of plant tissues has not been documented. Germination of *B. bassiana* can occur in the presence of free water or at relative humidities greater than 90% (Walstad *et al.*, 1970; Kuberappa and Jayaramaiah, 1987), both of which occurred in the present study. However, we did not detect germination of conidia on leaves at the top of the canopy for either crop, and whether germination occurred at the more favorable microclimate within the canopy is unknown. In several instances we did observe conidia within alfalfa leaf stomata (Fig. 6B) and whether stomata serve as possible sites of penetration requires investigation.

Conidia of *B. bassiana* are hydrophobic and were found to disperse better in oil than in water–Tween. Substantial clumping of conidia was observed in the oil emulsion even after homogenation but aggregation of conidia was observed for all three formulations on phylloplanes (Figs. 5A–5D, 6A, 6B). The attachment of co-

nidia to wheatgrass and alfalfa cuticle was considerable regardless of formulation and in several instances conidia were observed attached to cuticular hairs of alfalfa (Fig. 5D). Passive and nonspecific adherence of *B. bassiana* conidia to insect cuticles mediated by their hydophobicity has been reported (Boucias *et al.*, 1988). It seems likely that similar adherence occurs on plant cuticles, and we did not observe a reduction in numbers of conidia adherent to leaves for up to 4 days following their application. However, conidia appeared to congregate in intracellular depressions of both wheatgrass and alfalfa leaves (Figs. 6A, 6B). Although conidia appeared to be strongly attached to leaves, we found that conidia suspended in all three formulations were effectively removed from their surfaces by vigorous washing in buffer amended with Tween 80. For the oil emulsion formulation, recovery of cfu was greater than 100% and it seems likely that the high rate of recovery of conidia applied in this formulation resulted from the release of conidia from aggregations.

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

Most investigations into persistence of conidia on phylloplanes have focused on the infectivity of applied propagules over time. Gardner *et al.* (1977) showed that infection of fall armyworm larvae by conidia of *B. bassiana* applied onto soybean foliage was reduced by 5 days and nonexistent by 10 days. Similarly, infection of cabbage looper larvae by conidia applied onto leaves of collard and soybeans was significantly reduced 1 day

after application (Ignoffo *et al.,* 1979). Repeated application of conidia on potato phylloplanes provided inconsistent control of Colorado potato beetle (Hajek *et al.,* 1987). To test whether reductions in populations over time corresponded with infectivity of conidia, we fed grasshopper nymphs either wheatgrass or alfalfa leaves from the top of the canopy immediately, and 2 days after, application of conidia in trial two. Mortality ranging from 31 to 58% was observed in nymphs fed leaves immediately after application. Significant mortality of grasshoppers also was reported in North America and Africa following application of *B. bassiana* conidia onto foliage (Johnson *et al.,* 1988; Delgado *et al.,* 1991; Lobo Lima *et al.,* 1992). Our results suggest that the infectivity of conidia on wheatgrass and alfalfa leaves corresponds with reductions in conidial numbers. The importance of conidial dose on mortality has been demonstrated in a controlled environment (Goettel and Johnson, 1992); when conidia applied on lettuce disks were fed to nymphs of *M. sanguinipes* mortality was 100% at a dose of 10^5 conidia per nymph, 20–60% at a dose of 10^4 conidia per nymph, and 10–30% at a dose 10^3 conidia per nymph after 16 days (Goettel and Johnson, unpublished). Although nymphs were fed *ad libitum* in the present study, by 2 days postapplication of conidia populations on phylloplanes of wheatgrass and alfalfa were reduced by 99 and 75%, respectively, from the top of the canopy and the corresponding mortality in nymphs was reduced to 0–6%.

We observed much higher mortality not attributable to *B. bassiana* in nymphs fed leaves immediately after, than 2 days postapplication of conidia. Since the prevalence of other mortality in nymphs fed untreated leaves was not different from those fed leaves infested with conidia at Time 0, sublethal infection and predisposition of larvae to death can be excluded. An alternative explanation is that the additional 12-h starvation period predisposed nymphs to infection, a phenomenon reported previously for other insects (Lighthart *et al.,* 1988; Donegan and Lighthart, 1989). The influence of starvation and a number of environmental parameters on susceptibility of grasshoppers to infection by *B. bassiana* requires study.

Conidia of *B. bassiana* applied on phylloplanes of crested wheatgrass and alfalfa were found to be relatively short-lived at the top of the canopy for both crops. Slopes of conidial persistence were similar between trials although conditions of temperature, relative humidity, precipitation, and solar radiation (>300 nm) varied substantially. However, the degree of canopy coverage in alfalfa was greater than that of wheatgrass and mortality of conidia within the alfalfa canopy was reduced. This was attributed to protection from ultraviolet light and not to more favorable conditions of relative humidity and temperature. We observed that oil had little or no effect on persistence of *B. bassiana* conidia

relative to conidia applied in water or an oil emulsion. Mortality of grasshopper nymphs appeared to correspond with the conidial persistence data; by 2 days postapplication of conidia on phylloplanes populations were reduced by more than 75%. By increasing the concentrations of applied conidia, the effectiveness of *B. bassiana* for controlling grasshopper may be prolonged. An alternative approach could be the use of ultraviolet light protectants to increase persistence and this possibility merits investigation under laboratory and field conditions. If phylloplanes or aerial plant parts are targeted in a strategy to control grasshoppers with *B. bassiana,* in addition to virulence, isolates should also be selected on their ability to persist in these habitats.

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