1 **Efficacy of *Beauveria bassiana* formulations against the fungus gnat *Lycoriella***
2 ***ingenua***

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Abstract

Lycoriella ingenua Dufour (Diptera: Sciaridae) is a major pest species in commercial mushroom (*Agaricus bisporus*) production throughout the world. Grower demand for alternative control measures, following the recent withdrawal of a number of chemical control options, led to a label extension for use of the fungal biopesticide BotaniGard® ES, for control of mushroom flies. Semi-field trials were conducted to evaluate the efficacy of BotaniGard® ES, and two alternative formulations of *Beauveria bassiana* strain GHA (the active ingredient in BotaniGard® ES), for control of *L. ingenua*, and their effect on crop yield when incorporated at spawning. Data collected from two replicated trials demonstrated that incorporation of *B. bassiana* was not detrimental to mushroom yield, but was also ineffective in controlling the development of *L. ingenua* larvae in artificially infested compost. Subsequent laboratory bioassays demonstrated that *L. ingenua* eggs and larvae were not susceptible to infection by *B. bassiana* strain GHA whereas pupae were somewhat susceptible (41% mortality). Bioassays conducted on adult *L. ingenua* using 1 h exposure to a surface sprayed with BotaniGard® ES resulted in 100% mortality within 8 days and a mean survival time of 6 days, which was significantly different from the control population.

Keywords

Mushroom fly, Sciarid, *Agaricus bisporus*, Entomopathogen, Mushroom yield, Fungal infection, Biopesticide, BotaniGard® ES.

1. Introduction

Lycoriella ingenua (Dufour) (Diptera: Sciaridae) is a major pest species of commercial mushrooms throughout the world causing crop damage and reductions in yield (Park et al., 2006; Erler et al., 2011). *L. ingenua* damages mushrooms through direct larval feeding on developing mushroom mycelia (Cantelo, 1979; Lewandowski et al., 2004), larval competition with developing mushroom mycelia for nutrients in the compost (Binns, 1980), and negative effects of larval frass on mycelial growth (Hussey and Gurney, 1968). Furthermore, *L. ingenua* adults and larvae are associated with the vectoring of *Trichoderma aggressivum* (Samuels & W. Gams) (Hypocreales: Hypocreaceae), which causes severe epidemics of “green mold” and consequently leads to additional crop losses (Shamshad, 2010),

The developmental time for *L. halterata* is 18 to 21 days at 24 °C, with the egg stage being 4 days, larval stage 12 days and the pupal stage being 4 days (Lewandowski et al. 2004). On average, adults live for about 6 to 12 days (Unpublished dataset). Current control efforts primarily rely on applications of conventional synthetic pesticides (Cantelo, 1979, 1983; Shamshad et al., 2008; Shamshad, 2010). However, insecticide options are limited due to removal of tolerance for many insecticides, and label restrictions on the number of applications per season and/or the total amount of active ingredient applied for those that remain in use. Targeting can also be difficult, because emerged larvae move away from the hatching site to feed in the caps and stems of mushrooms, where they are well protected. Additionally, repeated applications of conventional pesticides may produce undesirable effects, such as insecticide residues, reduced populations of natural enemies and insecticide resistance (Brewer and Keil,

1989; Bartlett and Keil, 1997). A label extension for BotaniGard® ES was recently obtained for use in Pennsylvania mushroom houses for control of *L. ingenua* and the mushroom phorid fly *Megaselia halterata* (Wood). The extension was granted based on preliminary efficacy data on control *M. halterata* from the industry (unpublished). Given the lack of information on efficacy against *L. ingenua*, and the need to evaluate the effect of BotaniGard® ES application on mushroom yield, field evaluations were conducted to determine the efficacy of BotaniGard® ES to control immature life stages of *L. ingenua* when incorporated into compost at spawning at registered label application rates. Mushroom yields were evaluated, to ensure that addition of *B. bassiana* to compost was not detrimental to mushroom growth and development as a result of mycotoxicity or competition. Additional laboratory-based ‘maximum challenge’ bioassays were also conducted on each life stage of *L. ingenua* with the aim of identifying an optimal application strategy based on the susceptibility of each developmental stage of the pest.

