ABSTRACT

Title of Document: INTERACTIONS BETWEEN THE INVASIVE BROWN MARMORATED STINK BUG, *HALYOMORPHA HALYS* (HEMIPTERA: PENTATOMIDAE), AND ENTOMOPATHOGENIC FUNGI

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Brown marmorated stink bug, *Halyomorpha halys,* is an invasive stink bug native to Asia. It is a highly destructive pest in both agricultural and ornamental systems. In an effort to identify a novel biological control, several strains of entomopathogenic fungi were tested against brown marmorated stink bug nymphs and adults. Wild-type fungi were found to cause very low mortality in bioassays, as well as resulting in little fungal growth or sporulation. The addition of diatomaceous earth and/or horticultural oil did not increase mortality, nor did the use of transgenic fungi. To test the proposed mechanism of brown marmorated stink bug's defense against fungal infection, trans-2-octenal and trans-2-decenal were bioassayed against several strains of entomopathogenic fungi. These compounds were found to inhibit fungal growth and prevent spore germination at low concentrations. Results of an experiment to determine if brown marmorated stink bug responded to fungal exposure were inconclusive.

INTERACTIONS BETWEEN THE INVASIVE BROWN MARMORATED STINK BUG, *HALYOMORPHA HALYS* (HEMIPTERA: PENTATOMIDAE), AND ENTOMOPATHOGENIC FUNGI

By

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Table of Contents

List of Tables....................v

List of Figures....................vi

Chapter 1: Effects of entomopathogenic fungi on the brown marmorated stink bug (*Halyomorpha halys*) and their potential as a biological control agent....................1

Introduction....................1

Methods....................7

Study organism - brown marmorated stink bug....................7

Study organisms - entomopathogenic fungi....................8

Wild-type fungal bioassays....................9

Wild-type fungal bioassays with additives....................10

Transgenic fungal bioassay....................11

Statistical analysis....................12

Results....................12

Discussion....................19

Chapter 2: Interactions between defensive compounds of the brown marmorated stink bug (*Halyomorpha halys*) and entomopathogenic fungi....................26

Introduction....................26

Methods....................30

Study organism - brown marmorated stink bug....................30

Study organisms - entomopathogenic fungi....................30

Defensive compounds....................30

Detection and quantification of stink bug defensive compounds in whole insects....................31

Effects of defensive compound volatiles on fungal growth..31

Effects of defensive compounds on fungal spore germination..32

Inducing brown marmorated stink bug defense via fungal exposure.................................33

Statistical analysis...35

Results...35

Discussion...40

Literature Cited...45

List of Tables

1 Mean (±SEM) percent mortality of brown marmorated stink bug adults and nymphs 3 and 7 days following exposure to entomopathogenic fungi and controls. Table includes result of 4 adult and 5 nymphal stink bug bioassays..15

2 Fungal growth and sporulation data for wild-type bioassays showing mean (±SEM) days to fungal growth and sporulation of brown marmorated stink bug adults and nymphs as well as mean (±SEM) percent of dead insects showing fungal growth and fungal sporulation. Table includes 4 adult and 5 nymphal wild-type fungal bioassays. Note that growth and sporulation data includes only stink bugs that died in fungal treatments during the bioassay (see Table 1)..16

3 Mean (±SEM) percent mortality of adult brown marmorated stink bugs for the wild-type fungal bioassay with additives, 3 and 7 days following exposure to entomopathogenic fungi and additive treatments...18

4 Mean (±SEM) percent mortality and fungal growth for brown marmorated stink bug adults following treatments with four transgenic *Metarhizium* strains of the ARSEF 1548 modified to produce spider toxins and the unmodified strain (1548 WT). Note that fungal growth includes only insects that died during the bioassay...19

5 Mean (±SEM) percent of petri dishes showing fungal inhibition 3 days after exposure to two brown marmorated stink bug defensive chemicals at 3 concentrations and a water control...37

6 Mean (±SEM) percent of petri dishes showing recovery of 3 fungal strains 7 days post removal of defensive chemicals. Data includes only petri dishes that showed inhibition of fungal growth (i.e. fungal growth did not occur by 3 days post exposure to defensive compounds)..37

7 Defense response (chemical type and quantity produced) from each of the responding individual brown marmorated stink bugs by fungal treatment, gender, and day of response. Quantity is presented as peak ion counts...40

List of Figures

1 Example of germinated spores showing mycelium (left) and un-germinated spores (right) of *Metarhizium* fungal strain F52...33

2 Mean (±SEM) percent spore germination of F52 (M), GHA (B) and 3581 (I) at three concentrations of trans-2-octenal plus a 0% control (water)...39

3 Mean (±SEM) percent spore germination of F52 (M), GHA (B) and 3581 (I) at three concentrations of trans-2-decenal plus a 0% control (water)...39

Chapter 1: Effects of entomopathogenic fungi on the brown marmorated stink bug (*Halyomorpha halys*) and their potential as a biological control agent.

Introduction

The brown marmorated stink bug (*Halyomorpha halys* [Stål], Hemiptera: Pentatomidae) is an invasive species of stink bug native to China, Taiwan, Japan and Korea (Leskey et al. 2012c). The earliest confirmed detection of BMSB in the United States was in 1996 in Allentown, PA (Hoebeke and Carter 2003). Brown marmorated stink bug readily disperses and to date has been found in 41 states and the District of Columbia (Leskey et al. 2012c).

The life history of the brown marmorated stink bug has been studied extensively. Typically, adults overwinter in homes and other man-made structures which has led to their status as a nuisance pest (Nielsen and Hamilton 2009b). Adults also overwinter in natural areas such as rocky outcroppings and dead standing trees (Lee et al. 2014). Shortly after adults emerge from overwintering sites in the spring, females begin laying eggs on the underside of leaves on suitable host plants (Nielsen and Hamilton 2009b). The eggs then hatch and go through five nymphal instars before molting into adults (Nielsen and Hamilton 2009b). The number of generations per year depends on location, but in the mid-Atlantic region there are 1-2 generations (Leskey et al. 2012a). As winter approaches, newly emerged adults enter reproductive diapause and begin to move into overwintering locations (Nielsen and Hamilton 2009b).

Brown marmorated stink bug is a highly destructive pest of agricultural crops and ornamentals (Hoebeke and Carter 2003). It feeds on all parts of the plant: leaves, stems, fruits, and trunks of trees as nymphs and adults (Hoebeke and Carter 2003; Martinson 2013). They

have an extraordinarily wide host range and feed on plants ranging from tree and small fruit, vegetables, field crops and ornamentals (Hoebeke and Carter 2003; Wermelinger et al. 2008; Bergmann, stopbmsb.org). Feeding damage appears as pitting and discoloration on foliage and can discolor and effect the consistency of fruit (Hoebeke and Carter 2003). Brown marmorated stink bug can also cause noticeable aesthetic damage to ornamentals. Amplifying the damage further is the fact that the typical life cycle of brown marmorated stink bug has been found to coincide with periods of susceptibility of fruit in apple, peach, pear, and soybean (Nielsen and Hamilton 2009a; Nielsen et al. 2011) causing economic loss. In 2010, damage from brown marmorated stink bug alone caused $37 million in losses to apple producers in the mid-Atlantic region. (Leskey et al. 2012c).

Due to the destructiveness of brown marmorated stink bug, its wide host range, and exceptional ability to disperse, an integrated management strategy is necessary to successfully reduce its population and damage. The use and efficacy of various pesticides has been evaluated (Bergmann 2014; Lee 2014; Leskey et al. 2014). Growers have had to increase the number of pesticide applications in response to brown marmorated stink bug (Leskey et al. 2012b). Although pesticide use has had success in some cropping systems, it does not provide long-term, sustainable management (Nielsen et al. 2008) and alternative control methods should be developed and implemented. Biological control efforts are currently underway (Jones et al. 2014; Hoelmer, pers. comm.) and if successful should provide long-term sustainable management of brown marmorated stink bug. Research is being conducted on both native and exotic Hymenopteran and Dipteran parasitoids and predators to evaluate their potential as biological control agents for brown marmorated stink bug. Both native and exotic Hymenopteran egg parasitoids show promise, however, to date there have been no releases of

exotic parasitoids (Hoelmer, per. comm.), and even though native parasitoids are attacking brown marmorated stink bug eggs, stink bug populations remain high enough to cause damage to crops (Jones 2014). Therefore, continued research on biological control strategies against brown marmorated stink bug is needed.

One avenue of biological control that has yet to be explored towards the control of brown marmorated stink bug is the use of entomopathogenic fungi. Entomopathogenic fungi colonize and kill their insect host by utilizing cuticle-degrading proteases that allow them to penetrate the insect cuticle and grow within the host insect's body (St. Leger et al. 1992). These fungi can be particularly effective at controlling sucking insects like Hemiptera that are not able to consume topically (i.e. foliar) applied pathogens due to their mode of feeding (St. Leger et al. 1992). Two of the most commonly used entomopathogenic fungi are *Metarhizium anisopliae* [Metchnikoff] (Hypocreales: Clavicipitaceae) and *Beauveria bassiana* [Bals.-Criv.] (Hypocreales: Clavicipitaceae), each of which has hundreds of identified strains. *Metarhizium* and *Beauveria* have been found to show promise as biological control agents to several pest insects in a number of plant systems such as cabbage looper in *Brassica* (Behle 2006), false-eye leafhopper on tea (Feng et al. 2004), red palm weevil in canary palms (Guerri-Agullo et al. 2011), leaf-folder in rice (Sivasundaram et al. 2008), kissing bug (Luz and Batagin 2005; Luz et al. 2012), whiteflies (Malsam et al. 2002) and cotton stainer bug (Santi et al. 2011). A few studies have examined the effect of fungi on stink bugs, including *Nezara viridula* [Linnaeus] (Hemiptera: Pentatomidae), *Piezodorus guildinii* (Hemiptera: Pentatomidae), *Euschistus heros* (Hemiptera: Pentatomidae) (Sosa-Gomez et al. 1997; Sosa-Gomez and Moscardi 1998), *Plautia crossota stali* (Hemiptera: Pentatomidae), *Glaucias subpunctatus* (Hemiptera: Pentatomidae) (Ihara et al. 2008) and, most

importantly, *Halyomorpha halys* (Ihara et al. 2008; Gouli et al. 2012), demonstrating varying levels of effectiveness in killing insects.

