Assessing deposition and persistence of *Beauveria bassiana* GHA (Ascomycota: Hypocreales) applied for control of the emerald ash borer, *Agrilus planipennis* (Coleoptera: Buprestidae), in a commercial tree nursery

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**A B S T R A C T**

Determining the deposition and field persistence of mycoinsecticides is essential in the development of effective and economical application strategies, including specifically the timing and frequency of spray applications. In this study we used three methods to evaluate the persistence of *Beauveria bassiana* strain GHA applied for control of the emerald ash borer (EAB), *Agrilus planipennis*, an invasive pest attacking ash trees, *Fraxinus* spp., in North America. Fungal inocula present on ash bark and leaves, collected at ≤1, 7 and 14 days after spraying, were quantified by use of molecular (real-time PCR assay) and culture-based methods (semi-selective wheat germ dodine agar). We also assayed fungus-sprayed leaves and bark against adult beetles to determine whether the level of inocula persisting in the field was sufficient to affect beetle survival. Our data quantified deposition of *B. bassiana* and documented fungal persistence. We observed significant decline in recovery of colony-forming units of *B. bassiana* within 1 week of application. The decline was more pronounced on leaves than on bark, and was also evident in loss of virulence of treated substrates to adult EAB with increasing time after application. However, sufficient inocula persisted on bark and leaves for 7–14 days to cause 40–57% and 17–38% mortality, respectively, making pre-emergent sprays a practical means to target adults during emergence, pre-oviposition feeding, or oviposition.

1. Introduction

The emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is an invasive pest first discovered in 2002 near Detroit, Michigan. The beetle has now spread to twelve additional states in northeastern and midwestern US as well as to Ontario and Quebec, Canada (www.emeraldashborer.info). Within these areas, more than 20 million ash trees (*Fraxinus* spp.) have been killed by the EAB (Poland, 2007). Potential economic damage may exceed $10 billion in a 25-state study area within the next 10 years (Kovacs et al., 2010). Adult beetles feed on foliage but the key damage is inflicted by larvae feeding on the phloem of ash trees; this disrupts the tree’s ability to transport water and nutrients. Beetles have a 1- or 2-year life cycle completed entirely in association with ash trees.

Current measures used against EAB include quarantines on the movement of ash trees or firewood, removal of ash trees within infested areas and application of systemic insecticides to selected trees. Alternative biological control strategies being evaluated against EAB include the use of parasitoids (Bauer et al., 2004, 2005, 2006) and fungal entomopathogens *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales) and *Metarhizium anisopliae* (Metchnikoff) Sorokin (Ascomycota: Hypocreales) (Liu and Bauer, 2006, 2008a,b; Vandenberg et al., 2008). These biological and microbial control agents provide environmentally friendly options for beetle management, particularly in ecologically sensitive areas. Initial studies of *B. bassiana* have shown promise: field applications of a *B. bassiana* strain GHA-based mycoinsecticide resulted in 42% less crown dieback and a 63% reduction in EAB emergence in treated versus untreated ash trees (Liu and Bauer, 2008a). Trials in greenhouse cages also showed that fungal treatments prior to EAB emergence significantly reduced longevity in both female and male adults (Liu and Bauer, 2008b). Furthermore, we have recovered viable *B. bassiana* strain GHA from the bark of ash trees sampled more than a year after application; these propagules may...
provide inoculum for possible infection of emerging beetles in spring (unpublished data).

In 2006 we began a multi-year study near Stockbridge, Michigan, evaluating the efficacy of field applications of \textit{B. bassiana} strain GHA \textit{(the active ingredient in Botanigard ES\textsuperscript{®}),} alone or in combinations with imidacloprid-based insecticide, against EAB in a commercial tree nursery. In this paper we assess the deposition and persistence of \textit{B. bassiana} applied to ash trees. We compare molecular versus cultural methods for quantifying viable fungus from field-collected leaflet and bark samples and we correlate the results with those obtained from assays of beetle adults confined on field-treated leaves and bark.