2. Materials and methods

2.1. Insect Rearing

Lycoriella ingenua eggs, first instars, pupae and adults were obtained from a 2-year-old laboratory colony maintained at the University Park Campus of Penn State University. This colony was initiated in 2012 using gravid adult female flies that had been aspirated from the mushroom production beds of spawned *Agaricus bisporus* compost in Berks County (PA, USA). Flies were reared in white popup cages (30 x 30 x 30 cm) with a single vinyl window (Raising Butterflies, UT, USA) containing plastic cups (500 ml) (Solo, MI, USA) filled with phase II mushroom compost with an added

commercial nitrogen supplement (100:1 w/w, compost:supplement). Cages were maintained in an environmental growth chamber at 21 ± 1 °C, $70 \pm 5\%$ RH, and a 12:12 (light:dark) photoperiod regime to allow the females to oviposit in the compost mixture in the cups. After 2 days, the cages were covered with plastic autoclave bags (VWR International, Atlanta, GA, USA; 61 x 76.2 cm) to prevent the compost from drying out. Adult flies emerged approximately 21 days later, at which point fresh compost cups were provided for egg collection from the emerging adults. New cages were then utilized until a continuously emerging colony was established as described in Andreadis et al. (2015).

2.2. Fungal production

Beauveria bassiana strain GHA (the active ingredient in BotaniGard® ES), was regenerated from -80 °C storage on Mirobank® microporous beads, placed onto potato dextrose agar (PDA) and incubated at 25 °C for 10 days until fully sporulated. Conidia from this culture were suspended in sterile 0.05% Tween 80 in de-ionized water to a concentration of approximately 1×10^8 conidia ml⁻¹. This suspension was used to inoculate 75 ml CSYE liquid media (4% glucose, 1% KNO₃, 0.5% KH₂PO₄, 0.1% MgSO₄, 0.005% CaCl₂, 0.2% yeast extract in de-ionized water) in 250 ml capacity Erlenmeyer flasks, 1 ml per flask. Flasks were incubated on a rotary shaker (160 rpm) at 24 °C for 3 days.

One kg of Barley flakes (Grain Millers, Iowa, USA) was added into a mushroom spawn bag (Unicorn, Garland, Texas, USA) along with 600 ml tap water, and the contents mixed by hand to ensure even absorption of the water. Each spawn bag was then placed inside an autoclave bag for protection and autoclaved for 30 min at 121 °C. Once

cool, the bags were inoculated under aseptic conditions with 75 ml of the liquid culture plus 75 ml of sterile water to achieve a final moisture content of approximately 48%. The inoculated bags were carefully massaged to ensure even distribution of the inoculum, then sealed and incubated on shelves for 10 days at 25 °C. Following incubation, the bags were opened in a reverse flow cabinet (Labconco, USA) and the contents transferred to brown paper bags for drying. The paper bags were placed in a dehumidified room for 4 days (24-30 °C), until the sporulated substrate reached <20% moisture content. The conidia were then harvested from the barley flakes using a Mycoharvester (Acis, Devon, UK).

The harvested conidia were placed in glass dishes and further dried in a desiccator over dry silica gel at room temperature (approx. 20 °C). Once the conidia powder reached 5% moisture content, it was sealed in foil laminated sachets and stored at 5 °C until use. Spent grain was transferred to autoclave bags and placed in the freezer until use. Prior to use, spent grains were ground in a commercial coffee grinder to obtain a homogenous granular powder similar in size to ground coffee.

