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Bioassay method for assessing the virulence of *Beauveria bassiana* against tarnished plant bug, *Lygus lineolaris* (Hem., Miridae)

H. Liu, M. Skinner and B. L. Parker
Entomology Research Laboratory, The University of Vermont, Burlington, VT, USA

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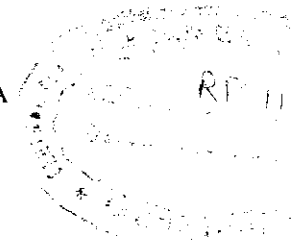

Abstract: A standard bioassay method for assessing the pathogenicity of *Beauveria bassiana* (Balsamo) Vuillemin (GHA strain) against second instar tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois) (Hem., Miridae) was developed. Several types of inoculation methods, assay containers and incubation times were tested. Our goal was to minimize control mortality and maximize treatment mortality. Five inoculation methods (immersing broccoli florets or bean pods, spraying broccoli florets or bean pods, and immersing insects) and four types of plastic containers (114-, 171-, 228- and 455-ml) were tested. Immersing insects directly in a fungal suspension was the most effective inoculation method, which resulted in a treatment mortality of 70–81.3% at a concentration of 1×10^7 conidia/ml. The 114-ml plastic container was the most suitable assay container when 10 tarnished plant bug nymphs were treated together, resulting in a control mortality of only 6% 12 days after treatment. Within the first 6 days after treatment, 71.1% of the insects were killed, compared with a total mortality of 81.3% after 12 days. Nymphs infected with the test fungus changed colour from green to black. Mycelial outgrowth and sporulation on the cadavers demonstrated that most nymphs died of fungal infection. A total of 61.1 and 80.5% of the cadavers showed signs of mycelial outgrowth 9 days after death among those that were surface sterilized and those that were not, respectively.

Key words: *Beauveria bassiana*, *Lygus lineolaris*, bioassay, biological control, entomopathogenic fungi

1 Introduction

Tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois) (Hem., Miridae), is the primary insect pest of June-bearing strawberries in the eastern US and Canada (Schaefers, 1981; Vincent et al., 1990). Feeding on receptacle achenes by first-generation nymphs causes 'catfacing' and apical seediness of berries (Riggs, 1990; Kovach et al., 1993). These berries are often unmarketable (Kovach et al., 1993). Insecticides are the only way to manage this pest in commercial plantings (Vincent and Lachance, 1993). Tarnished plant bug has developed insecticide resistance in other crops (Cleveland and Furr, 1979; Cleveland, 1985; Snodgrass, 1994, 1996a,b; Snodgrass and Elzen, 1995). Concern for human health and environmental quality has stimulated research for alternative management methods.

The entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycotina, Hyphomycetes) has been isolated from >200 insect species (Humber, 1998). It is a promising candidate for microbial control of many important pests such as the migratory grasshopper, *Melanoplus sanguinipes* (F.), Colorado potato beetle, *Leptinotarsa decemlineata* (Say), and the diamondback moth, *Plutella xylostella* (L.) (Inglis et al., 1997; Poprawski et al., 1997; Vandenberg et al., 1998). It also has potential for use against *L. lineolaris*. In cotton, Naturalis-L® (Troy Bioscience, Phoenix, AZ), a *B. bassiana* -based product, applied at 1.1 l/ha, reduced *L. lineolaris* nymphal and adult populations by 53.8 and 20.2%, respectively (Snodgrass and Elzen, 1994). Similar trials were conducted using Mycotrol™ WP (Mycotech Corp., Butte, MT), another *B. bassiana* formulation. Although there was no significant decrease in *L. lineolaris* population density following treatment with Mycotrol, there was a decreased rate of injury to berries at the first picking (Kovach, 1996). Twenty-five percent of the berries in the control plots were damaged, whereas 3–6% were damaged in the treated plots. In canola, 91.5% adult mortality was achieved in field cages 7 days post-treatment (Steinkraus and Tugwell, 1997). However, these trials have been carried out using commercial formulations only. Data are not available on the relative virulence of the isolates used in these formulations compared to other, non-commercial strains. Information is also not available on the relative efficiency of different formulations against this pest. Selection of the most virulent strain and formulation is important for the

consistent and effective management of *L. lineolaris*. This study was designed to develop a simple, rapid, reliable and inexpensive bioassay method for evaluating the virulence of entomopathogenic fungal isolates against *L. lineolaris* nymphs.

