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Development of a novel bioassay for estimation of median lethal concentrations (LC_{50}) and doses (LD_{50}) of the entomopathogenic fungus *Beauveria bassiana*, against western flower thrips, *Frankliniella occidentalis* ☆

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Abstract

To conduct laboratory experiments aimed at quantifying secondary acquisition of fungal conidia by western flower thrips (*Frankliniella occidentalis*), an efficient assay technique using *Beauveria bassiana* as the model fungus was developed. Various application protocols were tested and it was determined that the percent mortality did not vary among protocols. Peak mortality of second-instar nymphs, under constant exposure to conidia, occurred 5 days post-inoculation. Second-instar thrips that were exposed to conidia within 24 h of the molt to second instar were more susceptible to *Beauveria bassiana* than thrips exposed after times greater than 24 h post-molt. Conidia efficacy, which was monitored at 24 h intervals, did not differ significantly within 72 h. A test of the final bioassay system was conducted in a series of assays aimed at determining the LD_{50} of *B. bassiana* technical powder against second-instar western flower thrips. It was determined that *B. bassiana* (strain GHA) is highly effective at very low doses (LD_{50} of 33–66 conidia/insect).
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1. Introduction

The western flower thrips (WFT), *Frankliniella occidentalis* Pergande, causes substantial economic losses to greenhouse crops via feeding damage and virus transmission (Hausbeck et al., 1992; Loomans et al., 1995; Murphy et al., 1998). Their ability to rapidly develop resistance to synthetic chemical insecticides (Brødsgaard, 1994; Immaraju et al., 1992; Zhao et al., 1994) has stimulated research on alternative methods of control. One such alternative for thrips management in greenhouses is the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin. Applications of this pathogen have been reported to reduce populations of thrips in crops of greenhouse cucumbers, chrysanthemums, gerbera daisies, roses, and carnations (Bradley et al., 1998; Jacobson et al., 2001; Ludwig and Oetting, 2002; Murphy et al., 1998; Shipp et al., 2002).

The thigmokinetic, cryptic nature of thrips has the potential to make direct targeting with *B. bassiana*

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conidia difficult in some crops. This raises questions regarding the capacity of thrips to acquire lethal doses of conidia via treated substrates and the potential for control by this inoculation mechanism. To better understand the pathogen–insect interaction, some fundamental questions related to conidial acquisition by thrips from treated foliage need to be addressed.

The ability of thrips and other minute insects to escape from conventional assay cups led to the development of a successful assay chamber known as the Munger cell (Munger, 1942), and at least two additional versions have been modified from the original design (Morse et al., 1986; Tashiro, 1967). Munger cells are comprised of three pieces of Plexiglas, which are stacked and held tightly together by a pair of binder clips. Drawbacks to the use of Munger cells, especially with respect to conducting extensive or routine bioassays of thrips pathogens, include lack of disposability, inconvenience of cleaning and sterilization between uses, and labor-intensive setup and maintenance.

The initial objective of the current research was, therefore, to develop a thrips bioassay protocol employing an easy-to-use, inexpensive/disposable holding chamber that would produce bioassay results with acceptable levels of accuracy and precision. The bioassay system elaborated here is based, in part, on protocols (unpublished) developed by one of the authors (MB).

The main objectives in the bioassay development process were to develop efficient protocols for exposing second-instar thrips, one of the more difficult life stages to infect due to molting, to foliar spray deposits and for quantifying the number of conidia acquired by thrips feeding on the treated foliage. Toward these objectives, studies were conducted to (1) compare the efficacy of alternative bioassay techniques, (2) determine the minimum incubation time at constant $25 \pm 1\,°C$ required for accurate assessment of treatment effects (insect mortality due to fungal infection), (3) characterize the age-dependent mortality of second-instar thrips to identify the sample time providing the best estimate of effective acquired doses of *B. bassiana* conidia, and (4) investigate effects of conidial residue age on fungal infectivity.

The bioassay chamber and protocols developed were ultimately employed to characterize the pathogenicity of *B. bassiana* strain GHA against second-instar WFT under controlled laboratory conditions. Second-instar thrips were selected as the target life stage since this larval stadium is short and thus potentially difficult to infect. This specifically included an investigation of the capacity of WFT to acquire lethal doses of conidia from a treated leaf substrate and determination of the LC_{50} (conidia/mm^2 substrate) and LD_{50} (conidia/insect) of this well-known fungal pathogen.