Although strains of *Metarhizium* and *Beauveria* are known to kill a diverse array of insects, there are problems associated with their use that has led to somewhat poor adoption of entomopathogenic fungi as formulated biological controls. Problems with wild (or natural) strains of entomopathogenic fungi include low virulence which necessitates large inoculum loads, slow killing time and low overall mortality (Fang et al. 2012). Genetic modification of these fungi can potentially be used to ameliorate these issues. By incorporating select proteins in the fungal genome, these fungi can be engineered to express any number of desirable traits. This approach has been used several times primarily to increase the lethality of fungus against a target organism. By modifying *Metarhizium* to express a scorpion neurotoxin, fungal toxicity increased 22-fold against tobacco hornworm and 9-fold against the yellow fever mosquito (Wang and St. Leger 2007). *Beauveria* modified to express a similar scorpion neurotoxin and cuticle degrading proteases was shown to be significantly more effective against several Lepidopteran larvae than wild type fungi (Lu et al. 2008). These examples strongly suggest that "designer fungi" can be engineered to more efficiently kill certain organisms and overcome unique aspects of their biology.

Other challenges accompany the use of entomopathogenic fungi that can affect their virulence against insects. For example, a number of abiotic factors can cause a reduction in the effectiveness of the fungi. Sunlight has been demonstrated to reduce conidial viability in field settings (Behle 2006) and the rain-fastness of the fungi is heavily dependent on the type of formulation used (Inyang et al. 2000). Non-oil based formulations are susceptible to low humidity, which can result in a reduction in fungal growth and desiccation of the fungus (Santi et

al. 2011). Also, some fungi have difficulty penetrating the insect cuticle, reducing their ability to enter the body and kill the insect in a timely manner.

Based on previously conducted studies, it should be possible to address some of these challenges by altering the formulation in which the entomopathogenic fungi are suspended. A number of studies have demonstrated the advantages of oil-based formulations over water based ones (Inyang et al. 2000; Feng et al. 2004; Polar et al. 2005). Biological oils improve the distribution of conidia on the insect, ensuring that more conidia have the opportunity to attach and grow (Malsam et al. 2002). The oil protects the conidia from UV radiation, which can degrade the fungus and reduce viability (Santi et al. 2011). Oil also provides a supplementary food source for the fungus and traps humidity, allowing the fungus to flourish and prevents desiccation (Luz et al. 2012). There is some evidence of synergistic effects between the entomopathogenic fungi and oil (Malsam et al. 2002) as well as repellency or attractiveness effects of some oils to insects (Luz and Batagin 2005). Diatomaceous earth, meanwhile, increases the efficacy of the fungi by acting as a desiccant and abrasive (Korunic 1998). Abrading the insect cuticle with diatomaceous earth allows the fungus to more easily enter the insect. Formulations with diatomaceous earth have been demonstrated to increase fungal efficacy in trials against *Triatoma infestans* [Klug] (Hemiptera: Reduviidae) (Luz et al. 2012). All of these factors suggest that oil- and/or diatomaceous earth- based formulations could improve the efficacy of fungi as a formulated biological control.

Although entomopathogenic fungi have been shown to be excellent candidates as biological control agents of some insects, they are not without challenges. Therefore, it is critical to identify strains of fungi that infect brown marmorated stink bug and assess their performance in terms of rate of stink bug mortality, time to kill, and time to sporulation of the fungus on dead

insects. To be viewed as an effective control, fungal pathogens should be able to reduce insect populations and therefore the damage that they cause in a time effective manner. Similarly, fungal pathogens that have quick or abundant sporulation after killing their host are more likely to disperse and provide continued suppression of the pest. Identifying entomopathogenic fungal strains against brown marmorated stink bug and measures to increase the virulence of fungi should ultimately increase the likelihood of adoption of entomopathogenic fungi as a biological control against this important invasive stink bug. Therefore, the overall objectives of this study were to evaluate strains of entomopathogenic fungi as potential biological control agents of brown marmorated stink bug and identify measures to increase their virulence. Specifically, we examined formulations of wild-type (natural) fungi, wild-type fungi with additives in their formulation, and genetically modified or transgenic fungi for their efficacy in killing adult and nymphal brown marmorated stink bugs.

Methods

Study organism – brown marmorated stink bug

To evaluate strains of fungi as biological control agents for brown marmorated stink bug, bioassays were performed by treating adult and nymph stink bugs with fungal solutions and then monitoring them for mortality and fungal growth over time during 2013 and 2014. Stink bugs used in these bioassays came from three sources. Source varied between bioassays depending on availability, but all treatments within a bioassay used stink bugs from the same source. Stink bugs were collected from the field, taken from a laboratory-reared colony or taken from overwintering stock in the laboratory. Laboratory-reared colony stink bugs were kept in cages and provided with green bean plants, sunflower seeds, and water. The cages were kept in

environmental chambers and maintained at 25°C and 70% RH with a 16:8 light: dark cycle. Overwintering stink bugs were kept in cardboard buckets with damp paper towels and stored at 8°C until use. To bring the stink bugs out of their overwintering state, they were moved to colony conditions for two weeks before being used in a bioassay. Field collected stink bugs were placed in cages and maintained under colony conditions prior to use for 1 to 7 days.

Study organisms - fungal strains

The three primary genera of entomopathogenic fungi tested were *Metarhizium*, *Beauveria* and *Isaria*, which are representative of fungi known to kill insects and all of which have commercial products available. Fungal strains are denoted with M (for *Metarhizium*), B (for *Beauveria*), I (for *Isaria*) or O (for other fungi of unknown genus) for clarity. The fungal strains used for these studies were drawn from a number of sources. The majority of the strains came from laboratory-maintained stocks that originated from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) and are identified with an ARSEF designation code (1548 [M], 2547 [M], 1055 [M], 3581 [I] and 10386 [O]). One strain each of *Metarhizium* (F52), *Beauvaria* (GHA) and *Isaria* (3581) were obtained from M. Jackson at the USDA-ARS Peoria, IL. Strains 1548 (M), 2547 (M), 1055 (M) were obtained from R. St. Leger at the University of Maryland, College Park, MD. Strain 10386 (O) came directly from ARSEF and is of unknown genus. Another strain tested of unknown genus was isolated from the brown marmorated stink bug laboratory colony and is labeled "Unknown Fungus" in all tables and figures. Botanigard (BioWorks, Victor, NY), a commercial fungal pesticide utilizing the GHA (B) strain, was also evaluated in order to look at both a commercial version of the strain and a lab-reared sample of the same strain. These fungi were selected in order to have representatives

from a number of different genera, with special attention paid to *Metarhizium*, as it has been demonstrated to be an effective generalist, leading to its popularity. Strains 1548 (M), 1055 (M), 3581 (I) and 10386 (O) were selected as they had been isolated from Hemiptera, with 1548 (M), 3581 (I) and 10386 (O) being isolated from Pentatomidae and 10386 (O) being isolated from *Halyomorpha halys* in particular. Strain 2547 (M) was selected as preliminary tests performed suggested that it may be effective at killing brown marmorated stink bug.

Wild-type fungal bioassays

To evaluate the effect of wild-type fungi (fungal strains as they were found in nature) against brown marmorated stink bug, fourbioassays were run against adult stink bugs and five were run against nymphal stink bugs over time. Nine strains from three genera of fungi, plus water and Tween controls (=11 treatments) were evaluated. Not all treatments were run in all bioassays due to limitations in stink bug availability. The following wild-type fungal genera and strains were tested:

Metarhizium: ARSEF 1548, ARSEF 2547, ARSEF 1055, F52 (4 treatments)

Beauveria: GHA - USDA, GHA - Botanigard (2 treatments)

Isaria: ARSEF 3581 (1 treatment)

Unknowns: ARSEF 10386 (unidentified fungus isolated from brown marmorated stink bug in New Jersey), Unidentified Fungus (isolated from brown marmorated stink bug lab colony) (2 treatments)

Fungi for each treatment were plated on potato dextrose agar (PDA) (Sigma-Aldrich, Lenexa, KS) media in standard 90mm petri dishes and allowed to grow for 14 days at 27°C. To create the spore suspensions, conidia were scraped from plates into a 0.01% Tween 80 solution

(later bioassays used autoclaved deionized [DI] water instead of Tween 80) and diluted to a concentration of 1×10^7 conidia/mL. Control treatments were 0.01% Tween 80 solution and/or DI water. The stink bugs were treated by submerging them in the spore suspension or control for 3 seconds and then placing them in clear plastic 354 mL Dart® PresentaBowl containers (Randleman, NC). Stink bugs in containers were provided with a water source and sunflower seeds, both of which were replaced as needed. For all bioassays, between four and six replicates were used for each fungal strain and control treatment. Each container (replicate/treatment) contained five adult or five nymphal brown marmorated stink bugs (sub-samples). Air holes were cut in the top of the container and all containers were then placed in growth chambers set to colony conditions outlined above. All stink bugs were monitored daily for mortality and fungal growth and sporulation. Mortality is the major indicator of fungal effectiveness, with fungal growth and sporulation being used as an indicator of which dead stink bugs died as a result of fungal infection. Dead stink bugs were removed from their containers and placed in individual Solo® 29.57 mL clear plastic cups (Lake Forest, IL) which were also kept in the growth chambers. Dead stink bugs were then monitored daily for fungal growth (= presence of mycelium) and sporulation (= presence of spore stage). After two weeks, all remaining live insects were discarded and the dead insects were monitored for fungal growth and sporulation for an additional 7 days. Mean percent dead insects that showed fungal growth was also calculated.