2.3. *Field evaluations*

Two separate mycotoxicity cropping and efficacy trials were conducted in May and August 2014 respectively. The first trial included one *B. bassiana* treatment and a control to evaluate the potential utility of the spent grain waste product, following production and extraction of the active ingredient (pure conidia powder of GHA) of BotaniGard® ES. This preliminary trial also provided useful information on evaluation methodology, which was subsequently incorporated into the following field trial. In the August trial,

BotaniGard[®] ES (Laverlam International Corp., Buttle, MT, USA) was included at the label recommended application rate and compared with the spent grain waste product and pure conidia powder formulated in 0.05% Tween 80, all with equivalent conidia per kg compost. Both trials included evaluation of crop yield and effect on larval development and adult emergence of *L. ingenua* in artificially infested compost. Viability of all conidia suspensions was verified by plating on Sabouraud Dextrose Agar (SDA). Plates were incubated for 20 h at 25 °C and conidia evaluation under a microscope at 400X magnification. Conidia with a visible germ tube were counted as germinated and a total of 300 conidia were counted for each plate. All suspensions used in these experiments had >85% germination.

2.3.1. Preparation of phase I and phase II compost

Compost for the cropping trials was prepared at the Mushroom Research Center (MRC) at the Pennsylvania State University, University Park campus following a standard University wheat straw-bedded horse manure, dehydrated poultry manure, distiller's grain and gypsum formula.

2.3.2. Procedure for spawning, and application of B. bassiana strain GHA formulations

In mid-May 2014, phase II compost was weighed into 22.7 kg (50 lb) capacity tubs. For ease of mixing and incorporation of the spawn, supplement and *Beauveria* treatment, two tubs were filled with 11.35 kg (25 lb) phase II compost. Commercial, off-white hybrid *Agaricus bisporus* millet spawn was added at a rate of 100 g per 11.35 kg compost, along with 102 g, of a commercial, delayed-release supplement (a proprietary

blend of plant-based lipids, carbohydrates and protein) and 100 g "Ground Spent *Beauveria* Substrate" (GSBS) containing 4.4×10^8 conidia g^{-1} substrate. The compost was then thoroughly mixed by hand until the components were homogenously incorporated throughout the compost. The two 11.35 kg tubs were then combined into a single tub and compressed using a homemade hydraulic press. Treated tubs contained 8.8×10^{10} conidia per 22.7 kg tub and control tubs received spawn and supplement only. A total of 9 tubs from each treatment were monitored for cropping evaluation and four separate replicate cups containing 200 g sub samples of the mixed compost from each treatment were prepared for artificial infestation with *L. ingenua*.

In August 2014, phase II compost was weighed into tubs as described in the 1st field trial above. All procedures were the same except three *Beauveria* treatments were included in addition to a control. In this trial, BotaniGard® ES was prepared according to the label recommendations as a compost drench. 4 ml BotaniGard® ES was added to 400 ml tap water and 200 ml of this formulation was added to each of two 11.35 kg tubs, along with 100 g spawn and 102 g supplement, and thoroughly mixed prior to combining the two tubs to make a single 22.7 kg tub. GSBS was added as described as in the 1st trial above, except 200 ml tap water per 11.35 kg compost was also added to ensure equivalent moisture content to the BotaniGard® ES treatment. A third *Beauveria* treatment containing pure conidia extracted from the GSBS production batch and formulated in 0.05% Tween 80 in water was also incorporated at the same 400 ml per 22.7 kg compost volume application rate. Control tubs received spawn, supplement and water only. A total of 12 tubs from each treatment were observed for yield evaluation and

five replicate cups containing 200 g subsamples of compost for each treatment were prepared for artificial infestation with *L. ingenua*.

2.3.3. *Spawn run and cropping*

After spawning, tubs were placed randomly on stainless steel racks and covered with plastic to increase carbon dioxide concentration in the substrate and to promote vegetative mycelial growth and placed in environmentally controlled growth chambers for a 16-day spawn run. On day 16, agricultural lime-amended peat moss was mixed with water to achieve approximately an 82% moisture mixture. A commercially provided casing inoculum (CI) was added to the peat mixture at a rate of approximately 0.9 kg/m² growing area. Mushrooms were harvested, counted and weighed daily when the mushrooms were mature (prior to veil stretching and once the stipe was elongated and straight). Harvesting was initiated on day 15 and continued for 3 weeks (3 flushes). Water was applied from the day of casing through the end of the cropping cycle when needed based on the experience of the MRC Manager.

