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Mortality and reduced brood production in walnut twig beetles, *Pityophthorus juglandis* (Coleoptera: Curculionidae), following exposure to commercial strains of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium brunneum*

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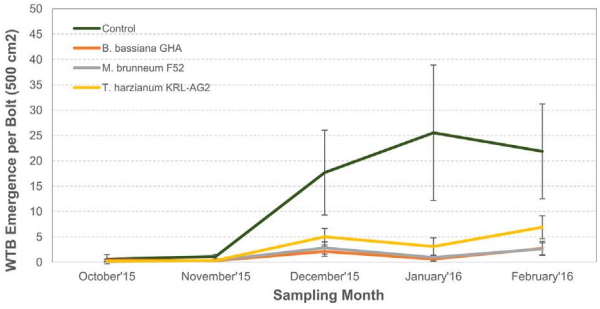
GRAPHICAL ABSTRACT



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ABSTRACT

Thousand cankers disease (TCD), caused by the walnut twig beetle (WTB), *Pityophthorus juglandis*, and its associated fungal symbiont, *Geosmithia morbida*, is a disease of economic and ecological concern on eastern black walnut, *Juglans nigra*. Numerous attacks and gallery formation by the WTB and subsequent development of cankers caused by the fungus result in progressive crown dieback. The disease can kill affected trees in a few to several years following initial infestation and very few management options are available for preventing or reducing impact of TCD on black walnut trees. Since advanced development of TCD requires multiple WTB generations and numerous beetle attacks, control strategies that reduce beetle attacks and brood production, without completely eliminating infestation, could still significantly benefit tree health and survival. We evaluated the use of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium brunneum* against the WTB. Laboratory and field studies showed that WTB adults are susceptible to commercial strains *B. bassiana* GHA and *M. brunneum* F52. Exposure of beetles to sprayed walnut bolts resulted in reduced brood production, primarily due to the death of parental adults from fungal infection prior to egg laying. Spraying walnut bolts with *B. bassiana* GHA and *M. brunneum* F52 prior to field exposure to natural WTB populations reduced emergence of next generation adults by up to 98% and 96%, respectively, due to a combination of fewer beetle attacks and

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mortality among those that tunneled. These results demonstrate the potential use of entomopathogenic fungi in the integrated management of TCD in walnut trees.

1. Introduction

The walnut twig beetle (WTB), *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae), a bark beetle endemic to Mexico and the southwestern continental United States (US) (Bright, 1981; Rugman-Jones et al., 2015), attacks several species of native and cultivated walnut trees, *Juglans* spp., including the economically important black walnut, *J. nigra* L. Walnut twig beetles attack large diameter twigs and branches and the main stems, and feed on the phloem (Graves et al., 2009). Males initiate tree colonization by constructing nuptial chambers in the bark and releasing pheromones to attract females, and a male is usually joined by two or more females (Kirkendall, 1983). Mated females then tunnel and lay eggs individually along the wall of a gallery. Larvae tunnel and feed perpendicular to this gallery, along the phloem. There are three larval instars and the life cycle can be completed within 7 weeks (Dallara et al., 2012; Tisserat et al., 2009), with the potential for two or more overlapping generations per year (Tisserat et al., 2009).

The WTB is associated with the fungus *Geosmithia morbida* Kolařík, Freeland, Utley & Tisserat (Ascomycota: Hypocreales), which is deposited as adult beetles feed on the phloem and in or around the galleries (Cranshaw and Tisserat, 2008; Kolařík et al., 2011). Fungal infection results in the development of cankers, or localized areas of dead bark, cambium and underlying wood, on tree branches (Graves et al., 2009; Seybold et al., 2013). Numerous beetle attacks and the subsequent development of cankers caused by *G. morbida* essentially girdle stems and branches when the cankers coalesce (Seybold et al., 2013; Utley et al., 2013). The disease, called thousand cankers disease (TCD), can lead to tree mortality within a few years of initial detection of symptoms (Cranshaw and Tisserat, 2008; Seybold et al., 2013).

