Disinfestation of *Beauveria bassiana* from Adult *Lygus hesperus*[1] Using Ultraviolet-C Radiation

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Abstract. The western tarnished plant bug, *Lygus hesperus* Knight, is an important pest of crops in the western U.S. Research on the biology of *L. hesperus* often relies on access to healthy insects from laboratory cultures. However, maintenance of colony vigor often requires periodic introductions of field-collected specimens or re-initiation of the colony. Either approach poses a risk of introducing diseases with the field-collected insects. We examined the potential utility of exposure to ultraviolet light (UV-C) as a way to sterilize adult *L. hesperus* previously treated with spores of *Beauveria bassiana* (Balsamo) Vuillemin, and also examined the influence of exposure to UV-C on subsequent reproduction by lygus. Mortality of *B. bassiana* conidia increased rapidly with increased exposure to UV irradiation, which also delayed germination of conidia at exposure times ≥2 minutes. Based on assays 48 hours after exposure to UV, 6.45 minutes of exposure to UV-C (≈130 μW cm^{-2}) reduced germination of conidia to approximately 1%. When *L. hesperus* adults treated with *B. bassiana* were exposed to UV-C for 2 hours, infection by the pathogen was greatly decreased but not eliminated. Similar exposure of untreated *L. hesperus* adults did not reduce subsequent fecundity. Our results suggest that routine exposure of field-collected adults to UV-C irradiation may reduce the probability of introducing *B. bassiana* into laboratory colonies with little or no adverse reproductive consequences.

Introduction

The western tarnished plant bug, *Lygus hesperus* Knight, is an important pest of numerous field, fruit, and vegetable crops in the western U.S. (Wheeler 2001). Timely completion of research on the biology of *L. hesperus* often relies on access to an adequate supply of vigorous and healthy insects typically provided from laboratory cultures. Efforts to maintain insect quality in laboratory colonies often rely on periodic introductions of field-collected insects. In the case of *L. hesperus*, shifts in physiological and behavioral characteristics of cultured insects may not be prevented by periodic introductions (Brent and Spurgeon 2011), and some research objectives require the entire colony be re-initiated after only a few

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generations (Spurgeon 2012). Unfortunately, periodic insect introductions and re-initiation of laboratory cultures present risks of coincidental introduction of entomopathogens from field-collected insects.

Beauveria bassiana (Balsamo) Vuillemin, an entomopathogenic fungus endemic to the San Joaquin Valley of California, frequently infects *L. hesperus* in the field (McGuire 2002). During the summer and fall of 2010, infection by *B. bassiana* was detected in the lygus colony at the Shafter Cotton Research Station on multiple occasions despite careful inspection of field-collected insects segregated from the colony for 7-10 days after their collection. Each incidence of infection necessitated disposal of the infected colony and laborious efforts to sterilize areas of the laboratory devoted to insect rearing. The serious consequences in terms of cost and effort to eliminate a disease after pathogen introduction into an insect colony would justify exploration of ways to minimize the probability of initial infection.

It is well known that ultraviolet (UV) radiation is an environmental factor that inactivates spores of entomopathogenic fungi (Gardner et al. 1977, Ignoffo et al. 1977, Inglis et al. 1997). The most potent inactivating UV in natural sunlight is UV-B (280-320 nm) because UV-C (germicidal UV, 200-280 nm) is almost completely filtered out by the atmosphere (Hunt et al. 1994). However, the action of UV-C on microorganisms is similar to that of UV-B (Chelico et al. 2005), and UV-C lamps are commonly used in microbiological laboratories to limit contamination of sterile work space. Because of the known sensitivity of *B. bassiana* to UV radiation, it seems possible that exposure of lygus bugs to UV-C may at least reduce numbers of any existing spores, and thereby minimize the opportunity for development of disease. Our objectives were to determine 1) the sensitivity of *B. bassiana* conidia to UV-C, 2) whether the incidence of infection of adult *L. hesperus* treated with *B. bassiana* could be reduced by exposure to UV-C, and 3) to assess the consequences of UV-C exposure on subsequent reproduction by *L. hesperus*.