2. Materials and methods

2.1. \textit{Beauveria bassiana} strain GHA field application

A block of 218 mixed 5- to 7-year-old green, \textit{Fraxinus pennsylvanica} Marsh. (Lamiales: Oleaceae), and white, \textit{Fraxinus americana} Linnaeus, ash within a commercial tree nursery near Stockbridge, Michigan, was used for this study. These and other trees at this site were used by us for related EAB studies. A randomized complete-block design was used for each ash species and each of the treatments. For this study, we sampled only trees from blocks treated with foliar sprays of \textit{B. bassiana} strain GHA (Botanigard ES\textsuperscript{®}) or with sprays of the formulation’s carrier (without fungus). The site initially had a light infestation of EAB and had no history of mycoinsecticide or fungicide treatment. Treatments were applied three times at 2-week intervals from early June to July of each year from 2006 to 2009. The first application coincided with initial emergence of adult beetles. Spray treatments were applied using two methods to ensure coverage of each tree. Boles were sprayed to a height of 2 m with a wand fitted with two nozzles (TX VS6) mounted at 90° to a backpack sprayer (R&D, Belspray Inc., Opelousas, LA) with CO\textsubscript{2} propellant. The sprayer was calibrated to deliver 30 ml at 30 psi during two spray passes on each tree bole. The crown and upper branches were sprayed with the same mixture using a 60-gallon (227-l) skid mounted hydraulic sprayer (Model SK-61-4R, FIMCO, North Sioux City, SD), with a handgun nozzle (5500 adjustable conejet nozzle, Spraying Systems, Carol Stream, IL) with 1.8 mm opening, calibrated to deliver 1 l in approximately 10 s at 100 psi. Sprays were timed and calibrated so that each tree received an estimated $2 \times 10^{11}$ conidia of \textit{B. bassiana}.

2.2. Sampling schedule and sample collection

Leaf, bark and soil samples were collected prior to the first spray application in June 2006 to sample indigenous \textit{B. bassiana} populations in the test site and to verify the absence of GHA. Identification of isolates collected only from soil revealed three common indigenous genotypes (Castrillo et al., unpublished data) and the absence of GHA (Castrillo et al., 2008). The absence of any indigenous \textit{B. bassiana} on ash trees allowed the use of colony forming unit (CFU) counts to quantify GHA present on leaves and bark.

After each spray application leaf and bark samples were collected in 2006. The entire upper or lower surfaces of three leaflets for washing were processed in the field upon collection as described below.

Bark samples (3.6 cm$^2$) were collected from the north side of the same trees sampled for foliage, at about 1.2–1.4 m from the ground, using a wood chisel (1.9 cm). The chisel was disinfected with 10\% bleach solution between samples. Each sample was transferred to a 50-ml sterile conical polypropylene tube and stored in a shaded cooler until processing later the same day. Six leaflets selected for pressing directly onto culture medium were processed in the field upon collection as described below.

Leaflet and bark samples were vortexed in 20 or 10 ml of 0.2\% aqueous Tween 80, respectively, two to three 10-fold dilutions were prepared and 200-μl aliquots of each dilution were plated on a semi-selective medium: wheat germ dodine agar (WGDA; Sneh, 1991). Three replicate plates were inoculated for each wash and subsequent dilution. Plates were sealed with parafilm strips and incubated at room temperature ($24 \pm 2 ^\circ C$). CFU were counted after 5–7 days and corrected for wash volume and dilution factor prior to analysis.

To examine fungus deposition on upper versus lower leaflet surfaces, six additional leaflets from the same sampled trees were collected in 2006. The entire upper or lower surfaces of three leaflets each were gently pressed directly onto WGDA plates to transfer fungal deposits onto the medium. The leaflet was pressed again onto a second plate and the two counts added for each sample. Care was taken not to move the leaflet during pressing to avoid smearing, which would prevent quantification of distinct CFU. The plates were sealed with parafilm strips, stored in a cooler for transport and incubated at room temperature ($24 \pm 2 ^\circ C$) for 5–7 days, when CFUs were counted.