2. Materials and methods

2.1. General methods

2.1.1. Rearing and selection of thrips for bioassays

Western flower thrips adults collected from a Cornell University greenhouse were used to establish a colony. Thrips were reared in square plastic containers (16 cm × 16 cm × 6 cm) with snap-on lids. A hole (10.5 × 10.5 cm) was cut in the lid and covered with thrips-proof organdy (mesh openings of 95 μm) (Sefar America, Kansas city, MO) for ventilation. The containers were held at $25 \pm 1\,°C$, under a 14:10 h light:dark (L:D).

Red kidney beans (*Phaseolus vulgaris*) were planted and allowed to grow in a thrips free growth chamber for 10–12 days at $26 \pm 1\,°C$. The first pair of true leaves was excised from each plant, and the petioles were placed in water to maintain leaf turgor. Two incisions were made in the top of the plastic wrapper of an absorbent cotton pad (American Fare Nitetime unscented maxipad, Kmart, Troy, Michigan), which was then saturated with water. The petioles of the excised leaves were wrapped in a small amount of moist cotton and inserted into the pad (one petiole per incision). One pad (with two petioles) was added to each rearing container, followed by 25–30 adult female WFT. The thrips were allowed to oviposit for 48 h before being removed via aspiration to a fresh container provisioned with a pad and leaves. Females were transferred to fresh containers 5–6 times.

Three days after removal of the adult females, the thrips populations in the containers were comprised primarily of second-instar nymphs. Groups of 12 early-second instars were collected via aspiration into modified 1 ml centrifuge tubes. Identification of early-second instars was based on the size of the abdomen relative to the head and thorax. The tubes (groups of 12 nymphs) were randomly assigned to the different treatments associated with an assay.

2.1.2. Fungal preparations

Conidial preparations of *B. bassiana* strain GHA were made using an unformulated (technical) powder produced by Emerald BioAgriculture (Butte, MT) using proprietary solid-substrate culture methods and ingredients (Bradley et al., 1992; Wraight et al., 1998). The technical powder contained $1.09 \times 10^8 \pm 8.61 \times 10^6$ conidia/mg. The conidial powder was stored at 4 °C following the manufacturer's instruction. Silwett L-77, an organosilicone surfactant (Loveland Industries, Greeley, CO), was added to deionized water at a concentration of 0.01%. Aliquots of 20–40 ml were pipetted into 50-ml plastic centrifuge tubes, and selected amounts of conidial powder were added. One gram of glass beads (2 mm diameter) was added to each tube, and the tube was agitated on a wrist action shaker (Model BT, Burrell

Scientific, Pittsburgh, PA) set at maximum speed (6.7 oscillations/s) for 15 min.

2.1.3. Bioassay protocols

To a one-ounce polystyrene portion cup (Fabri-Cal, Kalamazoo, MI) five items were added in the following order: a filter paper disk (2.5 cm diam.), two bean leaf disks (2 cm diam.) with the abaxial surfaces facing one another, an additional filter paper disk, and a group of 12 early-second-instar western flower thrips (Fig. 1). Deionized water (35 μl) was pipetted onto each piece of filter paper to humidify the assay cup and slow leaf desiccation. The cup was then sealed with Parafilm and capped with the plastic snap-on lids provided by the manufacturer. Cups were held at 25 ± 1 °C for 5 days under a natural light regime (incubator with glass door) in a laboratory with unshaded windows. After this time they were opened, and thrips mortality assessed. Hereafter, the layered paper and leaf-disk elements of each bioassay unit will be referred to as the leaf "sandwich."

2.1.4. Fungus application

All fungal treatments were applied using a Burgerjon Spray tower (Burgerjon, 1956) fitted with an air-atomizing nozzle (Fluid Cap 2850 + Air Cap 70) mounted in a 1/4 J nozzle body (Spraying Systems, Wheaton, IL). The nozzle was connected to a regulator valve providing a constant airflow of 10 L/min. The spray targets (leaf disks or filter paper disks with thrips) were placed in the lid of a petri plate containing a wet piece of filter paper, and were positioned on a rotating turntable (33 rpm) during application. Spray deposition at the level of the target surface was approximately 0.01 μl/mm^2 (resulting from spraying of a 5 ml aliquot). The spray deposits on the treated leaf disks were allowed to dry prior to the introduction of thrips.