2.3.4. *Artificial infestation with L. ingenua*

In order to avoid infestation of the Penn State MRC with *L. ingenua*, artificial infestation of the compost was conducted at a separate facility using 200 g subsamples of the treated or control compost in 500 ml plastic cups as per colony rearing and maintenance.

In May 2014, four replicate compost cups for each treatment were inoculated with 200 freshly laid *L. ingenua* eggs. Eggs were collected by placing dishes of water agar

(Difco™ Agar Technical; Becton, Dickinson and Company Sparks, MD, USA) in a cage with gravid females overnight prior to the experiment. Females oviposited on the surface of the agar and 200 eggs were carefully counted and transferred to the surface of the compost for each cup. Cups were then placed individually into white pop-up cages (30 x 30 x 30 cm) with a vinyl window and arranged randomly in an environmental chamber maintained at 20 °C and 90% humidity. After approximately 21 days, the next generation of adult flies began to emerge from pupae in the compost. Emerging adults were collected and counted daily and their cumulative numbers were plotted over time.

In the second trial (August 2014), five replicate cups for each treatment were inoculated with 100 freshly laid *L. ingenua* eggs. The number of eggs added to each cup was adjusted to 100 as opposed to the 200 added in the first experiment to reduce the potential for miss-counting of eggs during the infestation process. All other experimental details were as per the May trial above.

2.4. Maximum challenge bioassays

BotaniGard® ES was tested against eggs, first instar larvae, and pupae of *L. ingenua*. Suspensions were prepared at a concentration of 1.08×10^8 conidia ml⁻¹, equivalent to the label recommended rate of 4 teaspoons/gallon water (0.95 ml BotaniGard® ES plus 186 ml water). Tap water was used for control treatments.

2.4.1. Eggs

Newly laid eggs (<24 h) were collected overnight by placing rearing cups filled with compost mixture in a colony cage with emerging adults. The next day, cups were

removed and eggs were transferred individually using a size 0 artist paint brush (Sapphire, Michaels, USA). Five replicate groups of twenty eggs were placed into 60 mm diameter Petri dishes (surface area 19.6 cm²) (Crystalgen, Inc., NY, USA) containing approximately 7 ml water agar (15 g L⁻¹ agar). Petri dishes were fixed inside a 0.25 m² spray area on the rear wall of a reverse flow laminar cabinet and the formulation was applied using a Preval aerosol sprayer (Coal City, IL, USA). The sprayer was held approximately 10 cm from the surface of the plates and passed across the line of 5 replicate plates (twice) at a rate of 0.5 m sec⁻¹, and a volume of approximately 75 µl per plate (verified by weight of dishes before and after spray). The resulting coverage was 4.13 x 10⁵ conidia cm⁻². Tap water was used for the control and applied identically to the BotaniGard® ES formulation. Following spray application, the Petri dish lids were replaced and the plates incubated at 21 ± 1 °C, for 10 days. The total number of hatched eggs for each plate was recorded at the end of the 10 day incubation. A total of three replicate assays were conducted over time, each having 5 replicate plates of 20 eggs, resulting in a total of 300 eggs in the treated and control groups respectively.

2.4.2. *First instar larvae*

Eggs were collected overnight and transferred to water agar as described in 2.4.1 above. The plates were incubated at 21 ± 1 °C until the larvae commenced emergence (3-4 days). Newly emerged first instar (< 24 h) larvae were collected with a size 1 paint brush (Artist's Loft™, MSPCI, TX, USA) and transferred in groups of ten (n = 5) into new Petri dishes with water agar. Each plate was weighed and sprayed as described for the egg assay above. Just prior to incubating the plates, 0.2 g phase II mushroom compost

was added to each plate to act as a food source for the larvae. Plates were incubated at 21 ± 1 °C. The plates were observed and cadavers removed daily, to prevent horizontal infection of surviving individuals. The total number of adults successfully emerging was recorded for each plate after 30 d. The developmental time from 1st instar to adult eclosion was monitored and the sex of each individual was determined to compare the developmental time for males and females from the treated and control populations respectively. A total of five replicate assays were conducted over time, each having 5 replicate plates (for treatment and control) of 10 larvae, giving a total of 250 individuals in the treated and control groups respectively.

