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Susceptibility of two hymenopteran parasitoids of *Agrilus planipennis* (Coleoptera: Buprestidae) to the entomopathogenic fungus *Beauveria bassiana* (Ascomycota: Hypocreales)

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ABSTRACT

Emerald ash borer (EAB), *Agrilus planipennis* Fairmaire, native to Asia, is killing ash trees (*Fraxinus* spp.) across 15 states and southeastern Canada. Integrated pest management using biological control is the only viable long-term approach for controlling the spread of EAB outside of host resistance. Three hymenopteran parasitoids, *Spathius agrili* Yang, *Tetrastichus planipennisi* Yang, and *Oobius agrili* Zhang and Huang were discovered attacking EAB in China and were approved for release in the United States in 2007. The objective of this study was to assess susceptibility of the larval parasitoid species *S. agrili* and *T. planipennisi*, relative to that of EAB, to *Beauveria bassiana*, an entomopathogenic fungus that infects and kills EAB adults when sprayed on ash bark or foliage. Adult EAB and parasitoids were exposed to *B. bassiana* inoculated ash twigs for 2 h and then monitored daily for death and signs of infection for up to 10 days. All EAB adults exposed to *B. bassiana* were fatally infected while mean survival for control EAB was 77%. Average survival in the treatment groups for *T. planipennisi* and *S. agrili* were 99% and 83%, respectively, indicating these parasitoids are relatively unaffected by exposure to *B. bassiana*. This research elucidates interactions between a fungal pathogen and two parasitoids of EAB, and provides data necessary to developing a successful multi-stage integrated management approach to control of EAB.

1. Introduction

Emerald ash borer,[1] *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is an invasive wood-boring beetle indigenous to northeastern Asia. Discovered in Michigan in 2002 (Haack et al., 2002), EAB is now documented in 15 states and two Canadian provinces (Emerald Ash Borer Information, 2011). EAB feed on ash trees, *Fraxinus* spp., and North American species are more susceptible than those native to Asia, resulting in widespread ash mortality throughout the infested areas in the United States and Canada (Liu et al., 2003; Rebek et al., 2008). EAB adults emerge in late May through July and feed on ash leaves throughout their 6–8 week lifespan (LSB, personal comm.). Two to three weeks after emergence, females begin to oviposit between layers of bark and in bark crevices (Bauer et al., 2004). After egg eclosion, larvae burrow into the phloem, where they feed and develop through four instars (Cappaert et al., 2005). At high densities, larvae consume and deplete the tree of the phloem, thereby disrupting the flow of photosynthates and starving the roots resulting in tree death via girdling.

Results of EAB natural enemy surveys in southeast Lower Michigan in 2002–2003 showed ~2% of larvae were infected with several entomopathogenic fungi including *Beauveria bassiana* (Balsamo) Vuillemin, *Isaria*[2] *farinosus* (Holm ex SF Gray) Brown and Smith, *Isaria fumosoroseus* (Wize) Brown and Smith, *Lecanicillium lecanii* (Zimmerman) Viegas, and *Metarhizium anisopliae* (Metschnikoff) Sorokin (Liu and Bauer, 2006). In subsequent studies, a commercial isolate of *B. bassiana*, strain GHA used in the commercial mycoinsectide Botani-Gard®, was identified as highly virulent against EAB adults when applied as a pre-emergent trunk spray (Liu and Bauer, 2008a,b) and/or when applied post-emergence to bark or foliage (Castrillo et al., 2010a). *B. bassiana* strain GHA has an infection rate of up to 83% in field studies when the mycoinsecticide is applied as a trunk spray prior to adult emergence, with conidial concentrations of 100×10^{13} per hectare (Liu and Bauer, 2008a,b). *B. bassiana* spores

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[1] EAB.

[2] Formerly known as *Paecilomyces*.

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persist on foliage and bark for at least 2 weeks after the initial spray (Castrillo et al., 2010a), making this fungus a viable biological control option where mycoinsecticide applications are feasible.