2.3. \textit{GHA} quantification: culture on selective medium

Leaflet and bark samples were vortexed in 20 or 10 ml of 0.2\% aqueous Tween 80, respectively, two to three 10-fold dilutions were prepared and 200-μl aliquots of each dilution were plated on a semi-selective medium: wheat germ dodine agar (WGDA; Sneh, 1991). Three replicate plates were inoculated for each wash and subsequent dilution. Plates were sealed with parafilm strips and incubated at room temperature ($24 \pm 2 ^\circ C$). CFU were counted after 5–7 days and corrected for wash volume and dilution factor prior to analysis.

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2.4. \textit{GHA} quantification: real-time PCR

A real-time PCR assay developed for quantifying \textit{B. bassiana} GHA conidia (Castrillo et al., 2008) was used in the third year of study. Three 1-ml aliquots of stock washings from treated leaf and bark samples (same ones plated on WGDA) were spun for each treatment in 2007, 2008 and 2009. Tree numbers were randomized, omitting trees in each treatment to minimize contamination in control samples from spray drift. In addition, control trees were sampled prior to collecting fungus-treated leaves to minimize cross-contamination among samples. Leaves were clipped from the upper third canopy of the north side of each tree, avoiding terminal branches, terminal leaves and terminal leaflets from each leaf. Leaflets of approximately equal size were chosen throughout. The mean surface area of sampled leaflets was 49.8 ± 13.4 cm$^2$ ($n = 50$). In 2006 we clipped two or three leaves per tree and sampled three leaflets per leaf for the samples collected <1, 7 and 14 days after each spray and 28 days after the third spray. Three leaves were collected to compare two techniques, leaf washing versus leaf pressing, of sampling the fungus using selective medium on culture plates. Initial data on CFU counts from leaf pressing <1 day after the first spray revealed high CFU numbers too indistinct to count for comparison with leaf washing. Consequently, only 7- and 14-day samples were collected for leaf presses and direct comparisons with leaf washes were made only for leaflets sampled 14 days after each spray. In 2007–2008, only one leaf with three leaflets was sampled per tree for washing. Each of three leaflets for washing was transferred to a 50-ml sterile conical polypropylene tube and stored in a shaded cooler until processing later the same day. Six leaflets selected for pressing directly onto culture medium were processed in the field upon collection as described below.
leaves from greenhouse-grown adults were held in groups of 10 in plastic containers and fed fresh infested logs, were used for the bioassays. The infested logs were 2.5. Assays against EAB adults: fungus on foliage

Three to 4-day old adults, collected daily upon emergence from infested logs, were used for the bioassays. The infested logs were collected the previous winter from ash trees in woodlots near Lansing, Michigan, and stored at 4 °C prior to incubation at room temperature for emergence (Liu and Bauer, 2006). Newly emerged adults were held in groups of 10 in plastic containers and fed fresh leaves from greenhouse-grown Fraxinus uhdei (Wenzig) Lingelsh every 2 days until bioassay (Liu and Bauer, 2006). For bioassays, beetles from different cohorts were randomly mixed before release into assay cages.

Green ash leaves were collected <1, 7 and 14 days post-spray from trees treated with fungus or carrier only as described above. For each treatment three leaves from each of three trees, each with three to five leaflets, were placed inside a total of nine assay cages (11 × 11 × 3.5 cm polystyrene box [Dadant & Sons, Hamilton, IL]). Each cage had two 0.5 cm holes in the lid for ventilation and a 12 cm hole on the bottom side to which a 10-ml floral water pik (Floral Supply Inc., Fruit Heights, UT) was glued at a slight angle for holding water and leaf petiole. Five EAB adults were released into each cage and allowed to feed on the field-collected foliage (n = 45 beetles for each leaf sampling time). After 1 or 24 h, beetles were transferred to clean dishes containing untreated F. uhdei leaves. Dishes were held at room temperature (24 ± 3 °C) at 15:9 h L:D. Dishes were examined daily for 7 days and dead beetles removed and transferred to WGDA plates for sporulation. F. uhdei leaves were replaced every 2–3 days. The entire assay was done twice for the 1-h exposure (once each in 2007 and 2008) and three times for the 24-h exposure (2007–2009).