2.4.3. Pupae

Petri dishes (5 cm diameter) containing water agar as described in 2.4.1 above, were covered with two disks of filter paper (Whatman[®] qualitative filter paper, Grade 1, 42.5 mm diam.). The filter paper assisted in maintaining a moderate humidity to ensure normal survival of pupae and emergence to adults (placing pupae directly on the agar surface resulted in poor pupal survival due to excessive moisture – unpublished results). One to two-day-old pupae were collected with forceps from rearing cups. Five replicate groups of 10 pupae were added to the surface of the filter paper. Each plate was weighed and sprayed as described for the egg assay above. The exposed Petri dishes were incubated at 21 ± 1 °C. The plates were observed and the total number of adults emerging from each plate was recorded after 10 days incubation. A total of four replicate assays were conducted over time, each having 5 replicate plates of 10 pupae, giving a total of 200 individuals in the treated and control groups respectively.

2.4.4. Adults

Evaluations on the potential efficacy of premise spray treatments using BotaniGard® ES for control of adult *L. ingenua* were also conducted. Since the assumption for adult control is that flies will acquire fungal conidia as a result of alighting on treated surfaces, such as light tubes and walls in mushroom houses, the evaluations were conducted by exposing adult flies to treated surfaces rather than direct spray application of the fungal formulations on the adults themselves.

BotaniGard® ES was diluted as described in section 2.4. HP™ Color-Laser Paper was sprayed with this spore suspension, using an artist's airbrush at a volume application rate of 20 ml formulation per m², resulting in a coverage of approximately 2.16×10^5 conidia cm⁻², water was used as a control treatment. Papers were laid on a metal rack and allowed to air dry overnight prior to use.

The dried, sprayed surfaces were carefully placed in World Health Organization (WHO) pesticide evaluation tubes (WHO 2013), with the sprayed surface facing inwards. Five groups of 20 newly emerged (< 24 h) female flies were aspirated into the treated or corresponding control tubes and allowed to move freely within the chamber for 1 h. Following exposure, adults were removed from the tubes using a mouth operated aspirator and individual flies were placed in clean 30 ml portion cups (Dart Solo, Harrisburg, PA USA) with lids and placed in a controlled environment cabinet at 21 ± 1 °C. Two filter paper discs (Whatman® qualitative filter paper, Grade 1, 42.5 diam. mm); one soaked in deionized water and one soaked in a 10% table sugar solution were placed in the bottom of each cup. Observations for mortality were made daily for 14 days. All

cadavers were subsequently incubated at 27 °C at high (>90%) humidity for 1 week to visually confirm death by mycosis. Adult assays were replicated five times over a period of 2 weeks, resulting in a total of 200 individuals in the treated and control groups respectively.

2.5. Larval survival on pure *B. bassiana* cultures

Five plastic Petri dishes (5 cm diameter) were lined with moist filter paper (Whatman® qualitative filter paper, Grade 1, 42.5 mm diam.) and seeded with a 1 cm diameter disk of *B. bassiana* culture on PDA using a cork borer. One, newly emerged (< 24 h) first instar larva was transferred to the surface the fungal culture in each Petri dish. The Petri dishes were covered with Parafilm M (Bemis Healthcare Packaging, WI, USA) and incubated at 21 ± 1 °C. This process was repeated five times over a period of five days, resulting in a total of 25 individual larvae. Dishes were observed daily, and data collected on the development of the larvae to pupae and pupae to adults.