2 Materials and methods

2.1 Insects

Lygus lineolaris were reared on broccoli, *Brassica oleracea* var. *italica* Plenck ('Packman' and 'Premium') and green bean, *Phaseolus vulgaris* L. ('Blue Lake') according to the methods of Snodgrass and McWilliams (1992). The eggs used to establish the colony were supplied by Dr G. L. Snodgrass (Southern Insect Management Laboratory, USDA-ARS, MS). In all assays, 1–2-day-old second instars were used.

2.2 Test fungus

For all tests described herein, *B. bassiana* (GHA strain, Mycotech Corp., Butte, MT) was used against *L. lineolaris* second instars as a spore suspension in sterile distilled water (SDW) with 0.005% Tween 80 as a wetting agent. Pure cultures of the fungus were isolated from the Mycotrol™ WP formulation by spreading 200 μl of the suspension (*ca.* 10^4 conidia/ml) onto quarter-strength Sabouraud dextrose agar containing 0.25% w/v yeast extract (SDAY). Plates were incubated in the dark at 20 ± 1°C for 10 days. Slant cultures were prepared from a single colony and after incubation for 10 days they were stored at 4°C. For all tests, cultures were established from these slants. For each of the tests, five culture plates were prepared by spreading 200 μl of a conidial suspension (1×10^6 conidia/ml) onto SDAY. Plates were held as above for 14 days to maximize spore production. Spores were harvested by flooding each plate with 10 ml of 2.5% Tween 80 in SDW and dislodging the conidia into suspension with a glass rod. The suspension was filtered through a double layer of sterile cheesecloth and centrifuged at 1700 rpm for 15 min. The supernatant was discarded and the conidia resuspended in 5 ml SDW. Counts of conidia were made on a 400× dilution from the stock suspension using a Neubauer haemocytometer. The stock was stored at 5°C for 24 h until spore viability was determined. Spores with germination tubes longer than their width were considered germinated (Hywell-Jones and Gillespie, 1990). Only batches with >90% viability were used. A spore suspension containing 1×10^7 conidia/ml SDW with 0.005% Tween 80 was prepared from the stock for the assays.

2.3 Fungal inoculation

Fungi can be applied directly to insects by spraying (Majchrowicz et al., 1990; Vandenberg, 1996), immersion (Hegedus and Khachatourians, 1996; Steinkraus and Tugwell, 1997), or showering them with spores (Milner and Soper, 1980; Feng and Johnson, 1991). Secondary substrates, such as a leaf surface, a food source, or an inert surface treated with a fungal suspension can also be used to inoculate insects indirectly (Inglis et al., 1997; Reithinger et al., 1997). The efficacy of direct and indirect inoculation methods was tested.

For direct inoculation, 10 second-instars were placed in a sterile 20-ml vial and 10 ml of fungal suspension was added. The vial was capped and inverted five times, over a 5-s period, to ensure that the insects were completely immersed. The suspension was filtered through a tea strainer (6 cm diameter) after which the treated insects were transferred with a fine camel's hair brush (#00) to a 114-ml translucent plastic container (5.5 cm diameter, 3 cm height) with a fresh broccoli floret as a food source. For the controls, insects were treated with 0.005% Tween 80 only. Each container was closed with a tight fitting lid and incubated at 20 ± 1°C, 50–60% RH, 15 : 9 (L : D) for 24 h. A vented lid with a 1-cm^2 window of 50-mesh screening (0.2 mm opening with 36% open area) replaced the original lid, and the treated floret was exchanged with a fresh, untreated floret, the base of which was wrapped in a layer of moist tissue paper and sealed with parafilm to prevent wilting. Every 2 days for 12 days, the floret was replaced with a fresh floret and nymphal mortality was evaluated. Nymphs that did not move their legs or antennae when probed with a fine brush were considered dead. Dead nymphs were counted and removed.

For indirect inoculation of fungi, two methods were tested with two types of substrate, bean pods (2-cm long) and broccoli florets (2-cm long). For the first method, the substrate (bean pod or broccoli floret) was immersed in 10 ml of the suspension for 5 s. For the second method, the substrate was sprayed on both sides with 2 ml of suspension using a hand-held spray atomizer. For the controls, the substrate was immersed in or sprayed with 0.005% Tween 80 only. Each treated substrate was air-dried for 15 min and placed in a 114-ml plastic container to which 10 second-instars were added. After 24 h, the treated substrate was removed from each container and replaced with an untreated broccoli floret. The same general experimental procedures described above for the direct inoculation assays were followed after treatment. Each method of inoculation was replicated five times (total of 50 insects) and the entire experiment was repeated once.