Wild type fungal bioassay with additives

Two additives, ThomasLabs Organic Food Grade Diatomaceous Earth (Tolleson, AZ) and Bonide All Seasons Horticultural and Dormant Spray Oil (Oriskany, NY), were tested in

conjunction with the fungal strains F52, GHA and 3581 (*Metarhizium, Beauveria,* and *Isaria,* respectively) to determine if additives influenced the effect of the fungi on adult brown marmorated stink bug mortality and related factors. Both additives were incorporated into the fungal solutions. Diatomaceous earth was added to treatments at a rate of 50g/L. The horticultural oil was added at a concentration of 7%. All other procedures were identical to those outlined above. A factorial treatment structure (4x4) was used in an effort to discern any main or interactive effects between the fungi and additives. There were four levels of additives (no additives, diatomaceous earth, oil, diatomaceous earth and oil together) and four levels of fungi (no fungi, F52 [M], GHA [B], 3581 [I]). In total, there were 16 treatment combinations and four replicates.

Transgenic fungal bioassay

Several transgenic strains were tested on adult brown marmorated stink bug in an effort to see if the addition of spider toxin genes would increase the efficacy of the fungi. The spider toxin proteins (referred to as As1a, Dc1a, Hv1a and Ta1a) were derived from *Apomastus schlingeri* [Bond & Opell] (Araneae: Euctenizidae), *Diguetia canities* [McCook] (Araneae: Diguetidae), *Hadronyche versuta* [Rainbow] (Araneae: Hexathelidae) and *Tegenaria agrestis* (Araneae: Agelenidae), respectively. These peptides were selected as they have demonstrated insecticidal properties across a number of different insect orders (Bende et al. 2014; Bloomquist 2003; Johnson et al. 1998; Skinner et al. 1992). Four transgenic *Metarhizium* strains (Hv1a-1548, Dc1a-1548, As1a-1548, Ta1a-1548; acquired from R. St. Leger, University of Maryland) as well as the wild-type strain they were derived from (ARSEF 1548) and a water control were

applied to brown marmorated stink bug adults using the same procedure outlined above for the wild-type fungal bioassays. In total, there were six treatments and four replicates.

Statistical analysis

For each of the wild-type and transgenic bioassays, mortality was analyzed at days 3 and 7 post inoculation using an ANOVA (SAS Ver. 9.2, 2011) to identify significant differences between the treatments (α=0.05). If a significant difference was found, Tukey's multiple means comparison test was performed to determine which treatments differed. Fungal growth and sporulation were analyzed similarly to look for significant differences between the fungal treatments with regards to the mean days to growth or sporulation and the percent of dead bugs showing fungal growth or sporulation. The wild-type bioassay with additives was analyzed at days 3 and 7 as a 4x4 factorial design (SAS Version 9.2), to test for significant interaction effects between treatments (α=0.05). If no interaction effect was found then the data were analyzed using a Dunnett's test to compare the treatments to the water control.

Results

Wild-type fungal bioassays

Across all four adult and five nymphal bioassays, only three of the fungal strains tested, ARSEF 3581 (I) in bioassay 5 ($F_{3,19}$=4.35, P=0.0172 at day 3; $F_{3,19}$=10.96, P=0.0002 at day 7), ARSEF 1548 (M) in bioassay 1 ($F_{3,19}$ =20.62, P<0.0001 at day 7) and ARSEF 2547 (M) in bioassay 1 ($F_{3,19}$ =20.62, P<0.0001 at day 7) showed significant differences between treatments in mean mortality of nymphs and no treatments showed an effect against adults (Table 1). Across all bioassays, mean percent mortality from fungal treatments at day 3 ranged from 0 to

30% in adults and 0 to 46.67% in nymphs. At day 7, mean percent mortality from fungal treatments ranged from 3.33 to 65% in adults and 30% to 93.33% in nymphs. Mean percent mortality of control treatments at day 3 ranged from 0 to 25% in adults and 0 to 40% in nymphs. At day 7, mean percent mortality of control treatments ranged from 0 to 66.67% in adults and 0 to 86.67% in nymphs. Of stink bugs that died, mean days to fungal growth ranged from 1.25 to 5.26 days in adults and from 0.42 to 3.8 days in nymphs (Table 2), however, there was no significant difference in mean days to fungal growth between treatments in any wild-type bioassay (P>0.05). Mean days to sporulation ranged from 3.66 to 9.11 days in adults and from 1.4 to 10 days in nymphs (Table 2). Strain 2547 (M) took significantly more days to show fungal sporulation than strain 1548 (M) in adult bioassay 1 ($F_{1,9}$=9.94, P=0.0161) but no other differences were found for adults (P>0.05). There were no differences in mean days to fungal sporulation for nymphs (P>0.05). Mean percent of dead insects that showed fungal growth ranged from 13.33% to 53.33% in adults and from 0 to 90% in nymphs (Table 2). The Unidentified Fungus (O) in bioassay 3 had a significantly greater mean percent of dead insects with fungal growth in both adults ($F_{1,9}$=20.86, P=0.0014) and nymphs ($F_{1,9}$=25.00, P=0.0007). Strain 3581 (I) showed significantly greater mean percent of dead insects with fungal growth in nymph bioassay 5 ($F_{1,9}$=12.95, P=0.0058). Mean percent of dead insects with fungal sporulation ranged from 0 to 40% in adults and from 0 to 66.67% in nymphs (Table 2). The Unidentified Fungus (O) showed significantly greater mean percent of dead insects with fungal sporulation than strain 2575 in adult bioassay 3 ($F_{1,9}$=6.00, P=0.0368). Strain 2547 (M) showed significantly greater mean percent of dead insects with fungal sporulation than strain 1548 (M) in nymph bioassay 1 ($F_{1,9}$=7.35, P=0.0239). Strain 3581 (I) showed significantly greater mean

percent of dead insects with fungal sporulation than strain 10386 (O) in nymph bioassay 5 ($F_{1,9}$=8.18, P=0.0188).

Wild-type fungal bioassay with additives

There was no fungal strain by additive interaction effect found between any of the treatment combinations in the bioassay on adult stink bug mortality at days 3 ($F_{9,301}$=1.43, P=0.1760) or 7 ($F_{9,301}$=0.36, P>0.9532). There was a significant effect of treatment at day 3 ($F_{15,48}$=3.68, P=0.0003). Treatments that differed significantly from the water control were the DE/Oil treatment (P=0.0493), the Oil treatment (P=0.0493), the 3581/Oil treatment (P=0.0050) and the F52/Oil treatment (P=0.0493) (Table 3). Despite a significant result from the F-test ($F_{15,48}$=2.64, P=0.0056), none of the means comparison tests were significant at day 7.

Transgenic fungal bioassay

The transgenic fungal bioassay was analyzed in the same manner as the wild-type bioassays. Neither the wild-type nor any of the transgenic fungal treatments showed a significant difference in their effect on adult stink bug mortality at days 3 ($F_{5,17}$=0.61, P=0.69) or 7 ($F_{5,17}$=1.44, P=0.26) (Table 4). Mean percent treatment mortality at day 3 ranged from 10 to 30% and at day 7 from 65 to 95%. Control mortality was 20% at day 3 and 60% at day 7. Mean days to fungal growth ranged from 2 to 8 days. Mean percent of dead insects with fungal growth ranged from 10 to 40%. There was no significant difference in mean days to fungal growth ($F_{4,7}$=1.50, P=0.3005) between fungal treatments. Analysis of the mean percent of dead insects with fungal growth showed a significant difference ($F_{4,14}$=3.33, P=0.0408); however, the means

comparison test showed no significant difference between treatments. None of the insects showed evidence of fungal sporulation over the course of the bioassay.

Table 1: Mean (±SEM) percent mortality of brown marmorated stink bug adults and nymphs 3 and 7 days following exposure to entomopathogenic fungi and controls. Table includes result of 4 adult and 5 nymphal stink bug bioassays.

		Mean % Mortality [1]			
		x□ % Mortality (Day 3)		x□ % Mortality (Day 7)	
Bioassay #	Treatment	Adults	Nymphs	Adults	Nymphs
1	1548 (M)	0	3.33 (±3.33)	10 (±4.47)	30 (±4.47)a
1	2547 (M)	3.33 (±3.33)	0	13.33 (±6.67)	43.33 (±9.54)a
1	Tween	3.33 (±3.33)	0	3.33 (±3.33)	0b
1	Water	3.33 (±3.33)	0	10 (±4.47)	0b
2	1055 (M)	10 (±10)	46.67 (±15.20)	36.67 (±12.01)	100 (±0)
2	GHA - Botanigard (B)	3.33 (±3.33)	40 (±7.30)	30 (±11.25)	90 (±6.83)
2	Tween	10 (±6.83)	40 (±13.66)	40 (±15.49)	80 (±7.30)
2	Water	6.67 (±4.21)	26.67 (±8.43)	33.33 (±9.88)	73.33 (±9.88)
3	2575 (M)	3.33 (±3.33)	40 (±7.30)	43.33 (±8.02)	93.33 (±4.21)
3	Unidentified Fungus (U)	10 (±6.83)	20 (±7.30)	56.67 (±9.54)	93.33 (±4.21)
3	Tween	10 (±4.47)	16.67 (±6.14)	66.67 (±8.43)	86.67 (±6.67)
3	Water	0	16.67 (±8.02)	33.33 (±8.43)	83.33 (±6.14)
4	F52 (M)	25 (±5.00)	25 (±12.58)	60 (±8.16)	70 (±12.91)
4	GHA - USDA (B)	30 (±12.91)	20 (±14.14)	65 (±9.57)	65 (±9.57)
4	3581 (I)	20 (±8.16)	40 (±14.14)	55 (±5.00)	80 (±0)
4	Water	25 (±18.93)	35 (±9.57)	45 (±18.93)	65 (±5.00)
5	10386 (U)	-	13.33 (±6.67)b	-	33.33 (±8.43)b
5	3581 (I)	-	30 (±8.56)a	-	73.33 (±8.43)a
5	Tween	-	23.33 (±3.33)b	-	33.33 (±6.67)b
5	Water	-	3.33 (±3.33)b	-	13.33 (±6.67)b

[1] Treatment means followed by different letters within a bioassay and life stage significantly differed from other treatments ($P \leq 0.05$). Treatment means not followed by a letter did not significantly differ from other treatments within a bioassay ($P > 0.05$).