2.5. Assays against EAB adults: fungus on foliage

To quantify acquisition of B. bassiana conidia by individual beetles, EAB were confined on leaves or bark, as described above, collected at <1, 7 or 14 days after spray. Five to 12 beetles were used for each treatment combination. After removal from treated substrates, each beetle was washed in 5 ml of 0.02% aqueous Tween solution and 200-μl aliquots plated on three WGDA plates. Plates were incubated, and CFU counted, as described above.

2.6. Assay against EAB adults: fungus on bark

Five or six adult EAB were confined for 4 h in a 5.5 cm diameter circular aluminum screen cage attached at a height of approximately 1.2 m to the north side of a green ash tree. The screening was held 0.5 cm away from the bark surface by means of high-density foam surrounding the cage area and attached to the screen. Thus, confined beetles were in contact with the bark and could move freely within the cages. They were confined in trunk cages at 1, 7 or 14 days after sprays. After removal from the screen cages, beetles were transferred to polystyrene cages, as described above, provisioned with fresh F. uhdei leaves. Beetles were incubated, examined and diagnosed as described above. There were three trees per treatment and two screen cages per tree. The entire assay was done twice, once each in 2007 and 2008.

2.7. Conidial acquisition by EAB

To quantify acquisition of B. bassiana conidia by individual beetles, EAB were confined on leaves or bark, as described above, collected at <1, 7 or 14 days after spray. Five to 12 beetles were used for each treatment combination. After removal from treated substrates, each beetle was washed in 5 ml of 0.02% aqueous Tween solution and 200-μl aliquots plated on three WGDA plates. Plates were incubated, and CFU counted, as described above.

2.8. Statistical analyses

Analysis of variance was done on log10-transformed CFU estimates from leaflet pressings, leaf and bark washings, and washings of beetles confined on one of those two substrates. Factors analyzed for leaflets and bark included, as appropriate, year, ash species, sequential spray number and days after fungus spray. For analysis of beetle washings included sequential spray number, days after fungus spray, and exposure time. Following exposure of adult beetles to field-collected treated leaves or bark, analysis of variance was done on arcsine square root-transformed proportionate mortality after 6 days. Factors analyzed included the year of the assay and the time since the last fungus spray. Beetle mortality was corrected for mortality among controls using Abbott (1925). All analyses were done using JMP software (JMP Software, 2007).

3. Results

3.1. GHA deposition and persistence on leaves

Estimates of B. bassiana CFUs obtained by pressing upper or lower leaflet surfaces on selective medium varied by sequential spray number (F = 8.0; df = 2, 210; P < 0.0005), the time of sampling after sprays (F = 81.9; df = 1, 210; P < 0.0001) and their interaction (F = 24.9; df = 2, 210; P < 0.0001) but not by ash species (green vs. white, F = 0.01; df = 1, 208; P < 0.92) or leaf surface (upper vs. lower, F = 0.04; df = 1, 208; P < 0.84) (Table 1). CFU estimates were higher 7 (vs. 14) days after fungus application for leaflets sampled after the first and second spray but not the third. Samples taken 28 days after the third spray still showed viable CFUs (2.4 ± 3.5 on upper leaflet and 6.8 ± 6.4 on lower leaflet; n = 18). No estimates were obtained from leaflets pressed onto medium 30 min after each spray application because these samples produced CFUs that were too numerous and were indistinct for counting. From the control trees, 65 of 216 leaflets tested positive for B. bassiana for all sample times combined, with an average of less than one CFU per leaflet (range 0–22).

Estimate of CFU obtained by washing leaflets collected in 2006 varied by sampling time (F = 17.3; df = 3, 68; P < 0.0001) but not by ash species (F = 2.4; df = 1, 67; P > 0.2) and spray number (F = 0.7; df = 2, 67; P < 0.6). Comparison of CFU counts from 14-day leaflets using the two sampling methods, leaf presses vs leaf washes, showed the latter with higher whole leaf total CFU than those from leaf presses (Table 1).