Fig. 1. Small plastic assay cups containing: a filter paper disk (2.5 cm diam.), two leaf disks (2 cm diam.) with the abaxial surfaces facing one another, an additional filter paper disk, and a group of 12 early-second-instar western flower thrips. Deionized water (35 μl) was pipetted onto each piece of filter paper and the cup was then sealed with Parafilm and capped with the plastic snap-on lids provided by the manufacturer.

2.1.5. Quantification of spray application rates and acquired doses

The actual application rates (conidia/mm^2) were quantified by counting conidia deposited on polystyrene Petri dish lids (90 mm diam.), which were sprayed simultaneously with the leaf or filter paper disks. The enumeration protocol was that described by Wraight and Carruthers (1999).

Conidial doses acquired by thrips were determined using the following protocol. Two narrow (2 mm wide) pieces of paper labeling tape (TimeMed Labeling Systems, Burr Ridge, IL) were affixed along the opposite edges of a glass cover slip (22 mm^2). A drop of lactic acid (85%) containing acid fuchsin (1 mg/ml) was placed in the center of the cover slip. Five second-instar thrips were removed from an assay cup with a small paintbrush and submerged in the stain. A second cover slip was then placed on top of the first, sandwiching the tape between them. The thickness of the tape was such that in the finished mounts, the bodies of the thrips were slightly compressed but not ruptured. A special microscope stage was constructed to facilitate counting of all conidia attached to the thrips. Two sets of three glass cover slips were glued together. The two sets of cover slips were then affixed 1.6 cm apart on a glass microscope slide. These sets of cover slips served as lateral supports for the cover slip assembly containing the thrips, holding it above the slide surface and allowing it to be easily gripped with forceps and turned over. This made it possible to count conidia on both the dorsal and ventral surfaces of the thrips. Conidia were counted under a phase contrast microscope at 400× magnification, and the number per insect was recorded. Conidia-like objects on the Silwet-sprayed (controls) thrips were quantified, and the mean numbers of conidia on fungus-treated insects were corrected for these "background" numbers by subtracting the mean number of conidia observed on the controls from the numbers observed on each *Beauveria*-treated insect. Numbers of these objects were in all cases very low (<4 objects per thrips). Thrips collected from colony containers containing heavily thrips-damaged bean leaves, which became necrotic and were colonized by saprophytic fungi, contained high numbers of *Beauveria*-like objects. By limiting the number of adult female thrips added to rearing containers, necrosis and thus saprophytic fungal growth was kept in check. This greatly decreased the number of *Beauveria* like objects

on the bodies of second-instar thrips used in dose acquisition assays.

Additionally, a Petri dish with yeast extract agar (1%) was sprayed along with the leaf disks in each test. The inoculated plates were sealed with Parafilm and incubated for 24 h at 25 ± 1 °C. The spray deposits were then stained with acid fuchsin, and the first 100 conidia encountered under phase contrast microscopy (400×) were scored for germination. This procedure was conducted at five randomly chosen locations per Petri plate, and numbers of conidia in all assays were corrected for viability.

2.2. Bioassay-development tests

2.2.1. Treatment method

A series of tests was conducted to compare five different methods of exposing thrips to *B. bassiana* conidia. Methods included treating thrips both directly via topical sprays and indirectly via spray-treated foliage (see Table 1 for details). In all cases, fungal suspensions containing technical powder/ml were tested. Applications of these suspensions across all treatment-method tests translated to a mean (±SE) application rate of 4550 ± 710 conidia/mm^2.

Table 1
Effects of the mode of application of *Beauveria bassiana* strain GHA conidia on mortality of second-instar western flower thrips in a laboratory bioassay[a]

Treatment: conidia sprayed onto	Corrected % mortality (mean ± SE)[b]
Abaxial surfaces of both leaf disks[c]	75.9 ± 4.2
Adaxial and abaxial surfaces of both leaf disks[b]	69.8 ± 5.3
Thrips on filter paper disk (disk then placed on top of the top filter paper disk in bioassay unit)[d]	72.4 ± 6.5
Thrips on filter paper disk (disk then substituted for top filter paper disk in bioassay unit)[d]	69.1 ± 5.1
Thrips on filter paper disk (thrips then transferred to untreated bioassay unit)[e]	84.9 ± 5.0
Control[d]	19.9 ± 4.0
ANOVA statistics	$F_{[4,67]} = 1.85$; $P = 0.13$

[a] Bioassay unit comprising two filter paper and two bean leaf disks (all 2.5 cm and 2 cm diam., respectively) stacked in a small cup; the leaf disks were placed together (abaxial surfaces facing one another) and sandwiched between the two filter paper disks. All treatments applied at the concentration of 2 mg conidia/ml (4550 ± 710 conidia/mm^2).