Table 2: Fungal growth and sporulation data for wild-type bioassays showing mean (±SEM) days to fungal growth and sporulation of brown marmorated stink bug adults and nymphs as well as mean (±SEM) percent of dead insects showing fungal growth and fungal sporulation. Table includes 4 adult and 5 nymphal wild-type fungal bioassays. Note that growth and sporulation data includes only stink bugs that died in fungal treatments during the bioassay (see Table 1).

Bioassay #	Treatment	Fungal Growth				Fungal Sporulation			
		x̄[1] Days to Growth		x̄ % Dead with Growth		x̄ Days to Sporulation		x̄ % Dead with Sporulation	
		Adults	Nymphs	Adults	Nymphs	Adults	Nymphs	Adults	Nymphs
1	1548 (M)	1.68 (±0.31)	0.84 (±0.46)	53.33 (±8.43)	43.33 (±8.02)	3.66 (±0.62)b	NS	40 (±8.94)	0b
1	2547 (M)	2.27 (±0.46)	1.33 (±0.34)	36.67 (±6.14)	70 (±13.41)	5.66 (±0.42)a	4 (±1.00)	20 (±7.30)	16.67 (±6.14)a
2	1055 (M)	3.66 (±1.89)	3.8 (±0.81)	20 (±10.32)	33.33 (±6.67)	5 (±0)	9.5 (±4.09)	3.33 (±3.33)	13.33 (±6.67)
2	GHA - Botanigard (B)	4.4 (±1.03)	2.87 (±0.63)	33.33 (±9.88)	26.67 (±6.67)	NS	NS	NA	NA
3	2575 (M)	3 (±1.22)	NG[2]	13.33 (±6.67)b	0b	8.33 (±1.20)	NA	10 (±4.47)b	NA
3	Unidentified Fungus (U)	5.26 (±1.04)	3 (±0.83)	50 (±4.47)a	16.67 (±3.33)a	9.11 (±1.32)	10 (±0)	30 (±6.83)a	3.33 (±3.33)
4	F52 (M)	2.67 (±1.20)	2 (±1.00)	15 (±9.57)	10 (±5.77)	5 (±0)	NS	5 (±5.00)	0
4	GHA - USDA (B)	2.8 (±0.66)	1.4 (±0.50)	25 (±12.58)	25 (±9.57)	5 (±1.00)	3.75 (±1.60)	10 (±5.77)	20 (±8.16)
4	3581 (I)	1.25 (±0.49)	0.42 (±0.20)	40 (±16.33)	35 (±15.00)	4.14 (±0.96)	1.4 (±0.24)	35 (±12.58)	25 (±12.58)
5	10386 (U)	-	2.90 (±0.71)	-	40 (±14.60)b	-	6.12 (±1.80)	-	26.67 (±8.43)b
5	3581 (I)	-	1.48 (±0.45)	-	90 (±4.47)a	-	2.8 (±0.76)	-	66.67 (±11.15)a

[1] Treatment means followed by different letters within a bioassay and life stage significantly differed from other treatments ($P \leq 0.05$).

Treatment means not followed by a letter did not significantly differ from other treatments with in a bioassay ($P > 0.05$).

[2] NG and NS denote no fungal growth or no fungal sporulation respectively. NA denotes not applicable.

Table 3: Mean (±SEM) percent mortality of adult brown marmorated stink bugs for the wild-type fungal bioassay with additives, 3 and 7 days following exposure to entomopathogenic fungi and additive treatments.

Treatment	x□ % Mortality[1]	
	x□ % Mortality (Day 3)	x□ % Mortality (Day 7)
Water	5.00 (±5.00)	30.00 (±12.91)
DE [2]	20.00 (±8.16)	35.00 (±9.57)
DE/Oil	40.00 (±11.54)*	60.00 (±8.16)
Oil	40.00 (±8.16)*	55.00 (±12.58)
3581 (I)	20.00 (±0)	45.00 (±9.57)
3581/DE	5.00 (±5.00)	40.00 (±14.14)
3581/DE/Oil	20.00 (±0)	50.00 (±10.00)
3581/Oil	50.00 (±10.00)*	65.00 (±9.57)
F52 (M)	5.00 (±5.00)	35.00 (±5.00)
F52/DE	0 (±0)	50.00 (±10.00)
F52/DE/Oil	10.00 (±10.00)	65.00 (±12.58)
F52/Oil	40.00 (±21.60)*	70.00 (±10.00)
GHA (B)	5.00 (±5.00)	10.00 (±5.77)
GHA/DE	0 (±0)	25.00 (±15.00)
GHA/DE/Oil	20.00 (±8.16)	35.00 (±9.57)
GHA/Oil	15.00 (±5.00)	30.00 (±10.00)

[1] Treatment means followed by an "*" significantly differed from the control within a day ($P \leq 0.05$). Treatment means not followed by a letter did not significantly differ from the control ($P > 0.05$).

[2] DE = diatomaceous earth; Oil = horticultural oil

Table 4: Mean (±SEM) percent mortality and fungal growth for brown marmorated stink bug adults following treatments with four transgenic *Metarhizium* strains of the ARSEF 1548 modified to produce spider toxins and the unmodified strain (1548 WT). Note that fungal growth includes only insects that died during the bioassay.

Treatment	$\bar{x}$ % Mortality[1]		Fungal Growth	
	$\bar{x}$ % Mortality (Day 3)	$\bar{x}$ % Mortality (Day 7)	$\bar{x}$ Days to Growth	$\bar{x}$ % dead with Growth
1548 WT	20 (±14.14)	95 (±5.00)	4 (±0.87)	40 (±8.16)
As1a	10 (±5.77)	80 (±14.14)	2 (±0)	20 (±8.16)
Dc1a	15 (±9.57)	70 (±10.00)	2.67 (±1.11)	40 (±8.16)
Hv1a	30 (±10.00)	65 (±5.00)	3.25 (±1.43)	40 (±8.16)
Ta1a	20 (±8.16)	75 (±9.57)	8 (±0)	10 (±5.77)
Water	20 (±8.16)	60 (±14.14)	-	-

[1] There were no significant differences between treatments for any of the variables examined ($P>0.05$)

Discussion

Although entomopathogenic fungi can be useful in suppressing populations of some insect species, my results demonstrated relatively poor performance of the several strains of fungi from the three genera tested against the invasive brown marmorated stink bug. In general, results were inconsistent, levels of mortality were low, and of insects that did die, fungal growth and sporulation were variable. Additives of diatomaceous earth or oil to fungal formulations did not improve the performance of entomopathogenic fungi in killing brown marmorated stink bug, but oil itself did increase mortality of stink bugs compared to a water control. Also of interest is that the entomopathogenic fungal strain, *Metarhizium* 1548, that was genetically modified to produce various spider toxins, did not increase the pathogenicity of *Metarhizium* towards brown marmorated stink bug. These findings suggest that entomopathogenic fungi would likely make poor biological control agents towards managing the brown marmorated stink bug. In addition,

the overall poor performance of all three genera of fungi, even the transgenic strains, suggest some other factor may be influencing the ability of the fungi to infect and kill brown marmorated stink bug.

In searching for measures to control pest insects, the ideal tactic should provide quick knockdown of the pest population before it causes economic damage to a crop. Specifically, an optimally formulated fungal biological control for brown marmorated stink bug should result in high levels of mortality within one to three days. For example, feeding by brown marmorated stink bug in apples for one and three days can cause a significant amount of damage (5 and 30%, respectively), degrading the quality of fruit substantially (Leskey et al. 2014). In addition, it is preferred that once the fungus kills the insect there would be relatively quick development of fungal growth and sporulation to increase the likelihood that the pathogen would spread through the pest population.

In the wild-type fungal bioassays, mortality was low at days three and seven and overall incidence of fungal growth and sporulation on stink bugs that died was low relative to what would be indicative of an effective control method. Of the strains tested against brown marmorated stink bug, the effects of the *Isaria* strain 3581 appeared to be promising against nymphs (bioassay 5), however, this effect was not reflected in bioassay 4 where *Isaria* was tested against nymphs, demonstrating an inconsistency in *Isaria*'s pathogenicity. Poor performance of the fungal strains evaluated in my study is inconsistent with other studies that examined the effects of similar strains of entomopathogenic fungi against insects. For example, tarnished plant bug nymphs immersed in a *Beauveria* suspension reached 80% mortality after 12 days with 80.5% showing fungal growth by 9 days after death (Liu et al. 2003). My results suggest that the

wild-type fungi tested here are not sufficient on their own to kill brown marmorated stink bug at rates needed to be used as a biological control agent.

A possible explanation for poor performance of the wild type fungi may be related to humidity or the ability of the fungi to penetrate the stink bug's exoskeleton (Luz et al. 2012). I predicted that adding a horticultural grade oil or diatomaceous earth, alone or in combination, to the fungal formulations would protect the fungi from light and increase humidity or abrade the exoskeleton and aid in spore penetration, respectively, and increase the pathogenicity of the fungi. However, the addition of additives provided no synergistic interaction and results were similar to that of the wild type fungal bioassays. These results were surprising as other studies have found positive effects of additives. The addition of diatomaceous earth and horticultural oil to a *Metarhizium* formulation was found to kill 95% of *Triatoma infestans* nymphs within 10 days of exposure (Luz et al. 2012). Oil alone has also been shown to have synergistic effects with entomopathogenic fungi. The addition of sunflower oil to a *Metarhizium* formulation resulted in nearly 100% control of greenhouse whiteflies (Malsam et al. 2002). In my bioassays where there were significant treatment effects, it appears to be the result of horticultural oil directly which was present in each treatment with significant effects. Horticultural oil is known to have insecticidal properties and is commonly recommended as a pest control tactic (Beattie and Development Corporation).

Other studies have increased the pathogenicity of entomopathogenic fungi against insects by genetically modifying the fungi to produce scorpion neurotoxins (Wang and St. Leger 2007; Lu et al. 2008; Fang et al. 2012). These studies found that the addition of a scorpion neurotoxin AaIT significantly increased the efficacy of the fungi against yellow fever mosquitoes and several different Lepidoptera larvae. My study similarly examined genetically modified

Metarhizium except with four transgenic strains of ARSEF 1548 (M) that each expressed a different spider neurotoxin. Unlike the above studies using transgenic fungi, transgenic fungi in this study resulted in no greater mortality of stink bugs than the wild type *Metarhizium* 1548 or the water control. Similar to the wild type bioassays none of the treatments were particularly effective and we again see the same patterns in mortality. Of particular note here is the complete lack of fungal sporulation in any treatment even though there was fungal mycelia growth, an outcome unique to this bioassay.