Three parasitoid species that attack EAB were identified in China, evaluated for possible use as biocontrol agents of EAB, and approved for release in the United States (Federal Register, 2007). Of these, two are larval parasitoids, an ectoparasitoid *Spathius agrili* Yang (Hymenoptera: Braconidae) (Yang et al., 2005) and an endoparasitoid *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae) (Liu et al., 2003; Yang et al., 2006), and one is an egg parasitoid, *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae) (Zhang et al., 2005). In China, *S. agrili* may have up to four generations per year with parasitism rates ranging from 30% to 90% in the field (Yang et al., 2005). EAB larvae parasitized by *S. agrili* can yield up to 18 adult wasps, with an average of 8.4 wasps being reared for every EAB larva. *T. planipennisi* also has at least four generations per year and similar parasitism rates ranging from 32% to 65%, with an average of 35 (range 5–122) adults being reared from a single EAB larva (Liu et al., 2003, 2007; Yang et al., 2006). *O. agrili* were found to parasitize up to 62% of EAB eggs in China, with at least two generations per year (Liu et al., 2007). These parasitoid species were shown to have none to negligible potential impact on closely related non-target insects, and APHIS issued permits for their release in Michigan in July 2007 (Federal Register, 2007; Bauer et al., 2008a; Gould et al., 2009). In 2009, a USDA APHIS facility for rearing and distributing these parasitoids became operational (Bauer et al., 2008b, 2010), and during the 2010 field season, these species were released in eight additional EAB-infested states.

Information is lacking on the susceptibility of these parasitoid species to *B. bassiana*. Entomopathogenic fungi are most often generalists and *B. bassiana* is known to infect several insect families besides Buprestidae (e.g., Castrillo et al., 2008; De la Rosa et al., 2000; Ondiaka et al., 2008). However, individual strains vary in their virulence to EAB (Castrillo et al., 2010b; Liu and Bauer, 2006) and to groups of related hosts (Wraight et al., 2010). Overall, there is little research on the susceptibility of hymenopteran species to *B. bassiana*, and negative effects on the parasitoids will complicate, or prevent, simultaneous use of entomopathogenic fungi with parasitoids as biological control agents.

The purpose of this study was to determine susceptibility of *O. agrili, S. agrili* and *T. planipennisi* to *B. bassiana* in a laboratory setting, although results reported herein refer only to the larval parasitoids, *S. agrili* and *T. planipennisi*.[3] Assessing susceptibility will elucidate if *B. bassiana* can be used in areas that have been designated as release sites for parasitoids. If so, then EAB can be controlled at all life stages; i.e., at the egg and larval life stages with parasitoids, and as adults with *B. bassiana*.

2. Methods

2.1. Preparations for bioassay – ash twigs and holding cups

Ash twigs were cut from a mature *Fraxinus pennsylvanica* to an approximate length of 10 cm, with diameters ranging from ~1 to 2 cm. The surface area of each twig was estimated using the equation for surface area of a cylinder. These estimates were used for dosage estimation, described below.

Insect-holding arenas were 0.47 l plastic cups with lids cut with a 4 cm diameter hole to provide ventilation. Fine plastic screen was glued into place using a hot-glue gun for EAB and *S. agrili*. For *T. planipennisi*, a swatch of fine-mesh synthetic organdy was cut to fit on the cup, and the lid was used to hold the mesh in place.

[3] Bioassays for *O. agrili* yielded inconclusive results due to high control mortality.

2.2. Bioassay procedure

Before initial bioassays and after each experiment, all twigs were washed in a dilute (~0.03%) bleach solution. Twigs were separated into two groups, control and treatment, for all three repetitions of the experiment. Control twigs were hand-agitated in a 0.01% Tween 80 solution for approximately 2 min. and allowed to air-dry. For the experimental group, twigs were washed similarly in a spore suspension made from 1.0 l 0.01% Tween 80 and 2.14 g of dried *B. bassiana* spores (Mycotech technical powder, strain GHA, lot number TAG-I98) and allowed to air-dry. This was calibrated to deliver a dosage comparable to that used by Liu and Bauer (2008a,b).

Bioassays were repeated three times for EAB and *T. planipennisi* and twice for *S. agrili*. There were five replications of each treatment for each insect species for each bioassay. Each treatment arena consisted of a 0.47 l cup with a pair of randomly selected ash twigs, either control or treated, and 5–10 insects.

EAB served as the *B. bassiana*-susceptible insect for these bioassays. A total of 173 EAB adults were tested in the three bioassays; 85 beetles were exposed to Tween 80-dipped twigs and 88 were exposed to fungus-dipped twigs (5–6 beetles per replicate cup, 29–30 beetles in each bioassay).