2.6. Statistical Analyses

Data on survival of eggs, larvae and pupae following direct spray exposure to the label recommended dose of BotaniGard® ES as well as developmental time and mushroom yield data of the first cropping experiment were analyzed by Student's t-test. Mushroom yield data of the second cropping experiment was analyzed using one-way ANOVA, and differences between treatment means were tested using the Tukey Honestly Significance Difference test at 5% level of significance. All data were first inspected for normality and error variance for homoscedasticity and transformed using the arc-sine square-root

(percentage of survival) and square-root (developmental time) transformations, as necessary, before univariate analysis. Untransformed means ($\pm$ SE) are presented in results.

Cumulative adult emergence data from artificially infested compost were plotted against time for each treatment. Mean total emergence for the first cropping treatment was analyzed by Student's t-test. For the second cropping treatment mean total adult emergence was analyzed for significance using one-way ANOVA.

Daily adult survival data from laboratory bioassays were analyzed using Kaplan–Meier survival analysis with differences in median survival time (MST) between treatments compared using the log-rank test. All statistical analyses were done using SPSS Ver. 22 (IBM, 2013).

3. Results

3.1. Spawn run and cropping

Total yields for the first cropping experiment (May 2014) were not significantly different from one another though the control yielded slightly higher than the *Beauveria* treatment ($t = 0.947$, $df = 16$, $P = 0.368$). Likewise, yields for both treatments were similar for each break (Break 1: $t = 0.036$, $df = 16$, $P = 0.972$; Break 2: $t = 1.112$, $df = 16$, $P = 0.290$; Break 3: $t = 0.139$, $df = 16$, $P = 0.891$).

Similar yield results were obtained in the August 2014 trial. Total yields were not significantly different from one another though the control and GSBS formulations yielded slightly higher than the BotaniGard® ES and *Beauveria* conidia in Tween solution ($F = 0.945$, $df = 3, 44$, $P = 0.427$). Likewise, yields of all treatments were similar

for each break (Break 1: $F = 1.046$, $df = 3, 44$, $P = 0.382$; Break 2: $F = 1.493$, $df = 3, 44$, $P = 0.378$; Break 3: $F = 0.332$, $df = 3, 44$, $P = 0.802$). Confirming no effect on yield when *B. bassiana* is added at spawning, either as a drench or powdered formulation.

3.2. Adult emergence from artificially infested compost

Figure 1 shows cumulative daily emergence of adult *L. ingenua* from compost artificially infested with 200 *L. ingenua* eggs in May 2014. The pattern of adult emergence from the compost treated with GSBS was similar to the control. The mean total number of adults emerging from the treated compost pots 28 days after infestation was 212.0 ± 23.4 and 193.8 ± 19.4 for the treated and control pots respectively, with no significant difference in overall emergence between the two treatments ($t = 0.599$, $df = 8$, $P = 0.567$).

The second trial (August 2014) included the commercial formulation of BotaniGard® ES added at the label recommended rate and *Beauveria* conidia in a simple Tween water formulation, in addition to the GSBS and a control. In this experiment, compost pots were artificially infested with 100 eggs to reduce the potential for miss counting of eggs during the infestation process. Emergence of adults commenced 17 days after infestation and all treatments followed the same pattern of cumulative emergence for 7 days, after which no further adult emergence occurred (Figure 2). The mean total number of adults emerging from each treatment at the end of the experiment was 67.6 ± 1.1 , 64.2 ± 2.8 , 72.0 ± 6.8 and 63.2 ± 3.3 for GSBS, BotaniGard® ES, conidia in Tween water and control respectively, with no significant difference between any treatments or control ($F = 0.957$, $df = 3, 16$, $P = 0.437$).