One possible explanation towards explaining the generally poor virulence of the fungus is the possibility that the fungal strains I used were not viable and therefore incapable of infecting any insect host. To confirm viability of the fungi, a bioassay was performed against *Galleria mellonella* [Linnaeus] (Lepidoptera: Pyralidae), a common model for pathogenicity, using F52 (M) and GHA (B) and the same procedures as the wild-type bioassays above. The effect of fungal treatment was found to be highly significant at days 3 ($F_{2,56}$=33.76, P<0.0001) and 7 ($F_{2,56}$=68.90, P<0.0001) (T. Pike, unpubl. data) demonstrating that there were no issues with the fungi in regards to their ability to infect and kill an insect host.

Another factor that may have influenced the outcome of these studies is that stink bug mortality in the water and Tween control treatments was variable overall and high in several of the bioassays. High and variable control mortality made it difficult to discern significant treatment effects. While I was unable to determine why the control mortality was high, I was able to eliminate certain factors. One of the prevailing ideas was that dipping the insects in the treatment solutions (see Methods) may have caused some type of response from or a detrimental impact on the stink bugs that increased their mortality. However, a trial comparing mortality of stink bugs that were dipped in water to those not dipped in water found no difference in mortality

at 3 ($F_{1,5}$=0.43, P=0.54) or 7 ($F_{1,5}$=1.00, P=0.36) days after dipping (T. Pike, unpubl. data). Another possible explanation for high control mortality relates to the plastic containers that the stink bugs were kept in during the experiments. Despite the fact that the containers were ventilated, it is possible that the act of treating or handling the insects agitated them, prompting a defense response. Keeping them in close proximity (5 bugs/container) could have resulted in the stink bugs being killed by their own defensive compounds. This response has been noted when field collected stink bugs are kept in large numbers in the same collection container (P. Shrewsbury, pers. observ.). However, trials comparing the mortality of stink bugs when placed in plastic vs. cardboard containers, and one vs. five stink bugs per plastic container did not support this hypothesis. There was no significant difference in mortality between stink bugs placed in plastic vs. cardboard containers at days 3 ($F_{1,7}$=0.07, P=0.79) or 7 ($F_{1,7}$= 0.15, P=0.71) (T. Pike, unpubl. data) or between containers containing one vs. five adult stink bugs at days 3 ($F_{1,27}$= 0.18, P=0.6769) or 7 ($F_{1,27}$= 0.07, P=0.7953) (T. Pike, unpubl. data) demonstrating these factors did not influence control mortality in my studies. It is worth noting that even in bioassays with high mortality in the controls that lacked statistical differences, mean treatment mortality was generally not numerically greater than control mortality, suggesting that the fungal treatments did not contribute any additional mortality than was present in the controls.

To my knowledge there are only two other studies that have examined the effect of entomopathogenic fungi on brown marmorated stink bug. Gouli et al. (2012) evaluated five strains of entomopathogenic fungi and found generally poor mortality, with only four of the fungal treatments exceeding 80% mortality by 12 days and one exceeding 50% mortality by six days. Similar results were found in a study by Ihara et al. (2008) in which three stink bug species, *P. c. stali, G. subpunctatus* and *H. halys*, were treated with *Metarhizium* strain FRM515

and monitored for mortality. *P. c. stali* and *G. subpunctatus* reached 100% mortality 7 and 9 days after treatment, respectively, whereas *H. halys* reached only 50% mortality after 10 days (Ihara et al. 2008). Results of these studies support the findings of my study, suggesting that entomopathogenic fungi have relatively low pathogenicity against brown marmorated stink bug.

A possible mechanism that may explain the low virulence of the diversity of entomopathogenic fungal taxa on brown marmorated stink bug in my studies and those of Gouli et al. (2012), and Ihara et al. 2008 is that brown marmorated stink bug may have some type of defense against fungi. I hypothesize that defensive compounds known to be released by the brown marmorated stink bug, and believed to be used in defense against predators, may also have anti-fungal properties. A number of insects have been observed to produce defensive compounds that possess fungistatic effects, such as earwigs (Gasch 2013), sawfly larvae (Nagy 2009), chrysomelid leaf beetles (Pasteels 1992), bed bugs (K. Ulrich, Pers. comm.) and a number of Pentatomid species (Borges and Aldrich 1992; Fehlbaum et al. 1996; Sosa-Gomez et al. 1997; Fávaro and Zarbin 2013). This phenomenon would explain the low incidence of fungal infection on brown marmorated stink bug. Determining whether or not this is the mechanism underlying the apparent resistance to fungal infection by brown marmorated stink bug will be the subject of future work.

Overall, all genera and strains of entomopathogenic fungi tested for their effects on brown marmorated stink bug provided inconsistent and relatively low levels of mortality to stink bugs. These results were consistent for wild type fungi, fungi with additives in their formulations, and even transgenic fungi enhanced with spider toxins. Our findings suggest that entomopathogenic fungi would not serve as an effective biological control for brown marmorated stink bug. These data highlight the need for future studies to examine possible

mechanisms underlying the apparent resistance of brown marmorated stink bug to entomopathogenic fungi.

Chapter 2: Interactions between defensive compounds of brown marmorated stink bug (*Halyomorpha halys*) and entomopathogenic fungi

Introduction

Chemical defenses are present in and important for a number of insects. Many herbivorous insects rely on chemical defenses to evade or deter predators (Evans 1990; Eisner et al. 2005). The presence of chemical defenses have been well documented in several groups of insects like monarch butterflies, milkweed bugs (Duffey 1980; Trigo 2000) and chrysomelid leaf beetles (Pasteels 1992; Triponez 2007), among countless other groups. While some insects produce their own chemical defenses, many others sequester compounds present in the plants they consume to later deploy as a defense (Duffey 1980; Pasteels 1992).

Chemical defenses targeting predators work in a variety of ways. Among the most common are malodorous compounds present in groups such as earwigs (Gasch 2013) and some Hemiptera including stink bugs (Solomon 2013), as well as many others. While there is some variation between species, many Pentatomids produce a similar array of defensive compounds, including an assortment of alkane hydrocarbons, esters and aldehydes (Ho and Millar 2001; Marques et al. 2007; Solomon 2013). These compounds are secreted by the insects and their foul smell serves as a deterrent to predators. Some insects, such as the larvae of the western corn rootworm, contain toxic compounds in their hemolymph that discourage predators from feeding on them (Lundgren et al. 2010). Still other groups employ even more unique strategies, such as the larvae of the beet armyworm, whose oral secretions act as a surfactant which drives predators to groom themselves rather than continue to pursue the larvae (Rostás and Blassmann 2009).

In addition to their use in deterring predators, there are a number of studies that demonstrate the effectiveness of these defensive secretions against fungal infection. Assays of the defensive secretions of several species of earwig against *Metarhizium anisopliae* and *Beauveria bassiana* showed significant inhibition of fungal growth in concentrations equivalent to the contents of a single insect (Gasch 2013). Analysis of several species of nematine sawflies showed that in addition to secreting chemicals meant to deter arthropod predators such as ants, several chemicals in the defensive secretion also provide protection against fungal infection, critical for when the leaf-mining larvae drop to the ground to pupate (Nagy 2009). However, the relationship between entomopathogenic fungi and insect defense varies among different insects or insect groups. Fungal pathogens and host defense against infection are thought to be under reciprocal selective pressure providing a classic example of a co-evolutionary arms race (Ortiz-Urquiza and Keyhani 2013).

While an effective biological control for insects and other arthropods in many systems (Malsam et al. 2002; Feng et al. 2004; Luz and Batagin 2005; Behle 2006; Sivasundaram et al. 2008; Guerri-Agullo et al. 2011; Santi et al. 2011; Luz et al. 2012), entomopathogenic fungi have been demonstrated to be largely ineffective against stink bugs (Pentatomidae), with relatively long periods of time required to begin observing mortality in addition to low incidence of fungal germination (Sosa-Gomez et al. 1997; Sosa-Gomez and Moscardi 1998; Ihara et al. 2008; Gouli et al. 2012; Pike Chpt. 1 2014). While the reason for the poor performance of the fungi is not explicitly understood, one proposed explanation for the low mortality and virulence is the presence of fungistatic compounds on or within the insects that interfere with fungal growth (Sosa-Gomez et al. 1997; Sosa-Gomez and Moscardi 1998; Pike Chpt. 1 2014). The secretions of several species of stink bugs have been found to produce fungistatic effects. Thanatin, a

peptide present in the hemolymph of the spined soldier bug, *Podisus maculiventris* [Say] (Hemiptera: Pentatomidae), was found to be active at very low concentrations against a number of fungal species (Fehlbaum et al. 1996). Trans-2-decenal present in the scent glands of *Nezara viridula* [Linnaeus] (Hemiptera: Pentatomidae) was found to suppress germination of several entomopathogenic fungi at natural concentrations (Sosa-Gomez, Boucias, and Nation, 1997). The presence of such compounds could significantly retard the effects of the fungi and render them unsuitable as an avenue for biological control.

The brown marmorated stink bug (*Halyomorpha halys* [Stål], Hemiptera: Pentatomidae) is an invasive exotic pest of a range of agricultural crops and ornamental plants. Recent research evaluating the biological control potential of entomopathogenic fungi on brown marmorated stink bug has found the fungi to have low virulence (Pike Chpt. 1 2014). Experiments testing the effects of several wild-type strains of entomopathogenic fungi with representatives from a number of fungal genera found relatively low mortality in adult and nymphal brown marmorated stink bug as well as low rates of fungal growth and sporulation on dead insects. The addition of additives to improve fungal efficacy, such as diatomaceous earth and horticultural oil in conjunction with these fungi, and even the use of several transgenic strains of *Metarhizium* engineered to express spider neuropeptides, all produced similarly poor results.