[b] Mean mortality (±standard error; $n = 15$) recorded in bioassay units incubated 5 days at 25 ± 1 °C (12 early-second-instar thrips/unit; 5 units/assay; assays conducted three times). Percent mortality corrected for control mortality using Abbott's formula.

[c] Indicated surfaces of leaf disks were treated, the treated surfaces were allowed to dry, the treated disks were incorporated into bioassay units, and untreated thrips were added.

[d] Thrips were placed on wet filter paper disks and treated; the treated disks with thrips were then immediately placed in the bioassay units as indicated (treated surfaces up).

[e] Thrips were placed on wet filter paper disks and treated; the treated thrips were then immediately transferred to bioassay units using a fine brush; control thrips treated with 0.01% Silwet.

2.2.2. Incubation time

Abaxial surfaces of leaf disks were treated with 2 mg/ml of the technical powder. The treated disks were then added to the assay cups (two per cup as previously described) along with 12 second-instar thrips. Four or five replicate cups were held at 25 ± 1 °C and under the natural light regime for 3, 4, 5, and 6 days before being destructively sampled to assess thrips mortality. The same protocol was conducted using a lower conidial concentration (0.075 mg/ml, technical powder) and extending the post-inoculation monitoring period to a seventh day. Insects in control cups were provided untreated bean leaf disk, and cups were incubated for 6 days at the high rate and 5, 6, and 7 days at the low rate before determining percent mortality. The 6- and 7-day bioassays were conducted on three and two dates, respectively, using different generations of early-second-instar nymphs.

2.2.3. Age-dependent susceptibility of second-instar thrips

Conidial suspensions (2 mg/ml, technical powder) were applied to the lower surfaces of bean leaf disks, and the treated leaves were then placed in the assay cups in sandwich fashion and stored at 25 ± 1 °C under the natural light regime. To maintain leaf freshness throughout the bioassay described below, two sets of leaf disks were sprayed. The first set was sprayed at the start of the assay (hour zero) and another set 48 h later.

To obtain a large cohort of approximately even-aged individuals, 25–30 adult female thrips were added to each of four rearing containers and allowed to oviposit for 24 h. After most nymphs of the cohort molted to the second instar, a group of 12 early-second instars was selected and added to each of three assay cups. The thrips remaining in the rearing containers were maintained under the above-described conditions and allowed to continue development. For the following 88 h, at 8-h intervals, three additional replicate groups of 12 thrips were selected from the containers and added to new assay cups with treated leaf disks. The ages of the thrips collected at each interval were not precisely known given that the females were allowed to oviposit for 24 h and egg hatch occurred over an unknown period of time. Thus, the age of each consecutive group was designated as early-second instar (ESI) + 8 h, ESI + 16 h, ESI + 24 h, etc. The three replicate cups set up at each interval were maintained at 25 ± 1 °C for 5 days post-insect addition, at which time they were opened, and thrips mortality assessed. Controls with leaf disks treated with the carrier surfactant solution were set up for three of the age groups (ESI, ESI + 24 h, and ESI + 48 h). The entire experiment was replicated three times (once on each of three different dates).

2.2.4. Stability of conidia on bean leaf disks

Adult female WFT (25–30) were added to clean colony containers and allowed to oviposit for 24 h. This procedure was conducted on 4 consecutive days allowing early-second instars to be produced for 4 consecutive days. The lower surfaces of leaf disks were treated with *B. bassiana* at a rate of 2 mg/ml, 5 days after the first colony container was started. Treated disks were placed in assay cups as described above and stored at 25 ± 1 °C, under the natural light regime. At 24-h intervals, beginning at the time of application (0 h), 12 early-second-instar nymphs were added to each of 4–5 assay cups on each of 4 consecutive days. Following this procedure, the conidial inocula were allowed to age while the age of the thrips at the time of exposure remained approximately constant. Mortality was assessed after the assay cups plus thrips had been held for 5 days at 25 ± 1 °C, under the natural light regime. The bioassay was conducted on three dates using different generations of second-instar thrips.