2.4 Bioassay containers

To determine the most suitable size of container that can be used for the bioassays, we tested four types of translucent plastic containers with volumes of 114, 171, 228 and 455 ml (Sweetheart Inc., Chicago, IL). A fresh broccoli floret (2-cm long) was immersed in 10 ml of the fungal suspension (1×10^7 conidia/ml) for 10 s and air-dried for 15 min. The length of time that the floret was immersed in the fungal suspension was increased from 5 to 10 s because the treatment mortality obtained in the first series of assays was low. For the controls, florets were immersed in sterile 0.005% Tween 80 only. A treated floret was placed on sterile tissue paper in each assay container and 10 nymphs were added. The same general experimental procedures described above for the direct inoculation assays were followed after treatment. Each container was replicated five times within an assay and the entire assay was repeated once (total of 50 insects per test container per assay).

2.5 Disease progression

Studies were done to define how long an assay should run to effectively quantify mortality due to fungal treatment, and evaluate the effects on fungal outgrowth of surface-sterilizing compared with non-sterilizing cadavers. Using the direct immersion procedures described above, nymphs were treated with a standard fungal concentration of 1×10^7 conidia/ml 0.005% Tween 80 and transferred to a 114-ml container. The controls were treated with 0.005% Tween 80 only. Five replicates (10 insects per replicate) were set up for each trial

and the experiment was repeated three times over three consecutive days, thereby providing replication within and between times. Food was replaced and mortality assessed as described previously.

When the mortality was assessed each day, cadavers from the controls and fungal treatments were removed and divided into two equal groups. Those in the first group were transferred individually to a Petri dish lined with a moist sterile filter paper to provide the humid conditions necessary to promote fungal outgrowth. Cadavers in the second group were surface-sterilized to prevent possible posthumous contamination. To surface-sterilize a cadaver, it was immersed in 70% ethyl alcohol for 5 s, rinsed in SDW and placed in 1% sodium hypochlorite. After 10 s it was rinsed in two to three changes of SDW and transferred to a Petri dish as described above. All dishes were sealed with parafilm to maintain >95% RH (LACEY and BROOKS, 1997) and incubated in the dark at 20 ± 1°C. Fungal outgrowth and sporulation were recorded daily for 9 days.

2.6 Data analysis

Mortality data to evaluate inoculation methods, assay containers and disease progression were converted to percentages and subjected to arc sine transformation before analysis. Each was first analysed using analysis of variance (ANOVA) ($\alpha = 0.05$). Mortality data were then subjected to Duncan's multiple comparison (SAS INSTITUTE, 1996) to determine if there was evidence of significant differences among them. Probit analysis (SAS INSTITUTE, 1996) was used to analyse the time-course of nymphal mortality. A two-sample *t*-test was used to compare the rate of mycosis development on surface-sterilized and non-sterilized cadavers (SAS INSTITUTE, 1996).

Table 1. Mortality of Lygus lineolaris *nymphs exposed to* Beauveria bassiana *GHA strain at a standard dose of 1×10^7 conidia/ml using different inoculation methods*

Inoculation method	Mortality 12 days after treatment (%) (mean ± SEM)*	
	Control •	Treated
Immersing broccoli floret	4.0 ± 1.6ab	22.0 ± 3.9b
Immersing bean pod	7.0 ± 2.1ab	15.0 ± 3.4bc
Spraying broccoli floret	9.0 ± 2.3a	10.0 ± 3.3c
Spraying bean pod	9.0 ± 2.8ab	21.0 ± 4.3b
Immersing insect	2.0 ± 1.3b	70.0 ± 4.7a

* Means followed by the same letter within a column are not significantly different ($\alpha = 0.05$, Duncan's multiple comparison) (SAS INSTITUTE, 1996).

Table 2. Mortality of Lygus lineolaris *nymphs exposed to* Beauveria bassiana *GHA strain on broccoli florets after immersion in a standard dose of 1×10^7 conidia/ml for 10 s and held in different bioassay chambers*

Chamber size (ml)	Mortality 12 days after treatment (%) (mean ± SEM)*	
	Control	Treated
114	6.0 ± 3.1a	54.0 ± 5.8a
171	25.0 ± 5.8b	54.0 ± 5.2a
228	16.0 ± 2.2b	55.0 ± 6.9a
455	9.0 ± 4.1a	49.0 ± 8.0a

* Means followed by the same letter within a column are not significantly different ($\alpha = 0.05$, Duncan's multiple comparison) (SAS INSTITUTE, 1996).