Recent research has found that brown marmorated stink bug possess chemical defensive secretions. Moreover, brown marmorated stink bug defensive secretions were found to contain trans-2-decenal (Solomon et al. 2013; E. Tomasino, Pers. comm.), an aldehyde also present in *N. viridula* that was demonstrated to suppress fungal germination (Sosa-Gomez et al. 1997; Solomon 2013) and trans-2-octenal (E. Tomasino, Pers. comm.). Bed bugs (Hemiptera: Cimicidae) have also been shown to contain trans-2-hexenal and trans-2-octenal, chemically

similar compounds that also inhibit fungal growth (K. Ulrich, Pers. comm.). It is therefore possible that chemicals like it could be the mechanism underlying the low virulence of entomopathogenic fungi against brown marmorated stink bug. Determining the defense mechanism resulting in low virulence against brown marmorated stink bug could provide an opportunity to circumvent that defense and deploy the entomopathogenic fungi in an effective manner. Therefore, the overall objective of this study is to determine if defensive compounds play a role in the poor performance of entomopathogenic fungi against brown marmorated stink bug. Specifically, my objectives are to: 1) confirm the major constituents of the defensive secretion of brown marmorated stink bug, 2) determine if these compounds have a fungistatic effect on entomopathogenic fungi and if so at what concentrations, 3) determine the effect of these compounds on the germination of fungal spores, and 4) determine if the presence of fungi on brown marmorated stink bug induce production of defensive compounds.

Methods

Study organism - brown marmorated stink bug

Stink bugs used for these experiments were taken from a laboratory-reared colony at the University of Maryland. Laboratory-reared colony stink bugs were kept in cages and provided with green bean plants, sunflower seeds, and water. The cages were kept in environmental chambers and maintained at 25°C and 70% RH with a 16:8 light: dark cycle.

Study organisms - fungal strains

The three strains of entomopathogenic fungi tested were F52 (M), GHA (B) and ARSEF 3581 (I), belonging to the *Metarhizium* (M), *Beauveria* (B) and *Isaria* (I) genera respectively, which are representative of fungi known to kill insects and all of which have commercialproducts available. Strains were obtained from M. Jackson at the USDA-ARS, Peoria, IL.

Defensive compounds

Selection of tested defensive compounds was based on personal communication with E. Tomasino (Oregon State University) as well as published literature on brown marmorated stink bug and related Pentatomids (Sosa-Gomez et al. 1997; Solomon 2013). Trans-2-octenal and trans-2-decenal were chosen as the primary defensive compounds to evaluate in these experiments. Tomasino (Pers. Comm.) found trans-2-octenal and trans-2-decenal, along with tetradecane and dodecane, produced at high levels when she assessed the defensive compounds of brown marmorated stink bug. Similarly, trans-2-octenal and trans-2-decenal were also found by Solomon (2013). Other studies examining the defensive compounds of pentatomids, and compounds with potential fungistatic properties, suggest that trans-2-octenal and trans-2-decenal may be important (Sosa-Gomez et al. 1997). Chemical standards for trans-2-octenal and trans-2-decenal were ordered from Sigma-Aldrich and used in the following experiments.

Detection and quantification of stink bug defensive chemicals in whole insects

To confirm the presence of and detect how much of the two defensive chemicals, trans-2-octenal and trans-2-decenal, are present in an individual brown marmorated stink bug adult, I analyzed them using gas chromatography-mass spectrometry (GC-MS). Four adult brown marmorated stink bug adults were crushed in 2 mL of hexane. One mL of this liquid extract was

added to 4 mL of sterile deionized (DI) water, collecting as little solid debris from the mash as possible then vortexed for 15 seconds. The top 1.5 mL hexane portion of the solution was removed and centrifuged for 1 minute at 13k rpm, 150 µL of the hexane fraction was stored at -20°C for later analysis by GC-MS. GC-MS was performed using an Agilent GC-MS 5975-7890 (Agilent Technologies Inc.) with an HP-5MS column (length: 30 m, diameter: 0.25 mm, film: 0.25 µm). The method ramped from 75°C to 325°C at 30°C per minute. Trans-2-octenal and trans-2-decenal were identified by GC retention times (compared to known standards) and mass spectra. These compounds were quantified by comparing the total area under the curve for each peak in a single-ion trace (m/z=70) gas chromatogram to a standard curve. The standard curve was generated with the standards of each compound at known concentrations ranging from 1 mg/L to 100 mg/L.

Effects of defensive compound volatiles on fungal growth

Defensive chemicals were assayed against F52 (M), GHA (B), 3581 (I) in order to determine if the chemicals inhibit fungal growth and if so at what concentration and if fungi recover from inhibitory effects. Petri dishes with potato dextrose agar (PDA) were inoculated with fungi that had been grown for 14 days at 28°C. The fungal suspensions used for the inoculations were diluted to a concentration of $1x10^{7}$ conidia/mL. Immediately following inoculation, 9 mm discs of filter paper were then adhered to the underside of the lid of the petri dishes using double-sided tape. The filter paper was then treated with 5 µL of either trans-2-octenal or trans-2-decenal at concentrations of 100, 10, or 1% (with the 10 and 1% solutions being diluted in DI water) or with a DI water control. In total, there were 21 treatments, each replicated three times. Petri dishes were then sealed with Parafilm and allowed to grow at 28°C in an environmental chamber. After three days, presence or absence of fungal growth was noted

on all petri dishes. On petri dishes where no fungal growth was observed, the chemical was removed by replacing the lid of the petri dish with a clean lid and the petri dish was resealed. Petri dishes with the chemical removed were monitored daily for one week in order to determine if fungi no longer exposed to trans-2-octenal or trans-2-decenal petri dishes recovered or not (presence or absence of fungal growth, respectively).

Effects of defensive compounds on fungal spore germination

The defensive compounds, trans-2-octenal and trans-2-decenal, were also assayed at varying concentrations against fungal suspensions in liquid media to directly observe their effects on fungal spores of F52 (M), GHA (B), and 3581 (I). Fungal suspensions were prepared in potato dextrose broth at a concentration of $5x10^5$ conidia/mL. 2mL of the fungal suspension was added to 35 mm petri dishes in order to create a relatively standard number of spores within the petri dish. The spore suspensions were then treated with defensive chemicals at the rates and concentrations listed in the above bioassay by micro-pipetting them into the petri dish and then swirling briefly. In total, there were 21 treatments, each replicated three times. Petri dishes were sealed with Parafilm and allowed to grow at 28°C in an environmental chamber. The petri dishes were photographed daily for two days at 400x magnification. From the photographs taken on day 2, the proportion of germinated spores per petri dish was determined by counting the number of germinated and non-germinated spores in the entire image. Germination was defined as the presence of an observable germ tube (Fig. 1).

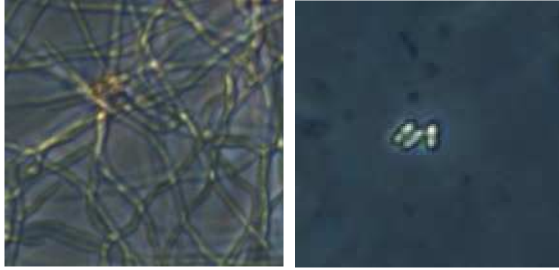

Figure 1: Example of germinated spores showing mycelium (left) and un-germinated spores (right) of *Metarhizium* fungal strain F52.

Inducing brown marmorated stink bug defense via fungal exposure

Defensive compound emissions of live adult brown marmorated stink bugs were analyzed using a Time-of-Flight mass spectrometer, AccuTOF (JEOL, Peabody, MA, USA) equipped with a confined Direct Analysis in Real Time (cDART) ion source. Instruments were configured according to Li (Li 2013). The instrument was operated in the positive or negative ion mode with a resolving power of 6000 (FWHM). Mass spectra were acquired at a rate of one spectrum per second. Calibration for mass measurements was done using polyethylene glycol (average molecular weight=600) as the internal standard. In my experiments, the helium gas heater was set to 250°C and the glow discharge needle potential and grid voltage were set at 3.5 kV and 250 V, respectively. The AccuTOF MS orifice 1 was set to 80°C and 20 V, orifice 2 was set to 5 V and the ring set to 5 V.

To identify that the defensive chemicals trans-2-octenal and trans-2-decenal were being secreted by the adult brown marmorated stink bugs, the molecular weight of stink bugs' emissions were recorded using the AccuTOF during an agitation event. Adult brown marmorated stink bugs were acquired from a laboratory colony and maintained in conditions as noted above. Twenty male and twenty female stink bugs were each placed individually in 20 mL

glass vials which were capped with screw-top lids with gas-tight permeable septa. In order to induce a defense response, the insects were manipulated with a straightened paper clip until a response was observed with the AccuTOF. Spectra for each stink bug were recorded until the defense response subsided. Chemical standards of trans-2-octenal and trans-2-decenal were run through the AccuTOF to confirm the identity of the peaks seen in the stink bugs.

In an effort to determine if exposure to entomopathogenic fungi induced a defense response of brown marmorated stink bug, adult stink bugs were treated with fungi and their response observed over time. Fungal suspensions of F52 (M), GHA (B) and 3581 (I) were each prepared at a concentration of $1x10^7$ conidia/mL. Suspensions of deactivated fungi of the same concentration were also prepared by autoclaving live fungal suspensions for 20 minutes at 121^oC to assess if just the physical presence of fungal spores initiated a defense response. In total, there were eight treatments: undisturbed insects (control), water-treated insects, insects treated with one of the three live fungal treatments and insects treated with one of the three deactivated fungal treatments. All insects were placed in individual 10 mL glass vials after receiving their treatment. Insects in the undisturbed treatment were placed in vials with minimal manipulation. For all other treatments individual insects were placed in a narrow cup to restrict movement, and treatments were applied using a perfume sprayer at a rate of 0.5 mL of liquid per insect. In total, 48 males and 48 females were treated, divided between the eight treatments, resulting in six replicates per gender (2) per treatment (8). The secretions of each stink bug were measured using the AccuTOF at time=0 and every 24 hours thereafter for five days. Between measurements the stink bugs were placed in a growth chamber at 25^oC with 75% relative humidity and a 16:8 day/night cycle. In order to ensure that the stink bugs would survive the duration of the experiment (i.e. had enough air), the vial caps were fitted loosely onto the vials.