2.3. Virulence bioassays

Results of the above-described experiments led to development of a basic bioassay protocol, which was ultimately employed to characterize the pathogenicity of *B. bassiana* strain GHA against second-instar WFT. The assay was based on the general protocol described above, including the following specifications.

Stock conidial suspensions were serially diluted to produce suspensions containing 0.01, 0.025, 0.05, and 0.10 mg of technical powder per milliliter (conidia with ⩾96% viability). These suspensions were applied to the abaxial surfaces of leaf disks. Twelve leaves were arranged in a circular pattern on the turntable (so that all were equidistant from the turntable center) and sprayed simultaneously. The sprayed leaf disks were allowed to dry, then selected at random and placed in the assay cups. Six cups were assembled for each application rate and a spray carrier control (60 thrips per rate). Assay cups were incubated at 25 ± 1 °C in a growth chamber under natural light. One cup from each application rate and the control was removed 24 h post-treatment and the thrips were examined to assess conidial acquisition (i.e., quantify total number of conidia on the body of each thrips). The remaining cups were used to assess mortality on the fifth day post-treatment. The entire procedure (bioassay) was replicated three times using different generations of early-second-instar nymphs.

2.4. Statistical analyses

Computations for all experiments except the LD_{50} and LC_{50} estimates were performed using the statistical software package JMP version 4 (SAS Institute, 2000). Experiments were conducted three times with the exception of the experiment investigating incubation time at the low (0.075 mg/ml) dose, which was replicated only twice. Treatment mortalities were corrected for control mortality using Abbott's correction and were transformed using the arcsine square root function prior to analysis of variance (ANOVA). Mortality data from the bioassay-development tests were analyzed using two-way ANOVA (tests were conducted over time, and the test dates were considered as experimental blocks). Means separations, where applicable, were carried out using Tukey's HSD test. Estimates of the LD_{50} and LC_{50} of *B. bassiana* (strain GHA) technical powder were obtained using probit analysis, carried out by the statistical program POLO-PC (LeOra Software, 1987). Treatment mortalities were corrected for control mortality by the POLO program.

3. Results

3.1. Bioassay-development tests

3.1.1. Treatment method

No significant differences in mortality estimates were detected among the five methods of treatment tested in the bioassay ($F_{[4,67]} = 1.85$; $P = 0.13$). High rates of mortality (69–85%) resulted from all treatments (Table 1).

3.1.2. Incubation time

There was a significant effect of incubation time on the percent mortality of second-instar thrips in both assays ($F_{[4,45]} = 19.5$, $P < 0.0001$; $F_{[3,48]} = 3.76$, $P = 0.017$). However, mortality quickly reached a plateau, and no differences were observed among the rates of mortality recorded on days 5–7 or 4–6 post-treatment following exposure to the low- and high-rates, respectively (Table 2). An increase in mortality was apparent in both assays between day 4 (64–65% mortality) and day 5 (83–77% mortality); however, neither increase was statistically significant. There were no significant increases in mortality after day 5. The two assays produced similar maximum levels of mortality (85% versus 83%), despite the considerable difference in the application rates (106 ± 8 versus 3740 ± 276 conidia/mm^2).

3.1.3. Age-dependent susceptibility of second-instar thrips

Age of second-instar thrips (relative to the time after molt to the second instar) had a highly significant effect on mortality; $F_{[11,85]} = 13.37$, $P < 0.0001$. As insects aged from early-second instars towards the pre-pupae, they became less susceptible to infection by *B. bassiana.* Conidia acquired >24 h after the initiation of the assay produced only low levels of infection and mortality (mean < 20%) (Fig. 2).

Table 2
Effect of incubation time on mortality of second-instar western flower thrips exposed to *B. bassiana* conidia at two application rates[a]

Number of days incubated (days)	Percent mortality (mean ± SE)			
	2 mg/ml[b]	Control	0.075 mg/ml[b]	Control
3	61.0 ± 6.4 a	—	15.9 ± 4.5 a	—
4	65.2 ± 6.2 ab	—	64.3 ± 8.0 b	—
5	77.5 ± 5.5 ab	—	83.4 ± 3.8 bc	8.7 ± 2.8
6	83.3 ± 5.6 b	17.0 ± 6.8	85.1 ± 5.0 c	23.4 ± 8.3
7	—		84.0 ± 4.9 c	29.9 ± 12.1

[a] Thrips maintained on bean leaf disks treated with high and low concentration of *B. bassiana* strain GHA conidia; high concentration: 2 mg/ml (3470 ± 276 viable conidia/mm^2); low concentration: 0.075 mg/ml (106 ± 8 viable conidia/mm^2); control leaf disks were treated with carrier surfactant (0.01% Silwet). Means within a column followed by the same letter are not significantly different according to Tukey's HSD test ($\alpha = 0.10$).
[b] Percent mortality (mean ± SE; 2 mg/ml, $n = 13$; 0.075 mg/ml, $n = 10$) recorded in bioassay cups incubated 3–7 days at 25 ± 1 °C (12 early-second-instar thrips/unit; 4–5 units/assay; assays were conducted three times at 2 mg/ml and two times at 0.075 mg/ml).