Materials and Methods

Sensitivity of *Beauveria* Conidia to UV-C. Conidia of *B. bassiana* (strain GHA, Laverlam International, Butte, MT) were obtained from prepared stocks stored in 10% glycerol at -80°C. The stocks were harvested from colonies grown on Sabouraud dextrose agar (SDA, Becton-Dickson, Cockeyesville, MD) supplemented with 0.2% (w/v) yeast extract (Sigma Chemical, St. Louis, MO). Based on preliminary germination assays, preparations of conidia were diluted to a final concentration of 5×10^6 conidia ml^{-1} in 0.01% Silwet-L77 (GE Silicones, Friendly, WV).

The experimental arena was a biological safety cabinet (Labgard Class II, Type A/BC Laminar Flow, NuAire, Plymouth, MN) equipped with a UV-C lamp (TUV 30W/G30 T8, Philips Lighting, Roosendaal, Netherlands). A PMA 2100 light meter equipped with a PMA 2122 germicidal UV sensor (Solar Light, Glenside, PA) was used to map a grid of light intensity across the floor of the cabinet. Using the measurements as a guide, the sensor was used to identify an arc of positions at which the intensity of UV-C was ≈130 ± 5 μW cm^{-2}. The location of the arc was marked with tape on the floor of the cabinet.

Conidia in uncovered plastic Petri plates (60 × 15-mm) containing SDA with 0.2% yeast extract were exposed to UV-C. A 3 × 3-cm square of autoclaved cellulose (Cellophane P00, Innovia Films, Atlanta, GA) was positioned on the agar

surface in the center of each plate, upon which was spread 10 µl of conidial suspension. Before exposure of conidia, the UV lamp was started and allowed to equilibrate for 15 minutes, and the exhaust fan was operated to remove any ozone generated by the UV light. During exposure to UV, the UV sensor was positioned at the apex of the arc, and plates of conidia were positioned, centered on the line marking the arc, on either side of the sensor.

The experiment used six exposure times: 0, 0.5, 1, 2, 5, and 10 minutes. The plate assigned to the exposure time of 0 minutes (nonirradiated control) was placed on the floor of the cabinet but outside the arc of positions, where it remained for the duration of the exposure period (10 minutes). During the exposure period, the plate was covered by a 150-mm-diameter plastic Petri plate lid resting on three #6 rubber stoppers ≈3 cm above the floor of the cabinet. Suspension of the lid above the control plate blocked the UV irradiation but allowed free air movement over the plate as for plates assigned to the other exposure times. At the end of each of the designated exposure times, one plate was covered with a lid, sealed with Parafilm M (Pechiney Plastic Packaging, Menasha, WI), and removed from the cabinet. As each plate was removed, a reading from the UV sensor was recorded to ensure the UV intensity was consistent throughout the exposure period.

Following exposure to UV, the sealed Petri plates were held in an incubator at 28°C without light. The proportion of conidia germinating was determined by examination of ≥200 conidia at 400× magnification. Germination was examined at 24, 48, and 72 hours after exposure to UV and was signified by the presence of a germ tube longer than the diameter of the conidia. The experiment was conducted three times.

The relationship between exposure to UV and germination of conidia was described by fitting observed proportions of germination to a logistic function using the NLIN procedure of SAS (SAS Institute 2008). The logistic function was $Y = [e^{(B0 + B1*\mathrm{TIME})}/(1 + e^{(B0 + B1*\mathrm{TIME})})]$, where Y is the proportion of germinated conidia, TIME is the duration of exposure to UV-C, and $B0$ and $B1$ are estimated. Separate functions were fitted to observed germination at each of 24, 48, and 72 hours after exposure to UV. For exposure durations resulting in a high proportion of conidial germination (typically ≥50% germination, exposure ≤1 minute), accurate estimates of germination could not be obtained at 48 or 72 hours because of extensive hyphal growth. In those cases, the logistic functions corresponding to later observation times were fitted using the greatest proportion of germination previously observed. Exposure times necessary to reduce germination of conidia by 50 (LT_{50}) and 99% (LT_{99}) were calculated from the fitted curves.