However, 12 hours prior to each measurement caps were tightened to ensure any chemical emissions that might be produced were contained. Stink bug defensive secretions were quantified by creating calibration curves using known quantities of trans-2-octenal and trans-2-decenal standards and comparing them to the peak ion height of the emission output for each stink bug.

Statistical analysis

Two analyses were conducted to determine the effect of the defense chemicals trans-2-octenal (#1) and trans-2-decenal (#2) on fungal spore germination. Each defense chemical trial was analyzed as a 3x4 factorial with 3 levels of fungal strain and 4 levels of chemical concentration to examine the interactive effects between fungal strain and defense chemical concentration as well as the main effects of each. Due to low response, statistics were not conducted on the induced response of stink bugs following exposure to fungi.

Results

Detection and quantification of stink bug defensive chemicals in whole insects

Analysis of targeted defensive compounds extracted from whole insect samples of brown marmorated stink bug was found to contain a mean of 0.006 ($\pm 2.12\times10^{-4}$) µL of trans-2-octenal and a mean of 0.184 (± 0.038) µL of trans-2-decenal per insect.

Effects of defensive compound volatiles on fungal growth

For both the trans-2-octenal and trans-2-decenal treatments, all three fungi tested showed complete inhibition of fungal growth in all petri dishes at the 100% concentration treatment

(Table 5). All three fungi also showed no inhibition of fungal growth in all petri dishes at the 1% and 0% concentration treatments. In the 10% concentration trans-2-octenal treatment, mean percent of petri dishes without fungal growth ranged from 0 to 100%. In the 10% concentration trans-2-decenal treatment, mean percent of petri dishes without fungal growth ranged from 33 to 66%. When examining fungal recovery one week post removal of the defensive chemicals from the petri dishes no recovery was observed in the 100% concentration treatments of trans-2-octenal or trans-2-decenal in any petri dishes (Table 6). Fungal recovery in the petri dishes in the 10% concentration trans-2-octenal treatment ranged from 50 to 66%. All petri dishes treated with 10% concentration trans-2-decenal showed fungal recovery.

Table 5: Mean (±SEM) percent of petri dishes showing fungal inhibition three days after exposure to two brown marmorated stink bug defensive chemicals at 3 concentrations and a water control.

	Mean % Petri dishes without Fungal Growth						
	Trans-2-octenal Concentration			Trans-2-decenal Concentration			
Fungus	100%	10%	1%	100%	10%	1%	Control
F52 (M)	100% (±0)	66% (±33.33)	0 (±0)	100% (±0)	33% (±33.33)	0 (±0)	0 (±0)
GHA (B)	100% (±0)	100% (±0)	0 (±0)	100% (±0)	66% (±33.33)	0 (±0)	0 (±0)
3581 (I)	100% (±0)	0 (±0)	0 (±0)	100% (±0)	33% (±33.33)	0 (±0)	0 (±0)

Table 6: Mean (±SEM) percent of petri dishes showing recovery of three fungal strains 7 days post removal of defensive chemicals. Data includes only petri dishes that showed inhibition of fungal growth (i.e. fungal growth did not occur bythree3 days post exposure to defensive compounds).

	Mean % Petri dishes with Fungal Recovery					
	Trans-2-octenal Concentration			Trans-2-decenal Concentration		
Fungus	100%	10%	1%	100%	10%	1%
F52 (M)	0 (±0)	50% (±50.00)	N/A*	0 (±0)	100% (±N/A)	N/A
GHA (B)	0 (±0)	66% (±33.33)	N/A	0 (±0)	100% (±0)	N/A
3581 (I)	0 (±0)	N/A	N/A	0 (±0)	100% (±N/A)	N/A

*N/A denotes treatments where all replicates showed fungal growth by three days post treatment.

Effects of defensive compounds on fungal spore germination

For both the trans-2-octenal and trans-2-decenal treatments for all three fungal strains tested, spore germination decreased as defensive chemical concentration increased (Figs. 2 and 3). In the trans-2-octenal bioassay, there was a significant interaction effect between fungal strain and chemical concentration ($F_{6,22}$=18.50, P<0.0001) as well as significant main effects of

fungal strain ($F_{2,22}$=53.61, P<0.0001) and chemical concentration ($F_{3,22}$=382.86, P<0.0001) (Fig. 1). Spore germination decreased as trans-2-octenal concentration increased for all three fungal strains, however, the *Isaria* 3581 strain was suppressed more at lower concentrations of trans-2-octenal than the other fungal strains. Spore germination across all fungal strains ranged from 43 to 99% at the 0% concentration (control) and 47 to 99% at the 1% concentration. All three fungal strains showed no spore germination at the 10 and 100% concentrations. In the trans-2-decenal bioassay, there was a significant interaction effect between fungal strain and chemical concentration ($F_{6,22}$=4.03, P<0.0071) as well as a significant main effect of chemical concentration ($F_{3,22}$=25.48, P<0.0001) (Fig. 3) but not fungal strain ($F_{2,22}$=1.62, P<0.2202). The general pattern was that spore germination decreased as trans-2-decenal concentration increased. This pattern was strong for the *Beauvaria* GHA strain, whereas the *Metarhizium* F52 and *Isaria* 3581 strains varied in their response between the 0 and 1% concentration and the 1 and 10% concentration of trans-2-decenal, respectively. Spore germination across all fungal strains ranged from 43 to 99% at the 0% concentration (control), 27 to 92% at the 1% concentration and from 13 to 29% at the 10% concentration. All three fungal strains showed no spore germination at the 100% concentration.

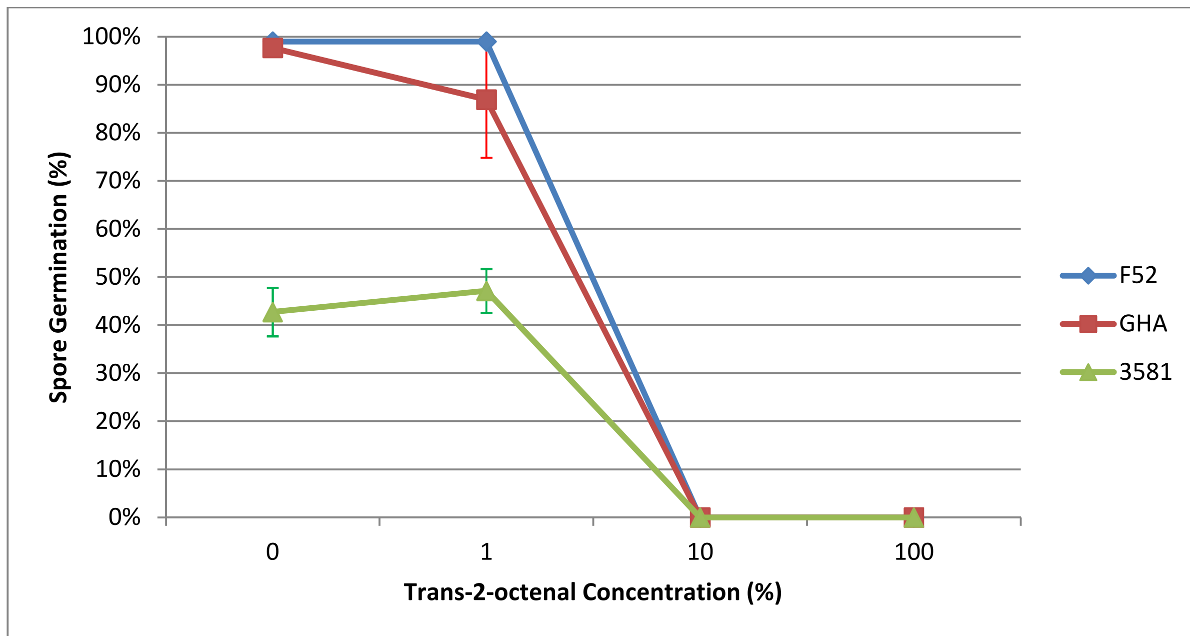

Figure 2: Mean (±SEM) percent spore germination of F52 (M), GHA (B) and 3581 (I) at three concentrations of trans-2-octenal plus a 0% control (water).

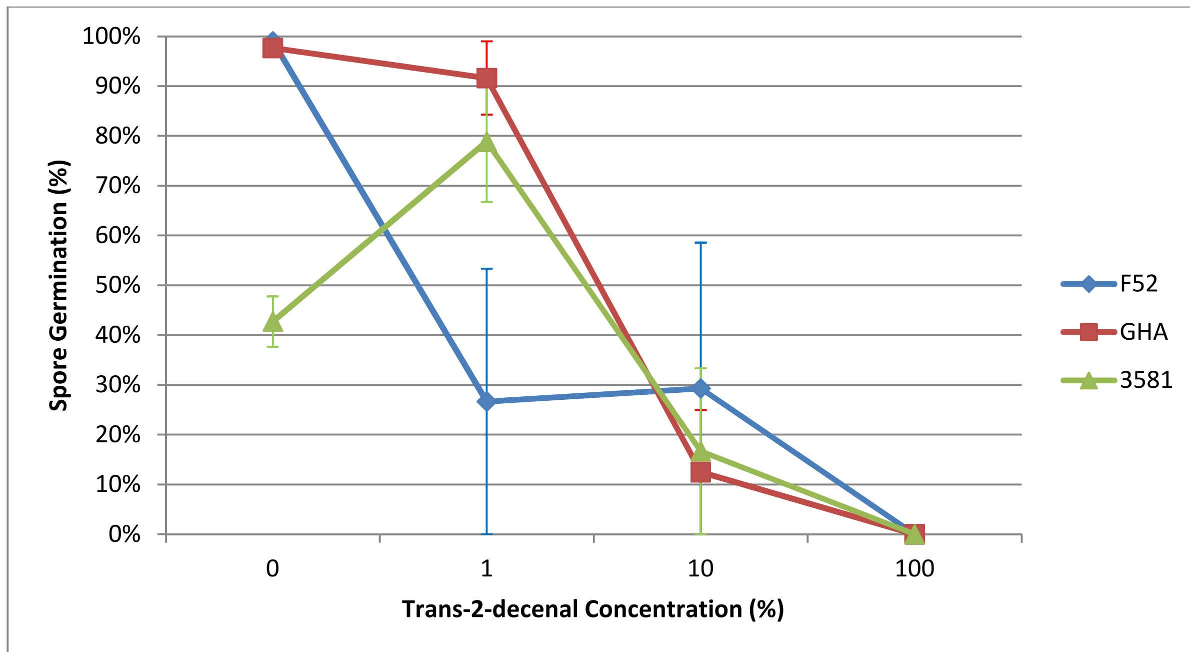

Figure 3: Mean (±SEM) percent spore germination of F52 (M), GHA (B) and 3581 (I) at three concentrations of trans-2-decenal plus a 0% control (water).