3.3. Maximum challenge bioassays

Figure 3 shows the survival of *L. ingenua* eggs, larvae and pupae following direct spray exposure to the label recommended dose of BotaniGard® ES. Survival was defined by successful transition to the next observable stage of development. Over 80% of treated and control *L. ingenua* eggs emerged to 1st instar larvae within the 10-day monitoring period ($t = 0.138$, $df = 28$, $P = 0.891$) and 83 and 89% of treated and control larvae successfully developed to adults ($t = 1.234$, $df = 48$, $P = 0.223$). The only significant difference in survivorship following direct spray application of BotaniGard® ES was observed in the pupal treatment, where 59% of treated pupae successfully emerged to adults in comparison to 75% for the controls ($t = 3.310$, $df = 38$, $P = 0.002$).

In addition to data on mortality, the developmental time (1st instar larva to adult) of male and female *L. ingenua* was evaluated following direct spray application of BotaniGard® ES in comparison to control populations sprayed with water. While male *L. ingenua* exhibited a shorter overall developmental time (18.2 ± 0.2 days) to females (19.8 ± 0.1 days) ($t = 10.679$, $df = 432$, $P < 0.001$), there was no difference in developmental time between treated and control larvae for both sexes ($t_{males} = 0.131$, $df = 174$, $P = 0.896$; $t_{females} = 0.950$, $df = 255$, $P = 0.343$) (Figure 4).

Survival of female adult *L. ingenua* following 1 h exposure to a paper surface treated with BotaniGard® ES is shown in Figure 5. Females exposed to BotaniGard® ES had a significantly lower mean survival time of 6.03 ± 0.1 days in comparison to 7.95 ± 0.2 days for the control ($\chi^2 = 84.030$; $P < 0.001$). Mortality in the treated population was confirmed by mycosis.

3.4. Larval survival on pure *B. bassiana* cultures

When reared on pure cultures of *B. bassiana* strain GHA, larvae fed on the fungal material and successfully progressed through the larval stages. 20 of 25 transferred larvae successfully developed to the pupal stage (Figure 6). Of the 20 pupae formed, 12 succumbed to *B. bassiana* infection (as confirmed by mycosis), and 8 adults emerged successfully having developed entirely from 1st instar larvae on the *B. bassiana* culture (Figure 6).

4. Discussion

While it appears that this series of evaluations was conducted in reverse order, starting first with field evaluations and followed by laboratory bioassays, our activities were driven by the recent label extension approval for BotaniGard® ES for use in mushroom houses, and grant funding support for field evaluations. Our field results indicated that application of *B. bassiana* strain GHA either as a granular addition or as a liquid drench at spawning did not affect mushroom yield regardless of formulation. However, mushroom primordia formation and development are sensitive to changes in the chemistry and environmental growth conditions, and are sensitive to petroleum-based products which are often used in biopesticides formulations, so careful evaluation would be required if applications were to be made later during the cropping cycle.

B. bassiana strain GHA has been shown to be highly effective against a wide range of insect pest species including the Russian wheat aphid (Hatting et al., 2004), western flower thrips (Murphy et al., 1998), diamondback moth (Shelton et al., 1998), and the

lesser grain beetle (Lord, 2005). However, no control of *L. ingenua* in artificially infested compost was observed for any of the GHA formulations we evaluated. Field efficacy is known to be affected by dose, timing, and the number of applications (Hatting et al., 2004; Cossentine et al., 2010). However, multiple applications will only improve control if the target insect is susceptible to infection in the first place. Our laboratory assays demonstrated that *L. ingenua* egg and larval stages are not susceptible to infections by *B. bassiana* strain GHA, even when directly sprayed with the BotaniGard® ES formulation, and in fact, *L. ingenua* could develop successfully to adults when reared from 1st instar larvae on pure cultures of *B. bassiana* strain GHA in agar plates. Some infection was observed in *L. ingenua* pupae following direct spray application, and after pupation on growing, sporulating cultures of GHA, but even this mortality was limited. In contrast, adult *L. ingenua* were susceptible to infection by GHA with a 100% infection rate as measured by mortality and confirmed by mycosis. Similar differences in susceptibility of immature and adult stages have been reported for house flies (*Musca domestica*). Watson et al. (1995) found that adult house flies were susceptible to two strains of *B. bassiana*, whereas house fly larvae were unaffected when exposed to the same dose rate as adult flies. Lecuona et al. (2005) achieved >90% mortality of adult house flies for five strains of *B. bassiana*, but were unable to infect 3rd instar larvae. Similarly, Daniel and Wyss (2009) achieved good control of adult European cherry fruit fly (*Rhagoletis cerasi*) using four genera of entomopathogenic fungi, including *B. bassiana*, but were unable to achieve larval mortality with any isolate. Likewise, Davidson and Chandler (2005) reported that there is no correlation between virulence of entomopathogenic fungi to larvae and virulence to adult onion flies (*Delia antiqua*). While GHA has been shown to