Disinfestation of *Lygus* Adults. Adult *L. hesperus* were obtained from a laboratory colony maintained for ≤4 generations on pods of green bean (*Phaseolus vulgaris* L.) and raw seeds of sunflower (*Helianthus annuus* L.) using the procedure described by Spurgeon and Cooper (2012). The colony originated from insects collected from local fields of alfalfa (*Medicago sativa* L.), and was maintained at ≈25-31°C and a photoperiod of 14:10 light:dark hours. Newly eclosed (0-1-day old) adults (≥90, mixed gender) were obtained for the experiments on the morning following removal of all adults from rearing containers the previous day. The adults were held individually in 18-ml plastic vials (Thornton Plastics, Salt Lake City, UT). Each vial contained a short section of green bean pod and was closed with a snap-cap lid, the center of which was replaced with nylon organdy. Vials of adults were held within an environmental chamber maintained at 27±1°C and a photoperiod of 14:10 light:dark hours for 4 days (adult age of 4-5 days).

On the day of the experiment, adults from the vials were partitioned among three 3.78-liter plastic buckets closed with screened lids (≥25 adults per bucket). The buckets were randomly assigned to treatments of 1) control, 2) treated with *B. bassiana* and not irradiated with UV, and 3) treated with *B. bassiana* and irradiated. Control and *B. bassiana* treatments were applied within a spray chamber described by McGuire et al. (2005). Before treatments were applied, the aluminum turntable of the spray chamber was chilled with frozen cold-packs for 15-30 minutes. The bucket assigned to the control treatment was chilled for 2 minutes in a standard laboratory freezer, and the chilled insects were decanted into a 100 × 15-mm Petri plate lined with filter paper. The plate containing the insects was sprayed with 5 ml of 0.01% Silwet-L77 and 1% glycerol in water. The sprayed insects were immediately returned to the bucket. Insects in the other two buckets were similarly handled except they were sprayed with 5 ml of a suspension of *B. bassiana* conidia (1×10^6 conidia/ml in 0.01% Silwet-L77). Based on the report by Leland et al. (2005), this concentration of conidia was expected to produce ≈50% mortality after 7 days.

After treatment, buckets of insects were placed in the biological cabinet, under the equilibrated UV-C lamp used for the conidial assays. During the exposure period, the buckets were closed with nylon tulle, which reduced the UV-C intensity to ≈85% of the fully exposed value. As in the conidial assays, the UV-C sensor was placed at the apex of the arc marked on the floor of the cabinet. Buckets of *L. hesperus* adults assigned to the control, or to the *Beauveria*-treated but not irradiated treatment, were placed outside the arc on the cabinet floor and covered with aluminum foil to block UV-C radiation. The remaining bucket (treated and irradiated) was randomly assigned to a position centered on the arc but on either side of the UV-C sensor. At 15-minute intervals for 2 hours, the buckets were shaken to dislodge the insects from the nylon tulle cover, and the reading from the UV-C sensor was recorded. The act of dislodging the insects from the cover was intended to facilitate UV exposure of both dorsal and ventral surfaces of the insects.

After the 2-hour exposure to UV, each bucket was chilled for 2 minutes in the freezer, and 20 insects from each bucket were individually transferred to numbered 18-ml plastic vials. Each vial contained a section of green bean pod, and was closed by a foam plug. Vials of insects were returned to the environmental chamber and maintained for 7 days. Each vial was examined daily for dead insects, and bean sections were replaced three times each week. When mortality was observed, the foam plug closing the vial was replaced with a snap-cap lid labeled with the day of death (post-treatment) and date. The bean section remained in the vial to provide humidity. Vials containing dead insects were examined daily for 6 days to detect eruption of mycelia or sporulation. The experiment was conducted three times.

Survival functions corresponding to the assigned treatments were compared using the LIFETEST procedure of SAS (SAS Institute 2008), controlling for repetition of the experiment using the STRATA statement. Statistical differences among the survival functions were declared based on the log-rank statistic. Pairwise comparisons of the survival functions were made controlling the experiment-wise error rate at $\alpha = 0.05$ by using the ADJUST = SIMULATE option in the STRATA statement. In addition, a contingency table analysis (PROC FREQ, SAS Institute 2008) was conducted to determine whether exposure to UV-C influenced the timing of appearance of fungal mycelia or of sporulation in *Beauveria*-infected insects. The analysis included only individuals that exhibited

evidence of infection during the 6-day period following insect death, and controlled for repetition of the experiment and time (days) post-death. Statistical differences between the two *Beauveria* treatments (irradiated or not irradiated) were assessed based on the Cochran-Mantel-Haenszel (CMH) row mean score statistic (Stokes et al. 2000).