Inducing brown marmorated stink bug defense via fungal exposure

Of the 96 insects tested and 480 observations taken over five days, only nine individual brown marmorated stink bugs exhibited a defense response by producing measurable quantities of trans-2-octenal and/or trans-2-decenal. Measurable trans-2-octenal responses ranged from a peak ion count of 84 to 258. Measurable trans-2-decenal responses ranged from a peak ion count of 146 to 6668. Of the nine responding individuals, seven were treated with live fungi and all three live fungal strains were represented by at least one individual stink bug responding.

Table 7: Defense response (chemical type and quantity produced as peak ion counts) from each of the responding individual brown marmorated stink bugs by treatment, gender, and day of response.

Day	Treatment	Gender	Trans-2-octenal response?	Trans-2-octenal Quantity	Trans-2-Decenal Response?	Trans-2-decenal Quantity
2	Untreated	M	Y	129	N	-
2	Live 3581 (I)	F	N	-	Y	2702
3	Deactivated 3581 (I)	F	N	-	Y	4922
3	Live 3581 (I)	M	N	-	Y	1026
4	Live GHA (B)	F	Y	258	Y	833
4	Live GHA (B)	F	N	-	Y	186
4	Live GHA (B)	F	N	-	Y	648
5	Live F52 (M)	F	N	-	Y	146
5	Live GHA (B)	F	Y	84	Y	6668

Discussion

Previous research on the virulence of entomopathogenic fungi on brown marmorated stink bug suggested that brown marmorated stink bug possess some form of defense against fungi (Ihara et al. 2008; Gouli et al. 2012; Pike Chpt. 1 2014). Based on studies that examined similar defenses in other Hemiptera, and more specifically Pentatomidae (Fehlbaum et al. 1996; Sosa-Gomez et al. 1997; Ho and Millar 2001; Marques et al. 2007; Solomon 2013), I predicted

that the mechanism underlying the apparent resistance of brown marmorated stink bug to entomopathogenic fungi was related to its defensive compounds. My experiments demonstrated that stink bug production of the defensive compounds trans-2-octenal and trans-2-decenal is a plausible mechanism towards explaining the generally low virulence of fungi. Mass spectrometry data confirms the presence of these compounds in quantities in individual stink bugs consistent with what was determined to inhibit fungal growth *in vitro*. My experiments also reveal the inhibitory effect of these two compounds on the fungal spores themselves, retarding their growth and even preventing germination entirely as chemical concentrations increase. Although it was not an overwhelmingly convincing result, exposure to entomopathogenic fungi appeared to induce a defense response, secretion of trans-2-octenal and trans-2-decenal, in brown marmorated stink bugs. In all, these data provide a likely mechanism for reduced performance of entomopathogenic fungi, and support the conclusion that fungi will likely not provide biological control of brown marmorated stink bug.

Analysis of whole insect extracts using GC-MS found that brown marmorated stink bugs contain the two defensive compounds of interest, trans-2-octenal and trans-2-decenal, and at concentrations that suggest they could result in a fungistatic effect. While neither compound on its own was present in the same quantities (0.5 µL) demonstrated in the two defense compound bioassays to inhibit fungal growth and sporulation to confidently say that it will inhibit the fungal growth to the same degree, their combined total of approximately 0.2 µL may be enough to elicit similar results. Despite the fact that the stink bugs contain less of the defensive compounds than what was produced in the fungal growth inhibition bioassay, two factors suggest that the performance could still be similar. First in the fungal growth petri dish bioassay, the fungi did not come in direct contact with the defensive compounds. Instead, growth was inhibited by the

presence of volatiles that dispersed within the volume of the petri dish. Thus, the fungi did not come in contact with the full 0.5 uL present in the 10% concentration treatment. Second, I did not bioassay concentrations of the defensive compounds between 0.5 µL and 0.05 µL. It is possible that testing a concentration range between those two values and including the 0.2 µL concentration that stink bugs may respond with the same level of fungal inhibition.

Of particular note here is that while this data shows how much of these compounds are present in a single brown marmorated stink bug, this number may not realistically represent how much is being released in a single agitation event. Currently, there is no published data on the mechanics of the stink bug scent glands that would say whether or not the stink bug is releasing only a portion of its defensive compounds or if it empties the glands in a single instance. Thus, I am unable to say if the entire 0.2 µL is being deployed for defense at once or not.

The fungal growth bioassay demonstrated that both trans-2-octenal and trans-2-decenal inhibited growth and that there were similar levels of growth inhibition between the two compounds. Both showed complete fungal inhibition at the 100% concentration and no fungal inhibition at the 1% concentration. Levels of inhibition varied between fungal strains and between the two chemicals at the 10% concentration. There is very little difference in the mean number of plates with fungal growth after three days, making it difficult to discern whether or not one compound is more effective than the other. However, the fungal recovery results suggest that the trans-2-octenal may be more effective, as a portion of the plates did not show recovery after removal of the compound, whereas all of the plates treated with trans-2-decenal showed recovery. In order to discern whether or not there is a real difference between the two compounds, repeating the experiment with more intermediate concentrations would give greater resolution to determine the point where the two diverge.

The fungal spore germination bioassay demonstrated that both trans-2-octenal and trans-2-decenal inhibited spore germination. When looking at fungal spores that were exposed to trans-2-octenal or trans-2-decenal, the effect that the two chemicals had on spore germination was striking. With trans-2-octenal, there is no spore germination at all at the 100 and 10% concentrations. At 1%, I began to see large amounts of spore germination. However, the germinated spores did not form the dense tangle of hyphae that was seen in the control treatment. A similar pattern can be seen in the trans-2-decenal treatments, though there was some spore germination at the 10% concentration. While F52 (M) and GHA (B) track very closely across treatments, 3581 (I) seems to differ, especially in the control and 1% treatments, in its response. This may be due to the control for the 3581 (I) treatments having lower spore germination than the controls of the other two fungi, rather than a real difference in response to the defensive compounds.

Results of the experiment that attempted to induce a defense response (secretion of trans-2-octenal or trans-2-decanal) via exposure of brown marmorated stink bug to the entomopathogenic fungi was generally inconclusive. With only nine responses (9.4% of insects tested) found over the course of the five day experiment, it is difficult to draw conclusions. However, the fact that the fungi were able to induce a response in even some of the insects was somewhat promising. There were more stink bugs in which a trans-2-decenal response was induced and these tended to be produced in greater quantities compared to trans-2-octenal; trans-2-octenal responses did not exceed 258 ion counts, whereas the highest trans-2-decenal response was 6668 ion counts. There seems to be more responses from females as opposed to males, but without more data to draw from it is difficult to conclude that females more readily release their defensive compound. Most promising is that eight of the nine stink bugs that responded were

treated with some kind of fungal treatment, with seven of those being treated with live fungi. Future studies will focus on a similar experiment that will have more replicates and better test whether exposure to fungus induce a defensive response in stink bugs.

The results seen in these experiments are consistent with studies done on other Pentatomids. Studies on the southern green stink bug demonstrated that trans-2-decenal was a compound in its defensive secretions and that it had fungistatic effects, reducing the germination of spores that adhered to the cuticle by 80-95% (Sosa-Gomez et al. 1997). Similar results in germination rates of spores were seen when I exposed spores to the defensive compounds *in vitro*. This provides further support that defensive compounds produced by stink bugs explain the generally low mortality seen in studies that tested entomopathogenic fungi on brown marmorated stink bug (Ihara et al. 2008; Gouli et al. 2012).

By preventing the fungal spores from germinating, brown marmorated stink bug is able to stop fungal infection at the cuticle or epicuticle layer. Preventing the growth of the fungus before it is able to penetrate the cuticle is critical to the survival of the insect. Investing energy towards preventing fungal infection at the cuticle or epicuticle potentially allows brown marmorated stink bug to avoid the metabolic investment of an internal immune response to fungal infection (Ortiz-Urquiza and Keyhani 2013), though many insects are capable of defending themselves from fungal infection after the fungi have penetrated the cuticle (Kurata 2006). Preventing or delaying fungal penetration of the cuticle is also advantageous to brown marmorated stink bug nymphs, which while perhaps unable to prevent all fungal growth on their cuticle are able to shed their cuticle and thereby escape infection, possibly another contributor to low nymph mortality seen in previous work (Pike Chpt. 1 2014).

Taken in aggregate, these results point towards trans-2-octenal and trans-2-decenal present in the brown marmorated stink bug's defensive secretions as being the mechanism underlying low virulence of fungi and by extension low stink bug mortality. Mass spectrometry work suggests that the amount of these compounds present in a stink bug is comparable to the amount necessary to inhibit fungal growth, as seen in petri dishes of fungi that have been exposed to various concentrations of the compounds. Low incidence of recovery also suggests that a one-time exposure to the defensive compounds is sufficient to stop the germination of fungal spores, potentially indefinitely. While not conclusive, evidence suggests that exposure to entomopathogenic fungi may induce a defense response in brown marmorated stink bug, further increasing its ability to defend itself from fungal infection. Further research will be required to confirm this mechanism of defense as well as potentially devise a way to circumvent it if entomopathogenic fungi are intended to play a role in biological control of brown marmorated stink bug.

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