CFU estimates from washing leaflet samples from 2007 and 2008 varied with the sequential spray number (F = 28.3; df = 1, 84; P < 0.0001), with time of collection after fungus application (F = 99.8; df = 2, 84; P < 0.0001), and with their interaction (F = 17.1; df = 2, 84; P < 0.0001). CFU estimates for samples taken <1 day after either spray were significantly higher than for the other times, and samples taken 14 days after the first spray were significantly lower (Fig. 1a; Tukey’s HSD, P < 0.05). No CFU were observed from washings of leaflets from control trees sampled at three time points after each of the first and second spray applications in each of the 2 years.

CFU estimates from beetles washed after exposure to field-inoculated ash foliage did not vary by exposure time (1 or 24 h; F = 1.15; df = 1, 23; P > 0.3) but did vary with the time the foliage was collected post-spray (F = 23.4; df = 2, 23; P < 0.0001). Mean log10 CFU estimates were 2.83 ± 0.25 (n = 15), 0.71 ± 0.24 (n = 9) and 0.24 ± 0.18 (n = 9) for ash leaves collected 1, 7 and 14 days post-spray, respectively, and were significantly higher for leaves collected at 1 day post-spray (Tukey’s HSD, P < 0.05). Among 60 beetles exposed to control leaves, two had one CFU each.
Table 1
Comparison of Beauveria bassiana colony-forming units (CFU) obtained from green and white ash leaflets using the leaf press versus leaf wash method in 2006.

<table>
<thead>
<tr>
<th>Sequential spray</th>
<th>Days post-spray</th>
<th>Leaflet press (log_{10} CFU ± SE)</th>
<th>Leaflet wash (log_{10} CFU ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower</td>
<td>Upper</td>
</tr>
<tr>
<td>1</td>
<td>1 &lt;1</td>
<td>1.7 ± 0.1</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.9 ± 0.2</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>2.7 ± 0.1</td>
<td>2.2 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>0.8 ± 0.1</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>1.6 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>1.4 ± 0.1</td>
<td>1.8 ± 0.1</td>
</tr>
</tbody>
</table>

1 Sprays occurred at 14-day intervals. For leaflet pressing the effects of ash species (green or white; \( P < 0.6 \)) and leaf surface (upper or lower; \( P > 0.8 \)) were not significant. For leaflet washes, the effect of ash species was also not significant (\( P < 0.6 \)). See text for ANOVA results.

Mean CFU estimates (log_{10}) were 4.96 ± 0.09 (\( n = 24 \)), 3.77 ± 0.18 (\( n = 18 \)) and 4.13 ± 0.09 (\( n = 22 \)) for bark samples 1.7 and 14 days after application, respectively. CFU estimates for samples taken 1 day post-spray were significantly higher than for the other times (Tukey’s HSD, \( P < 0.05 \)). Samples taken prior to the first sprays in 2007 and 2008 showed no CFU on white ash, indicating that GHA did not persist in these trees over the winter months. In contrast, GHA was still found on green ash, with an average CFU of 99.7 ± 115.5/bark sample and 16.7 ± 28.9/bark sample for 2007 and 2008, respectively.

CFU estimates from washed, individual beetles confined on fungus-sprayed bark varied with the time from fungus application (\( F = 4.5; \ df = 2, 54; \ P < 0.016 \)) but not with sequential spray number (\( F = 1.2; \ df = 2, 52; \ P < 0.3 \)). Mean CFU estimates (log_{10}) were 1.2 ± 0.2 (\( n = 35 \)), 1.6 ± 0.2 (\( n = 16 \)) and 0.3 ± 0.2 (\( n = 6 \)) for beetles exposed 4 h on treated bark 1, 7 and 14 days post-spray, respectively, and were significantly higher for samples taken at 1 and 7 days post-spray (Tukey’s HSD, \( P < 0.05 \)). We detected no CFUs from 31 beetles exposed to control bark samples.