Influence of UV-C Irradiation on *Lygus* Reproduction. Newly eclosed adults (60 of each gender) were obtained from the laboratory colony as previously described. The insects were divided among four 3.78-liter plastic buckets (30 adults per bucket) provisioned with green bean pods and shredded paper. Buckets of adults were maintained at 27 ± 1°C and a photoperiod of 14:10 light:dark hours within an environmental chamber until they were assayed at 4-5 days of age.

On the day of assay, adults from each bucket were separated by gender. Insects of each gender were divided between two clean buckets (a total of four buckets) closed with nylon tulle. The two buckets corresponding to each gender were randomly assigned to treatments of 1) UV irradiated and 2) not UV irradiated (control). The UV lamp was equilibrated with the fan operating as for the conidial assays, the UV sensor was positioned at the apex of the arc marked on the floor of the biological safety cabinet, and the buckets designated for irradiation were randomly assigned to positions on the arc on either side of the UV sensor. The two buckets assigned to the control treatment were covered with aluminum foil and placed on the floor of the cabinet outside the arc. The buckets were shaken to dislodge insects from the nylon tulle, and the reading of the UV sensor was recorded at 15-minute intervals during the 2-hour exposure period. Following exposure to UV, shredded paper and green beans were placed in the buckets of insects, which were returned to the environmental chamber for 2 days (until adults were 6-7 days old). On the second day after exposure to UV, males and females of respective treatments (irradiated or not irradiated) were combined to facilitate mating. Beginning on the following day, females were individually aspirated into 18-ml plastic vials with screened lids where they were examined for the presence of a spermatophore indicating recent mating (Cooper 2012). Mated females were provided a section of green bean pod, the ends of which were sealed with melted paraffin. Unmated females were returned to their corresponding bucket to be examined on succeeding days for mating. This process was continued until 10 mated females were obtained, except for the first repetition of the experiment when only seven females from the irradiated group survived to mate.

The green bean sections provided to mated female *L. hesperus* were replaced daily for 5 days. Each bean section removed from a female was placed in a new vial labeled with the treatment, female number, and day of oviposition. The bean sections containing eggs were held in the same environmental chamber as the adults. Beginning on the fifth day after removal from the mated female, each bean section was examined daily for 5 days, and newly emerged nymphs were counted and removed at each inspection. The experiment was repeated four times.

The daily fecundity of female *L. hesperus* assigned to the two treatments was compared by mixed-model repeated-measures analysis (SAS Institute 2008). Fixed effects included treatment (irradiated or not irradiated), day of oviposition, and their interaction. Random effects included repetition of the experiment and the repetition-by-treatment interaction that served as the error term for testing the main effect of treatment. A type-I autoregressive covariance structure was specified, and denominator degrees of freedom were corrected using the Kenward-Roger adjustment (Littell et al. 2006).

Results

Sensitivity of *Beauveria* Conidia to UV-C. The proportion of conidia that germinated decreased rapidly with increasing exposure to UV-C (Fig. 1). Predictions of germination based on assessments 24 hours after exposure of conidia indicated an LT_{50} of 1.36 minutes and LT_{99} of 3.87 minutes. In comparison, assessments at 48 and 72 hours after exposure indicated irradiation with UV-C delayed but did not prevent germination of some conidia. The delayed germination was observable only for UV exposure times ≥2 minutes, because extensive growth of mycelia corresponding to shorter exposure times prevented accurate estimates of germination at observation times >24 hours. Regardless of any non-lethal effects of the UV irradiation, germination at the longest exposure time (10 minutes) never exceeded 0.5%, and there was little additional germination after 48 hours. The LT_{50} and LT_{99} values predicted based on germination estimates at 48 (LT_{50}, 2.01 minutes; LT_{99}, 6.45 minutes) and 72 hours after exposure (LT_{50}, 2.17 minutes; LT_{99}, 7.36 minutes) were almost twice the predicted values based on germination estimates at 24 hours after exposure.