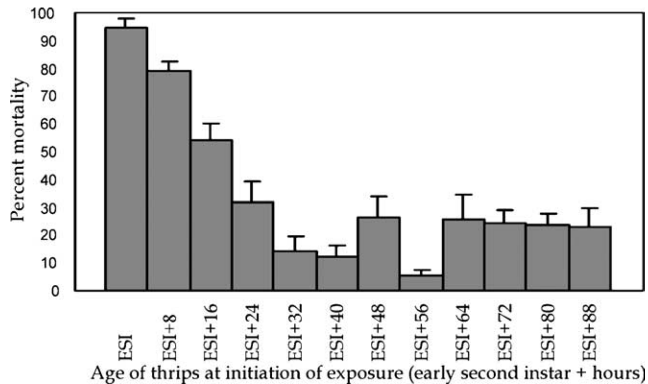

Fig. 2. Effect of western flower thrips age beyond early-second-instar (ESI) on susceptibility to *B. bassiana* strain GHA applied to bean leaf disks at the concentration of 2 mg conidia/ml (5136 ± 283 conidia/mm^2). Percent mortality (mean ± standard error; $n = 9$) in bioassay units incubated 5 days at 25 ± 1 °C (12 thrips/cup; 3 units/assay; assays conducted three times). Controls (leaf disks treated with 0.01% Silwet) were included on four occasions (ESI + 0, 24, 48, and 72 h), and treatment mortalities (from nearest age classes) were corrected for mean respective control mortalities of 11, 6, 8, and 16% using Abbott's formula.

3.1.4. Stability of conidia on leaf disks

There was no effect of conidial inoculum age on virulence against early-second instars ($F_{[5,6]} = 0.38, P = 0.77$). Conidia applied to leaf disks and stored up to 3 days prior to bioassay, exhibited virulence equal to conidia assayed immediately following application (Table 3).

3.2. Virulence bioassays

The median lethal concentrations (LC_{50}) of conidia, as determined by probit analysis, ranged from 34 to 107 conidia/mm^2 in the three replicate assays. probit regression slopes in these assays varied from 1.1 to 1.5 (Table 2). The median lethal doses (LD_{50}), based on

Table 3
Effect of *B. bassiana* inoculum age on efficacy against second-instar western flower thrips in a laboratory bioassay

Age of inoculum[a] (h)	Corrected percent mortality (mean ± SE)[b]	Control
0	76.1 ± 7.2	15.3 ± 3.1
24	69.1 ± 7.5	14.9 ± 1.9
48	71.4 ± 7.2	23.8 ± 12.8
72	60.3 ± 7.5	55.6 ± 12.2
ANOVA	$F_{[3,6]} = 0.38$; $P = 0.77$	

[a] Bean leaf disks were treated with conidia of *B. bassiana* strain GHA at the concentration of 2 mg/ml (4312 ± 271 conidia/mm^2), placed in clean bioassay cups, and stored at 25 °C for 0–72 h prior to introduction of thrips. Control leaf disks were treated with carrier surfactant (0.01% Silwet).
[b] Corrected percent mortality (mean ± standard error; $n = 13$) recorded in bioassay cups incubated 5 days at 25 ± 1 °C (12 early-second-instar thrips/cup; 4 or 5 units/assay; assays conducted three times). Percent mortality corrected for control mortality using Abbott's correction.

counts of conidia on second-instar thrips following 24-h exposure to treated leaf disks, ranged from 33 to 66 conidia per thrips; slope varied from 1.0 to 2.0 (Table 4).

4. Discussion

Two important goals of the bioassay development process were to design a bioassay system which was less labor intensive than existing Munger cell systems and to minimize the amount of insect handling to ensure healthy test subjects (low control mortality). The series of experiments examining treatment methods tested several different modes of conidia delivery and included different levels of insect handling and different numbers of sprays (spray treatment of both the axial and abaxial surfaces of leaf disks would require two spray applications).