be effective in controlling larvae of western cherry fruit fly (*Rhagoletis indifferens*) (Cossentine et al., 2010), a broad range of lepidopteran larvae (Wraight et al., 2010), and cabbage root fly larvae (Bruck et al, 2005), its ability to infect *L. ingenua* appears to be limited to adults and to a lesser extent pupae. *L. ingenua* pupae are most often found toward the upper surface layer of the compost and within the casing layer (Cantelo, 1988), so drench application of BotaniGard® ES could be effective in reducing the number of adults emerging from pupae, but with an expected survival of at least 59% this is unlikely to be an economic method of control.

Our results show that the adult life stage is the most susceptible to infection by *B. bassiana* strain GHA. While BotaniGard® ES is not intended as a premise spray for control of adult *L. ingenua*, we evaluated the potential for this method of use, since very few adulticides are available for control of *L. ingenua* populations. Further work will be required to investigate whether higher concentrations of BotaniGard® ES would result in faster mortality. Given that female *L. ingenua* are generally mated immediately upon emergence from the compost and will lay fertile eggs within 2 days of mating, further investigations will be required to determine whether fungal infection affects fecundity, and to evaluate the the survival/stability of the spray residue under operational conditions.

Ongoing investigations by this team are evaluating BotaniGard® ES for control of the mushroom phorid fly *M. halterata*, another serious pest in mushroom houses with few chemical control options available.

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Disclosure

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540 **Figure captions**

541 Figure 1

542 Cumulative emergence of adult *L. ingenua* from compost pots artificially infested with
543 200 eggs. Each data point represents SEM from five replicate pots. GSBS stands for
544 "Ground Spent *Beauveria* Substrate".

545

546 Figure 2

547 Cumulative emergence of adult *L. ingenua* from compost pots artificially infested with
548 100 eggs. Compost was treated with GSBS, BotaniGard® ES or *Beauveria* conidia in
549 Tween water or left untreated. Data point represents SEM from five replicate pots. GSBS
550 stands for "Ground Spent *Beauveria* Substrate".

551

552 Figure 3

553 Survival of *L. ingenua* eggs, larvae and pupae following maximum challenge direct spray
554 application of BotaniGard® ES under laboratory conditions. Survival was measured by
555 successful emergence of larvae from eggs or emergence of adults from larval and pupal
556 stages. Star denotes that survival is significantly different from control (Student's t test, P
557 < 0.05). Ns indicates not significant. Error bars represent SEM.

558

559 Figure 4

560 Duration of developmental time from 1st instar larvae to adult emergence for male and
561 female *L. ingenua* following maximum challenge direct spray application of
562 BotaniGard® ES under laboratory conditions. No significant differences on

developmental time were achieved (Student's *t* test, $P > 0.05$). Ns indicates not significant. Error bars represent SEM.

Figure 5

Cumulative survival of newly emerged adult female *L. ingenua* following 1 h exposure to paper surfaces either sprayed with BotaniGard® ES or water (control). Data point represents SEM.

Figure 6

Number of *L. ingenua* first instar larvae reaching pupation, and emerging as adults from remaining pupae, when reared on pure cultures of *B. bassiana* strain GHA on PDA plates.

Agaricus bisporus

Eggs

Larvae

Pupae

Adults

Lycoriella ingenua