3.3. GHA quantification using real-time PCR assay

Comparable estimates of GHA propagules were obtained from green ash bark sampled a day after spray application based on real-time assays and the culture plate method (Table 2). After 7 and 14 days estimates were 19- and 2 and a half-fold higher, respectively, for real-time PCR assays. On leaves, estimates were higher using the plating method for samples taken 30 min after the first and second spray applications, but were higher from real-time assays for subsequent samples taken, except for 14 days after the second spray. Numbers obtained using both methods showed a trend of declining GHA propagules after each spray application, except for an increase observed from 7 to 14 days for bark samples. This increase, however, did not correlate to higher mortality values among beetles exposed to bark sprayed 14 days versus 7 days. Mortality among beetles exposed to bark and leaves collected from 1- to 14-days post-spray showed a continuous decline (Table 2).

3.4. Beetle mortality after exposure to treated leaves and bark

Beetle mortality within 6 days after a 24-h exposure to field-collected ash foliage varied with foliage collection time post-spray...
Ash bark also varied with time post-spray (F(2,75) = 19.8; df = 2, 75; P < 0.0001) but not by year (F = 0.5; df = 2, 73; P < 0.6). After a 1-h exposure to fungus-sprayed leaves, beetle mortality varied with time of foliage collection post-spray (t = −4.7; df = 58; P < 0.0001). For both of these tests, mortality was significantly higher among beetles exposed to foliage collected immediately after spray (Table 3). Control mortality averaged 13% (SE = 0.9) for 3 yearly assays of 24-h exposure and 24% (SE = 8) for two assays of 1-h exposure.

Beetle mortality within 6 days after a 4-h exposure to treated ash bark also varied with time post-spray (F = 19.8; df = 2, 75; P < 0.0001) but not by year (F = 1.7; df = 1, 75; P < 0.2). Mortality was significantly higher when beetles were confined on bark 1 day after spray (Table 3). Control mortality averaged 24% (SE = 6) for 2 yearly assays; this relatively high level is probably due to the additional beetle handling necessary for these assays.

### 4. Discussion

Determining field persistence of mycoinsecticides is essential in the development of effective and economical application strategies, including specifically the timing and frequency of spray applications. To control the invasive EAB, the fungus B. bassiana GHA needs to be applied to target the emerging adults in early summer, during the maturation feeding period and before females lay eggs, to maximize impact on beetle populations. Emerging adults could come in contact with conidia sprayed on ash trunks as they emerge or while walking or ovipositing and on foliage during feeding (Liu and Bauer, 2006, 2008a,b). Thus, applications timed to coincide with beetle emergence and persistence of sufficient viable fungal propagules could result in an effective and economical control strategy. In this study we first tested two sampling methods to estimate deposition and persistence on ash leaves (Table 1). Leaf presses could provide estimates of both and allow comparison of CFU numbers on the upper versus the lower leaf surface. These data would be important since EAB normally land on the upper surface for feeding. Conidia on this surface, however, may be more exposed to UV light, with consequent reduction in viability (Inglis et al., 1993; Moore et al., 1993; Braga et al., 2002), and may be more likely to be washed off by rainfall (James et al., 1995). CFU estimates, based on our leaf pressing, of initial deposits from leaflets collected after spraying, however, were not possible to obtain because resulting colonies were too numerous to count accurately. Subsequent leaf pressing of samples collected 7 or 14 days after spray revealed lower numbers with no significant differences between upper and lower leaf surfaces at either time point. The lack of difference by 7 days post-spray may be due to greater initial deposition on upper leaf surfaces and a subsequent, equalizing decline due to UV exposure. While it may be expected for conidia on the lower surface to be better protected from UV rays, the dense and overlapping canopy in these ash trees could have provided shading to minimize UV damage to conidia on the upper leaf surface. During these sampling years, the level of EAB infestation was still relatively low and canopy decline minimal (unpublished data). The persistence of conidia on either surface for at least 14 days after application shows that pre-emergence sprays may provide sufficient inocula to infect adults as they feed. Tefera and Pringle (2003) found that feeding and movement on treated leaves during the course of feeding were the most important means of acquiring conidia by Chilo partellus Swinhoe (Lepidoptera: Pyralidae).