Disinfestation of *Lygus* Adults. Light output by the UV-C lamp was relatively stable during the 2-hour exposure of adult *L. hesperus* treated with *B. bassiana*, and recorded sensor readings ranged only from 127 to 132 μW cm^{-2}. The log-rank statistic indicated the survival functions corresponding to the treatments (control, treated with *B. bassiana* and irradiated with UV-C, or treated with *B. bassiana* and not irradiated) differed (χ^2 = 34.55, df = 2, P < 0.01; Fig. 2). Comparisons adjusted for multiplicity indicated mortality of insects treated with *Beauveria* but not irradiated was much greater than in other treatments (Fig. 2;

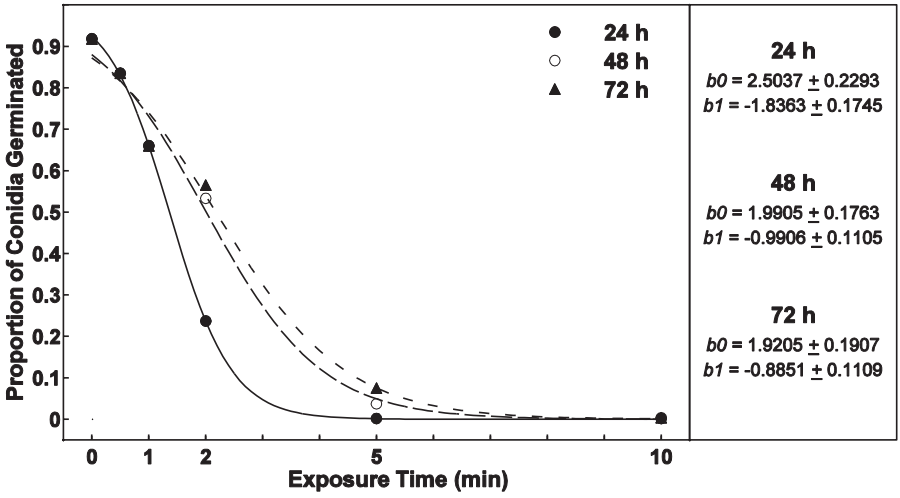

Fig. 1. Observed and predicted proportions of germination for *B. bassiana* conidia exposed to UV-C light. A separate logistic function was fitted to respective estimates of germination made at 24, 48, and 72 hours after exposure of conidia.

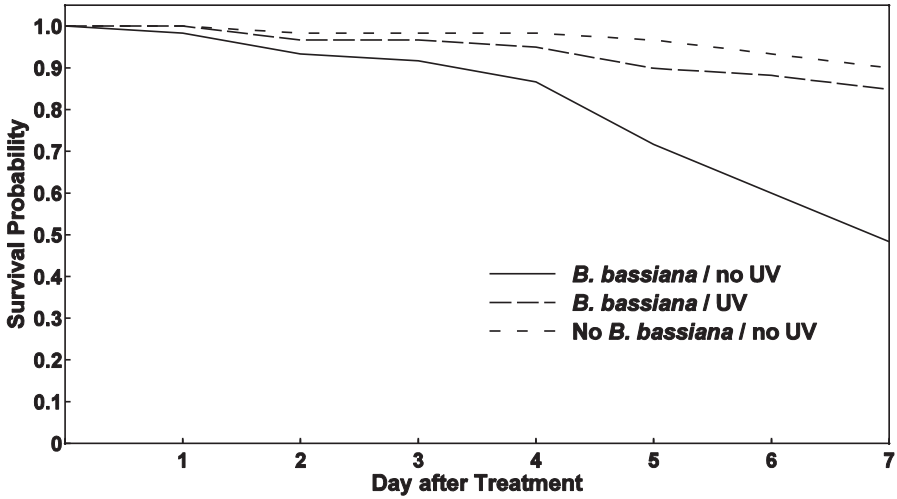

Fig. 2. Survival functions for adult *L. hesperus* treated with *B. bassiana*, treated with *B. bassiana* and irradiated for 2 hours with UV-C, or not treated and not irradiated.

compared with the control, $\chi^2 = 28.29$, df = 1, $P < 0.01$; compared with treated and irradiated, $\chi^2 = 21.46$, df = 1, $P < 0.01$). In contrast, the survival functions corresponding to the control and to the adults treated with *B. bassiana* and irradiated were not different ($\chi^2 = 0.51$, df = 1, $P < 0.76$). However, exposure to UV-C did not eliminate infection of insects treated with *B. bassiana*, because 5% of the insects exhibited hyphae and sporulation before the end of the experiment.