T. planipennisi were transferred to experimental cups via an aspirator. *S. agrili* were held at 4 °C for 10–15 min. to reduce activity and then transferred using a soft paintbrush. For each bioassay, ~50 adults of each parasitoid species, without regard to sex, were tested (five insects per replicate cup, five replicate cups per treatment, two treatments). After insects were sorted into experimental cups, pairs of twigs, control or inoculated, were added to each cup. All insects were kept in cups with treated twigs for 3 h (first assay) or 2 h (subsequent assays).

After the exposure period, insects were transferred to clean holding cups. EAB were supplied with fresh ash leaves, either greenhouse grown *F. uhdeii* (Wenz) Lingelsh, or field-collected green ash (*F. pennsylvanica*) every 2 days. *T. planipennisi* and *S. agrili* were supplied with honey and moist cotton. All insects were observed daily for up to 10 days. Dead insects were removed and placed in Petri dishes with a semi-selective medium (Sneh, 1991) to culture for presence or absence of *B. bassiana*.

2.3. Determining fungal dosage and contamination

To estimate fungal dosage and control contamination, one twig from each cup was chosen randomly for evaluation. Twigs were placed in a sterile 50 ml plastic screw-cap centrifuge tube and stored at 4 °C overnight. Twenty milliliter of 0.1% Tween 80 solution was added to each tube and the contents were vortexed for 30–60 s. The resulting suspensions were sampled undiluted (controls) or diluted in a 10-fold series (fungus-treated), and aliquots of 200 µl were spread on semi-selective medium. Plates were incubated at room temperature (20–24 °C) for 3–4 days and colonies counted.

2.4. Statistics

All analyses were completed in JMP 8.0 (SAS 2004). For EAB, an estimate of survival time was calculated using Weibull parameters. Matched-pairs analyses were completed for all species.

3. Results

3.1. EAB and parasitoid survival

One hundred percent mortality was observed for EAB exposed to *B. bassiana* inoculated ash twigs 6 days after exposure, Fig. 1.

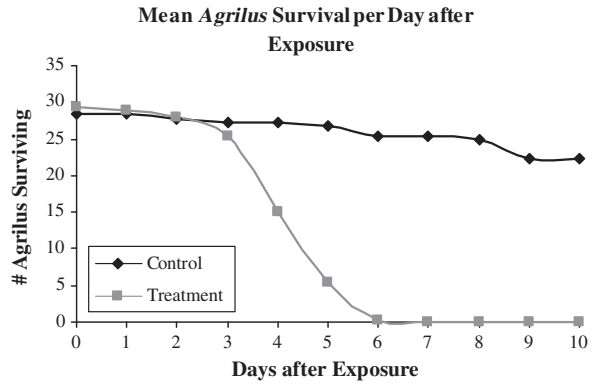

Fig. 1. Mean survival of EAB control beetles versus *B. bassiana* treated beetles over three bioassays with five replications of 29–30 beetles each per bioassay. Day 0 represents day of exposure to *B. bassiana* (treatment) or control.

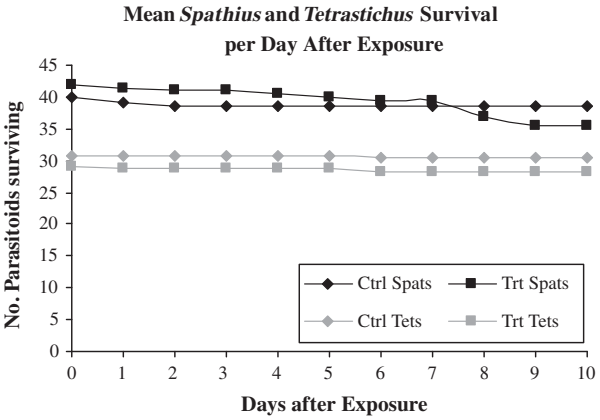

Fig. 2. Mean survival for two bioassays (five replications per bioassay) with *S. agrili* (Spats) and three bioassays (five replications per bioassay) of *T. planipennisi* (Tets). Day 0 represents day of exposure to *B. bassiana* (Trt) or control treatment (Ctrl).

This was significantly higher compared to 23% mortality 10 days after exposure in the control group ($t_{1,14} = -15.03$, $P < 0.0001$). All beetles that died in the treatment groups showed signs of *B. bassiana* infection after culturing, with fungus erupting from intersegmental membranes within 1–3 days after death. Dead beetles from control groups showed no signs of *B. bassiana* infection. The estimate of survival time to 50% mortality (ST_{50}) was 18.0 days for control beetles and 4.1 days for fungus-treated beetles.