3 Results

There was a significant difference in nymphal mortality depending on the inoculation method used ($F = 51.44$, d.f. = 4, 4, $P < 0.01$). Mortality was significantly higher among nymphs treated with a fungal suspension by immersion than by all other inoculation methods tested (table 1). Nymphal mortality among the controls was significantly less when insects were immersed than when the food source was treated (table 1). When analysed by multiple comparison, differences in control mortality were not significant among the four indirect inoculation methods. Among the fungal treatments, direct immersion of the insects in a fungal suspension resulted in the highest percentage of overall mortality, and spraying the broccoli florets produced the lowest levels of mortality among all of the indirect inoculation methods (table 1). Nymphal mortality increased from 22 to 54% when the immersion time of the broccoli floret in the fungal suspension was increased from 5 to 10 s (tables 1 and 2).

No significant difference in mortality was detected when treated nymphs were held in the four different-sized assay containers ($F = 4.94$, d.f. = 3, 3, $P = 0.111$) (table 2). However, control mortality was significantly lower in the 114- and 455-ml containers than in the 171- and 228-ml containers. Among the controls, nymphs held in the 171-ml container had the highest mortality (table 2).

Typical consistent symptoms of infection were observed among the treated insects. The appendages of the dead, infected nymphs were fully extended and a blackening of the cuticle was evident. In contrast, nymphs that died from other causes usually retracted their legs and maintained a bright green colour.

Mortality of nymphs was 81.3% 12 days after fungal treatment by immersion when held in the 114-ml containers, compared with a control mortality of 6.7% (fig. 1). There was a significant correlation between cumulative mortality and time (Pearson's goodness-of-fit test, $\chi^2 = 15.01$, d.f. = 4, $P < 0.01$) and mortality increased over time (fig. 1). The most rapid increase in cumulative mortality occurred from 2 to 6 days post-treatment (fig. 1). Within the first 6 days post-treatment, 71.1% of the insects died out of a final total of 81.3% over the entire observation period. Therefore, bioassays on nymphs using the immersion method should be run for at least 6 days to allow development and expression of mycoses at the standard dose level we used.

Microscopic examination of sporulation on the cadavers confirmed infection by *B. bassiana*. The highest rate of infection and mortality occurred between days 2 and 6, and the incidence of infection in dead individuals declined thereafter by day 12 (fig. 2). There was a significant difference in mycosis development between surface-sterilized and non-sterilized

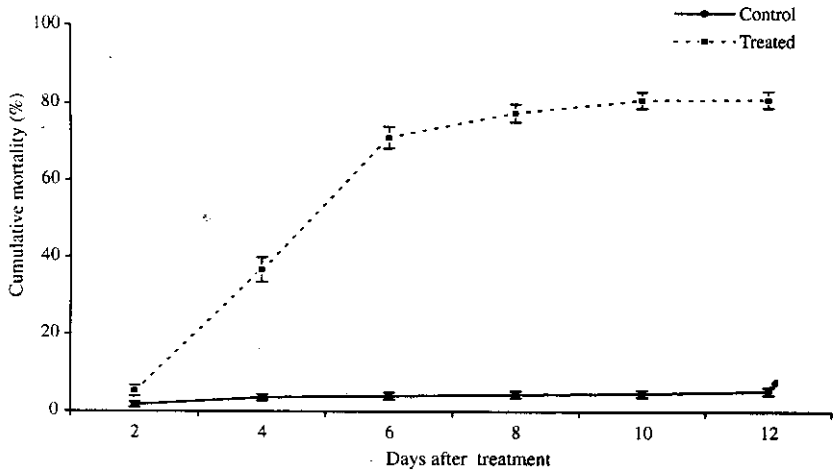

Fig. 1. Cumulative mortality of Lygus lineolaris *nymphs over time following exposure to* Beauveria bassiana *GHA strain at* 1×10^7 *conidia/ml via direct immersion and held in a 114-ml assay chamber. Each point represents the mean (± SEM) of five replicates per experiment, each containing 10 nymphs, and three experimental replicates*