Contingency tables did not indicate that UV-C irradiation of *L. hesperus* treated with *B. bassiana* altered the pattern of appearance of hyphae or of the occurrence of sporulation (Row Mean Score = 0.68, df = 1, $P = 0.41$). Hyphae were observed on the first day after death for most of the insects (78%) exhibiting symptoms of *B. bassiana* infection, and the fungus sporulated on 74% of the infected insects by the second day after death. Appearance of hyphae was delayed beyond 2 days after death in only 13% of the insects, and all of those individuals were in the treatment that was not irradiated.

Influence of UV-C Irradiation on *Lygus* Reproduction. Although most mated females (80.5%) were obtained on the day after adults of both genders were combined, and 93.5% were obtained by the second day, the last mated female was not obtained until the fourth day after the insects were combined. Observed mean (± SE) daily fecundity was 3.7 ± 0.90 day^{-1} for female *L. hesperus* that were not irradiated and 3.4 ± 0.91 day^{-1} for females irradiated for 2 hours by UV-C. Also, mean daily fecundity varied only from 3.1 ± 0.92 day^{-1} corresponding to the fourth day of oviposition to 4.1 ± 0.91 day^{-1} for the third day of oviposition. Analyses failed to indicate an effect of exposure to UV-C on daily fecundity ($F = 0.10$; df = 1, 3.40; $P = 0.77$). Evidence of differences in fecundity corresponding to the first 5 days after mating also were lacking ($F = 1.12$; df = 4, 261; $P = 0.35$). The absence of a significant interaction between treatment group (irradiated or not irradiated) and day

of oviposition (F = 0.80; df = 4, 263.3; P = 0.53) indicated interpretation of the other factors (treatment and day of oviposition) was straightforward.

Discussion

Our results show that exposure to UV-C rapidly decreases survival of *B. bassiana* conidia, but that delayed germination is also a product of such exposure. These observations are consistent with the report of Hunt et al. (1994), who noted both mortality and delayed germination when conidia of *Metarhizium flavoviride* Gams and Rozsypal were exposed to simulated sunlight. Because the difference in germination observed at 24 and 48 hours was substantial for the longer exposure times, similar studies should not base conclusions solely on germination 24 hours after exposure to UV irradiation.

Irradiation of *B. bassiana*-treated adult *L. hesperus* with UV-C did not eliminate infection of the insects in spite of exposure times well in excess of the estimated LT_{99}. Failure to eliminate infection probably was not caused by the relatively minor shading effect of the nylon tulle cover that enclosed the insects. It seems more probable that inability to eliminate infection was associated with inability to adequately expose all external surfaces of the insects to the UV radiation. Regardless, irradiation with UV-C considerably decreased the probability of infection compared with insects not irradiated.

Regardless of whether adult *L. hesperus* were irradiated, once the genders were combined, mating occurred more slowly than was reported by Brent (2010a,b). Under the conditions of our study, the adult *L. hesperus* should have been reproductively mature by the time the genders were combined (Spurgeon and Cooper 2012). An increased propensity to mate in response to long-term laboratory culture was previously reported for *L. hesperus* (Brent and Spurgeon 2011). Therefore, the more timely mating observed by Brent (2010a,b) likely resulted from use of a long-standing laboratory colony, whereas the insects in our study originated from recent collections in the field. Irrespective of the time required to mate, or the day of oviposition following mating, our results provided no evidence of a deleterious effect of exposure to UV-C on reproduction by *L. hesperus*.

Although our results indicate the ability of UV-C to kill *B. bassiana* conidia on the cuticle of *L. hesperus* adults, they do not imply that irradiation by UV would be effective against the pathogen once infection was initiated. However, irradiation should be useful for limiting the impact of conidia that may be dispersed from sporulating cadavers or passed from nets or other devices used to collect insects from the field. Therefore, we conclude that routine exposure of field-collected *L. hesperus* adults to UV-C irradiation may reduce the probability of introducing individuals infected with *B. bassiana* into insect colonies and that such exposure does not adversely impact subsequent reproduction.

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