We obtained higher CFU estimates from leaflet washing than pressing. The vortexing of leaflets in aqueous surfactant likely facilitated conidial dislodging from leaflet surfaces; sample dilution, with further vortexing, breaks up conidial clumps prior to plating. This method also provided an estimate of persistence over our entire study period. We found no significant difference in CFU counts from leaflets collected <1 day after each spray in 2007 and 2008, and examination of the <1 day sample after the first spray in 2006 also showed comparable numbers. Comparable values

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### Table 2

Comparison of 2008 Beauveria bassiana propagules on green ash leaves and bark detected by culture on semi-selective medium, real-time PCR assays, and bioassay against Agrilus planipennis adults.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sequential spray&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Days post-spray&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Culture method&lt;sup&gt;c&lt;/sup&gt; (CFU ± SE)</th>
<th>Real-time PCR&lt;sup&gt;d&lt;/sup&gt; (conidia ± SE)</th>
<th>Beetle assay&lt;sup&gt;e&lt;/sup&gt; (% mortality ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bark</td>
<td>1</td>
<td>1</td>
<td>5.56 ± 1.29 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>6.29 ± 3.33 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>70 ± 6</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1.26 ± 0.35 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2.45 ± 1.39 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>38 ± 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>13.93 ± 4.69 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>3.46 ± 4.52 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>17 ± 6</td>
<td></td>
</tr>
<tr>
<td>Leaf</td>
<td>1</td>
<td>&lt;1</td>
<td>6.85 ± 0.06 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.42 ± 0.49 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>100 ± 0</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2.7 ± 0.4 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>5.19 ± 1.99 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>33 ± 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>8.17 ± 5.6 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>5.30 ± 1.36 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>7 ± 6</td>
<td></td>
</tr>
</tbody>
</table>

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<sup>a</sup> Sprays occurred at 14-day intervals. 
<sup>b</sup> Bark samples were collected 1, 7 and 14 days after spray application; leaf samples were collected 30 min, 7 and 14 days after spray applications. 
<sup>c</sup> Bark and leaf washings were plated on wheat germ dodine agar plates (three plates/dilution) and colony-forming units (CFU) counted after 5–7 days. Aliquots of the bark and leaf washings were used for DNA extraction and were analyzed using a real-time PCR assay specific for B. bassiana GHA. The quantity of DNA detected in each sample was converted to estimates of conidia numbers by correlating a 6 series 10-fold dilution of B. bassiana GHA10<sup>7</sup> conidia and its DNA yield (Castrillo et al., 2008). 
<sup>d</sup> Adult A. planipennis were exposed to treated bark for 4 h in the field or to leaf samples for 24 h in laboratory assays. Leaf samples were collected at the same time as for washing or PCR assays. Percent corrected mortality 6 days after exposure (n = 60 beetles/treatment for bark; n = 45 beetles/treatment for leaves). SE = standard error. ND = bioassays were not done for leaf samples from the second spray application.

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### Table 3

Adult emerald ash borer mortality, corrected for control mortality, 6 days after exposure to leaf or bark samples collected from green ash at three intervals after application of Beauveria bassiana GHA.

<table>
<thead>
<tr>
<th>Days post-spray</th>
<th>Substrate</th>
<th>Exposure time (h)&lt;sup&gt;i&lt;/sup&gt;</th>
<th>% Mortality ± SE&lt;sup&gt;j&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1</td>
<td>Leaves</td>
<td>24</td>
<td>78 ± 7a</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>38 ± 7b</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>17 ± 4b</td>
<td></td>
</tr>
<tr>
<td>&lt;1</td>
<td>Leaves</td>
<td>1</td>
<td>50 ± 9a</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>11 ± 9b</td>
<td></td>
</tr>
<tr>
<td>&lt;1</td>
<td>Bark</td>
<td>4</td>
<td>83 ± 5a</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>57 ± 7b</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>40 ± 6b</td>
<td></td>
</tr>
</tbody>
</table>

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<sup>i</sup> Twenty-four h leaf assay was done three times from 2007 to 2009; other assays were done twice from 2007 to 2008.