The lack of significant differences in percent mortality among the five treatment methods indicates that thrips can readily acquire lethal doses of conidia from the surface of a treated leaf and that the indirect inoculation method (an easier, less time consuming protocol) is acceptable for most laboratory bioassay purposes. This result is not surprising considering the small size and thigmokinetic behavior of these insects. Juxtaposition of the treated leaf surfaces takes advantage of thrips behavior, but could conceivably overestimate acquisition of conidia from leaves of whole treated plants. Future investigation of acquisition from a single treated leaf surface might be warranted.

It would generally be presumed that maximum mortality would result from a combination of the direct and indirect treatment methods, i.e., spraying thrips on leaf disks and then leaving the inoculated thrips on the treated leaves. This treatment was initially explored;

Table 4
Median lethal doses (LD_{50}) and median lethal concentrations (LC_{50}) of *B. bassiana* strain GHA conidia applied against early-second-instar western flower thrips

	LC_{50}[a]	Slope	χ^{2}[b]	Concentration range[c]	LD_{50}[a]	Slope	χ^{2}[b]	Dose range[c]	Percent mortality range[c]
Assay 1	33.5	1.54	0.4	8–114	32.6	0.96	20.7	15–45	30–84
Assay 2	106.9	1.06	6.2	6–89	66.4	1.21	10.3	9–48	15–61
Assay 3	90.4	1.54	0.7	17–141	50.8	2.04	3.8	16–56	20–64
Means	76.9 ± 22.3	1.38 ± 0.2			49.9 ± 9.8	1.40 ± 0.3			

[a] Median lethal concentrations and doses estimated from replicated four-rate bioassays (12 thrips/bioassay unit; 5 units/application rate). Control mortalities recorded for assays 1-3 were 12, 13, and 12%, respectively; treatment mortalities were corrected for control mortalities by the POLO program.

[b] Heterogeneity χ^2 value (Finney, 1971) with two degrees of freedom given by PROBIT analysis.

[c] Range of concentrations (viable conidia/mm^2 of leaf-disk abaxial surface), doses (viable conidia/insect), and % mortality observed for each bioassay. Doses were determined from direct counts of conidia on five insects/application rate/assay following 24 h exposure to treated leaf disks.

however, we found it difficult to restrain movement of thrips on leaf disks. It was found, however, that thrips were unable to rapidly traverse the surface of wet filter paper. The highly textured, wet surface slowed thrips movement sufficiently to allow completion of spray treatments.

Determination of optimal incubation time for a bioassay system requires consideration of many factors. Generally, predetermined incubation periods for pesticide bioassays are relatively short compared to the potential survivorship of the test insects. Truncation of the monitoring period can significantly bias estimations of medial lethal doses or survival times, but this disadvantage may be outweighed by the obvious advantages of reducing assay time. It is common for small insects to be overcome within a few days by a virulent fungal pathogen developing under near-optimal temperature and moisture conditions. The experimental results summarized in Table 2 reveal that a 5-day incubation period at 25 °C is adequate for detection of most lethal infections.

It is also noteworthy that the leaf disks held up well over a 5-day period with the addition of 35 μl water to each piece of filter paper (70 μl/cup). The leaf disks remained hydrated and condensation in the small assay cup was minimized. After 5 days, however, some leaves began to degrade, exhibiting signs of decay (necrosis and growth of saprophytic fungi). This likely accounts for the marked increase in control mortality noted on days 6 and 7 in the low-dose bioassay (Table 2). Considering these results, a 5-day incubation period was identified as most appropriate and efficient for the thrips assay system described here.

Estimating the median lethal dose of a fungal pathogen acquired via secondary acquisition of the inoculum (conidia) is a difficult process that has seldom been attempted. This is particularly true for immature insects. Estimating the LD_{50} for larval stages requires not only a reliable method for enumeration of infectious propagules on the insect's body, but also a determination of when to sample the individuals exposed to treatment. In the described assay system in which conidia were applied to the leaf disks, we assumed conidial acquisition would be a continuous process; the longer the exposure, the more conidia the thrips would acquire. However, immature insects undergo periodic molting, which effectively removes the inoculum from the body. Thus, there exists a point during a stadium when further acquisition of conidia will not contribute to infection and mortality. This is especially the case with second-instar thrips, as the subsequent stage (the prepupa) does not remain on the leaf and begin the acquisition process anew.