Survival rates were very high for *S. agrili* and *T. planipennisi* (Fig. 2). Mean survival 10 days after exposure for *S. agrili* and *T. planipennisi* was 97% and 99%, respectively for control groups, while average survival for treatment groups was 83% and 97%, respectively. Survival of *S. agrili* was only marginally affected by treatment, with significant differences between control and treatment groups ($t_{1,9} = -2.88$, $P = 0.02$). *T. planipennisi* was not affected by treatment over the 10-day observational period ($t_{1,14} = -1.15$, $P = 0.27$).

Fungal infection on *S. agrili* cadavers was relatively high with 77% (10/13) of dead treatment insects showing *B. bassiana* growth. Zero control *S. agrili* showed *B. bassiana* growth. Total *S. agrili* mortality was less than 10% (16/164). Three *T. planipennisi* adults died throughout the study, two from the treatment group and one in the control group, and only one insect, from the treatment group, showed *B. bassiana* growth.

3.2. Fungal dosage and contamination

The overall average estimate of dosage was 2.0×10^7 spores/cm^2 on the twig surface for *B. bassiana* inoculated twigs. Plating of control twig washes revealed that contamination with *B. bassiana* was present in control bioassays in each repetition. However, control *S. agrili*, *T. planipennisi* and EAB insects exposed to contamination showed no difference in mortality and no signs of infection.

4. Discussion

Our results show *T. planipennisi* has very low or no susceptibility to infection by *B. bassiana* under our laboratory conditions. *S. agrili* appears to be slightly susceptible to *B. bassiana*, with an average difference of 13% more mortality in treatment groups compared to control groups. Our methods resulted in very high infection rates of EAB, resulting in 0% survival and 100% infection rates in treatment groups.

The fungal exposure method used was designed to mimic field exposure of insects to treated tree surfaces. The resulting dosage estimate approximates that used by Liu and Bauer (2008a,b) and that recommended for the commercial product Botanigard®. The high conidial concentrations obtained on twigs should have readily infected any insect that was susceptible. All 88 EAB exposed to the fungus-inoculated ash twigs died within 6 days. In contrast, our results indicate that *T. planipennisi* is not susceptible to *B. bassiana* as only two treatment insects died and only one of these exhibited signs of fungal infection. In total, only 3.4% of *T. planipennisi* died out of the 87 exposed. Our results indicate that *S. agrili* is more susceptible to *B. bassiana* than *T. planipennisi*, but significance may not be of substantial biological consequence in this case as only 13 *S. agrili* died out of the 84 exposed (15.5%), much less than EAB, which exhibited 100% mortality.

Laboratory tests on the egg parasitoid, *O. agrili*, were also conducted in this experiment; however, this parasitoid is extremely sensitive to environmental conditions, e.g., heat and sunlight. This resulted in low survival among control *O. agrili* (all insects died within 5 days) while 10% of treatment *O. agrili* survived. Further studies need to be done to determine susceptibility of *O. agrili* to *B. bassiana.*

Plated Tween 80 washes of the treatment and control twigs used for all bioassays did show that contamination in control bioassays by *B. bassiana* did occur. Control contamination was likely due to the fact that the technical powder used to make the fungal suspension is very dry and spores can easily become airborne. Contamination frequency did decline with the successive repetitions of the bioassay, likely due to increased laboratory sanitation as methods were perfected.

Field studies should be conducted in areas where parasitoids have already been released or in areas where parasitoid releases are being planned to further confirm that parasitoids are not susceptible to *B. bassiana*. Environmental factors were held relatively constant in these bioassays and some, such as humidity, temperature, and sunlight, which would vary highly in field conditions, can affect survival and virulence of *B. bassiana* (Shipp et al., 2003; Iskandarov et al., 2006; Bugeme et al., 2009; Castrillo et al., 2010a) and thereby influence not only efficacy to EAB, but possibly the susceptibility of parasitoids.

The necessity to control EAB at all stages of life is vital for a successful biological control program. The potential of using both parasitoids and entomopathogens to slow the spread of EAB is promising and should be pursued. Our results indicate that application of *B. bassiana* during times of adult emergence or activity may be an appropriate companion treatment along with *S. agrili* and *T. planipennisi* parasitoid releases.

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