<sup>j</sup> Analyses done on angular-transformed proportion mortality, corrected for controls. n = 120–140 beetles per treatment. SE = standard error. Means within each substrate and exposure time followed by the same letter are not significantly different (Tukey’s HSD or t-test, P < 0.05).
observed in 2007 and in 2008 indicate no significant propagule accumulation on leaflets, with three sprays, over a 6-week period each year.

The number of \textit{B. bassiana} CFU on ash leaves as measured by leaf pressing and by washing showed significant decline from <1 day after each spray to 7 and 14 days afterwards. But even though the level of inoculum was reduced to 2- to 5-fold less, mortality was still observed among beetles exposed to these leaves (Tables 2 and 3). Cases were also observed when CFU estimates remained comparable between 7 and 14 days, indicating that despite exposure to sunlight and rainfall during the summer season, sufficient conidia may remain to cause some beetle mortality. Surprisingly, CFU were still obtained from ash leaves approximately 28 days after the last spray application in 2006. This persistence may be partly attributed to the fungus formulation, an emulsifiable oil suspension, which has been shown to exhibit greater rainfastness than wettable powder-formulated conidia (Wraith and Ramos, 2002; Wraith et al., 2001).

Persistence on bark showed the same trend, with CFU numbers showing significant decline from initial spray applications to 7 or 14 days later. In contrast to leaflets, however, the estimates remained comparable from 7 to 14 days after each spray (Fig. 1). Better persistence on bark may be due to the presence of cracks and crevices on ash bark in which conidia get lodged during high-pressure spray applications. Although we did not find significant differences among CFU estimates on white versus ash bark sampled 1, 7 and 14 days post application, GHA was recovered in green, but not white, ash bark from pre-spray samples collected after winter. Green ash typically has a more complex bark structure than white ash when trees are younger. However, older trees of both species show greater surface complexity that may enhance persistence of \textit{B. bassiana}. We observed persistence of GHA on older green ash trees for over a year since the last application in a separate study (unpublished data).

Data from the real-time PCR assays showed the same declining number of GHA propagules over time after field applications (Table 2). Estimates, however, differed from the culture method because of the type(s) of propagules detected and possible variability from aliquots sampled. The culture method directly samples from the washings while real-time PCR assay requires DNA extraction from aliquots followed by amplification. Any sample volume losses during either of these processes for molecular assays could increase the variability of estimates. In addition, the real-time PCR assay was developed to estimate conidial numbers from the amount of DNA detected, while the culture method measured CFU, which may reflect more than one conidium and could contain hyphal bodies. Estimation from samples collected 7 and 14 days after spray may be further complicated if fungal conidia germinate and multiply. This may explain the increase in CFU counts from 7 to 14 days from bark samples (Fig. 1). Under humid conditions, conidia lodged in bark cracks or splits may germinate producing hyphae and mycelia that increase the number of detectable propagules present. Wraith also observed an increase in \textit{B. bassiana} GHA propagules on potato plants after rainfall (pers. comm.). Although these additional propagules complicate the sampling for persistence, fungal growth improves the likelihood of sufficient inocula being available to target beetle populations during the summer.

Our data showed a positive correlation between estimates of viable fungal propagules and beetle mortality. We observed a significant decline in CFU within 1 week post-spray, correlated with a decline in mortality among beetles confined on leaf and bark samples collected at those times. Persistence of conidia on bark was evident in higher mortality among beetles confined on bark at 7 and 14 days post-spray (Table 3). Currently we are evaluating the impact of three biweekly fungus applications, in concert with other control tactics, on beetle survival in the field and the condition of infested trees.

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References