Gerin et al. (1994) reported that the duration of the second-instar of western flower thrips reared on beans at 25 °C is ca. 3 days (73.4 h). We estimate that the early-second-instar thrips introduced into the assay cups had been second instars for an average of 12 h, as the cohort developed from eggs deposited over a 24 h period. This, in addition to the fact that significant infection resulted only from those conidia acquired during the initial 24 h of the assay, suggests that thrips are not susceptible to infection via conidia acquired during the last half (last 36 h) of the second instar. Most of this time can be accounted for as the period required for conidial germination and infection. For standard viability determinations, *B. bassiana* conidia are typically incubated for 16–24 h on agar-based media. Germination on the insect integument may be slower, due to limited free moisture, and considering the additional time required for penetration, a reasonable hypothesis would be that conidia acquired within 24–36 h of the molt to third instar would be largely ineffective at achieving infection. The results presented in Fig. 2 strongly support this hypothesis. This narrow window of susceptibility of immature insects is a recognized phenomenon (Vandenberg et al., 1998; Vey and Fargues, 1977), with clearly important implications with respect to the thrips biocontrol potential of slow-acting fungal pathogens.

This phenomenon may explain the observation of similar maximum levels of mortality in the low- and high-rate assays of the incubation time study (Table 2). The peak mortality of 83% at the high rate may reflect the error associated with identifying early-second-instar

nymphs. Approximately 15% of the individuals selected may have been older than intended, with low susceptibility to infection.

It is also possible that the drop in susceptibility of aging second instars was related to a decline in the virulence of conidia over the course of the experiment, or to an increase in control mortality. However, in the study investigating the stability of conidia on bean leaf disks (Section 2.2.4) it was determined that conidial virulence did not decrease significantly within a 72-h period (Table 3), suggesting that the drop in susceptibility was not due to a decline in virulence of conidia. Additionally, the uncorrected treatment mortality after exposure to 72 h old conidia (82%) may be artificially inflated due to high levels of control mortality (56%), presumably a result of a decrease in leaf disk quality. After correction for control mortality, treatment mortality was not significantly lower than treatment mortalities of thrips exposed to younger conidia. This supports the conclusion that the molting process was responsible for the decline in susceptibility of second-instar thrips.

Early-second-instar western flower thrips are susceptible to the GHA strain of *B. bassiana* at a very low dose, approximately 42 conidia/insect and 62 conidia/mm^2 substrate. Conidia were acquired from treated foliage, which suggests that secondary acquisition alone may be sufficient to manage thrips populations.

There have been few attempts to quantify the number of conidia on the integument of an insect by direct observation of conidia using standard phase contrast microscopy. Typically doses of conidia delivered to test insects have been estimated by counting colony forming units of a suspension that had been plated on agar, or by counting the number of conidia in a given volume of suspension using a hemacytometer. Given that a known volume of suspension is applied to an insect, it is possible to approximate the dose. The technique used in this study relies on direct observation of the conidia, and should work well for small insects that are semitransparent. Without light transmission through the insects' body, dose quantification becomes more difficult.

The culmination of these tests resulted in a reliable bioassay system that was used to determine median lethal concentrations and doses of *B. bassiana* against early-second-instar WFT. Accuracy of the bioassays in terms of goodness-of-fit to the probit model (within-assay variability) was excellent, as indicated by the low heterogeneity χ^2 values from the LC_{50} regressions. The higher chi-squares in the LD_{50} determinations are not surprising given that doses on the individual insects were highly variable and sample sizes (number of thrips used to quantify dose) were small (five insects/application rate/assay). Variability among the LC_{50} and LD_{50} estimates from the repeated assays was greater than might be desired, but not excessive in the context of the variability typically observed in repeated bioassays of fungal pathogens.

These experiments were all conducted in the laboratory, under conditions that are favorable to *B. bassiana*. Further investigations under production style conditions need to be carried out to evaluate the potential of *B. bassiana* for WFT control. The study reported here resulted in the successful development of a thrips bioassay system with several advantages over existing techniques. The small plastic assay cups sealed with parafilm contain thrips as well as Munger cells, are more easily set up and manipulated, and are inexpensive enough to be disposable (eliminating any need for cleaning and sterilization). Bioassays of *B. bassiana* based on this design produced data, which showed good fit to the classical probit model.

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