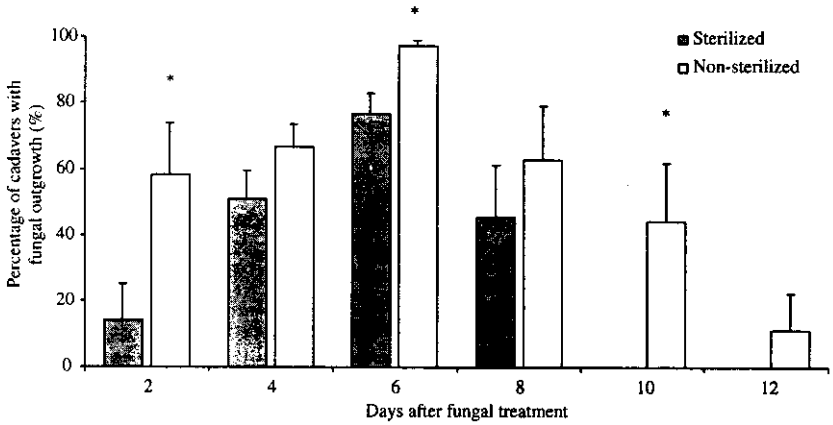

Fig. 2. Mean percentage (± SE) of sterilized and non-sterilized Lygus lineolaris *cadavers with evidence of* Beauveria bassiana *outgrowth and conidiation. Nymphs were treated by immersion in* B. bassiana *GHA strain* (1×10^7 *conidia/ml). Asterisks indicate that the treatment means on a given day are significantly different* ($\alpha = 0.05$, *two-sample t-test)*

cadavers ($t = 3.25$, d.f. = 106, $P < 0.01$). Fungal outgrowth was observed in a mean 61.1 and 80.5% of the sterilized and non-sterilized cadavers, respectively. Posthumous surface sterilization appeared to significantly influence the interpretation of fungal efficacy. No mycosis was observed in the controls.

4 Discussion

The pathogenicity of *B. bassiana* against *L. lineolaris* can be evaluated reliably by immersing the insects directly in a suspension of fungal conidia. Treating a broccoli floret or bean pod by immersion in a fungal suspension (1×10^7 conidia/ml) was not an effective way of inoculating *L. lineolaris* nymphs. A higher dose, longer immersion time, or use of more effective wetting agents may result in higher levels of substrate coverage with an increase in treatment mortality.

Among the containers tested, the 114-ml plastic container was the most suitable for bioassay as treatment mortality was maximized while control mortality was minimized. Various studies (Bariola, 1969; Khattat and Stewart, 1977; Slaymaker and Tugwell, 1982; Snodgrass and McWilliams, 1992) and our own observations (unpublished data) have shown that natural mortality of *L. lineolaris* was relatively high even under favourable laboratory rearing conditions, with only around 70% of the first instars surviving to adulthood. Therefore, minimizing control mortality is critical for effectively and efficiently evaluating fungal pathogenicity. Because of its small size, the 114-ml container also has the advantage over other chambers tested when temperature-controlled incubation space is limited.

A minimum incubation period of 6 days post-treatment is needed to assess the efficacy of *B. bassiana*. Selection of a suitable incubation time is critical to a bioassay procedure taking into consideration the infection cycle of the test fungus. Bioassays may be run for as long as 2 weeks due to the slow infection process of some entomopathogenic fungi (Goettel and Inglis, 1997). With the *B. bassiana* strain we tested, an incubation period of 12 days was used to ensure that all mortality resulting from infection would be expressed. However, because there was no significant increase in cumulative mortality after day 6, it would have been possible to shorten the bioassay period for this particular isolate. Use of a shorter bioassay period has the added benefit of minimizing mortality due to 'natural' causes, which can

interfere with the evaluation and interpretation of fungal assay data.

Observation of fungal outgrowth or conidiation on insect cadavers is usually used to verify death by fungal infection (Moorhouse et al., 1993; Vandenberg et al., 1998). Results of this study showed that surface sterilization of cadavers with alcohol and sodium hypochlorite significantly inhibited this process. Because the test insect was small, and soft-bodied, the sterilizing agents, alcohol and sodium hypochlorite, may have penetrated the cuticle of the cadaver, thereby killing the invading fungus. Therefore, care should be taken when this practice is used in the interpretation of fungal efficacy in a bioassay. For laboratory bioassays, death as a result of fungal infection may be significantly underestimated if it is based solely on fungal outgrowth on cadavers after surface sterilization.

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Author's address: MARGARET SKINNER (corresponding author), Entomology Research Laboratory, The University of Vermont, 661 Spear Street, Burlington, VT 05405-0105, USA. E-mail: mskinner@zoo.uvm.edu