The WTB and its associated pathogen have recently spread from its historic range in New Mexico, California and Arizona to other western states (Cranshaw, 2011; Tisserat et al., 2009; Tisserat et al., 2011). WTB and *G. morbida* were detected in Tennessee in 2010 (Grant et al., 2011) and since then have been found in six other eastern states (Seybold et al., 2012a; Wiggins et al., 2014), where *J. nigra* is important both economically and environmentally (Leslie et al., 2010; Randolph et al., 2013). Moreover, among the nine *Juglans* spp. inoculated with *G. morbida* by Utley et al. (2013), *J. nigra* was observed highly susceptible to canker development, in contrast to *J. major*, which is native to the southwestern US and the original host described for the beetle (Cranshaw and Tisserat, 2008). Recently, TCD was discovered in Europe, in *J. nigra* and *J. regia* trees in northeastern Italy, where both are also grown for lumber (Montecchio and Faccoli, 2014; Montecchio et al., 2014).

Efforts to control the disease and its spread include county- and state-level quarantines and other regulatory restrictions on walnut logs and walnut wood products with bark from infested areas. The movement of beetles and associated *G. morbida* results from human-mediated transport and natural dispersal and colonization of available hosts (Newton and Fowler, 2009; Rugman-Jones et al., 2015). Interstate and intercontinental movement, however, is more likely due to the former, given the beetle's limited flight capacity (Kees et al., 2017). Potential control measures to help prevent spread of WTB and TCD include phytosanitary treatments such as insecticide dips or heat treatments that kill the beetle, fungus, or both in infested log materials (Audley et al., 2016a,b; Mackes et al., 2016; Mayfield et al., 2014).

Another control strategy might involve preventing or suppressing WTB infestations in live host trees. Currently, however, there are limited options to control beetle populations in infested trees, especially for *J. nigra*. Because black walnut trees are sources not only of wood products but also of edible nuts, chemical pesticide application may be restricted by regulations applied to "nut crops" (Cranshaw and Tisserat, 2010). Biological control agents including entomopathogenic fungi may represent a more environmentally acceptable alternative. A preliminary survey of pathogens associated with WTB showed that the fungus *Beauveria bassiana* (Ascomycota: Hypocreales) is a naturally occurring pathogen in WTB populations in Knox County, TN, with fungal infection present in 15% of the beetles examined (Juzwick, unpublished data). Mycoinsecticide applications could augment naturally occurring levels of fungal infections in the field, and may significantly reduce beetle populations when applied under a regime optimized to target peak beetle flights and at an effective rate and spray schedule.

In an effort to evaluate the potential of biological control fungi against WTB, we compared the susceptibility of WTB adults to two commercially available entomopathogenic fungi and assessed the impact of mycoinsecticides based on these fungi on beetle attacks and on beetle gallery establishment and brood production. We also tested the use of a commercially available mycoparasitic fungus, *Trichoderma harzianum* strain KRL-AG2 (Ascomycota: Hypocreales), to examine its potential impact on *G. morbida*.

2. Materials and methods

2.1. Source beetles for laboratory assays

Walnut twig beetles were obtained from naturally occurring populations in *J. nigra* trees in Knox County, TN, in 2014 and 2015. Uninfested logs (7–15 cm diameter) were first collected from a plantation of mature *J. nigra* trees in McDowell County, NC (35.688398 °N, −81.885745 °W), 70 miles west of any known WTB infestations. The logs were cut to 0.6 m lengths and the ends were sealed with melted paraffin wax to minimize drying. The following day the cut logs were baited with a WTB bubble cap lure (Cat. No. 300000968, Contech Enterprises, Victoria, BC) and hung in the crown of mature, WTB-infested *J. nigra* trees in Knox County, TN. Bolts were hung by attaching an eye-screw at one end, tying a rope to the eye screw, shooting the rope over tree branches using a throw line with a sandbag and a slingshot (Jameson LLC, Clover, SC), and then raising the logs into the crown (Mayfield et al., 2014). In 2014, beetles were trapped at four locations: Burkhart Road (36.079571, −83.857823), Emory Road (36.094568, −83.907921), Hinton Drive (35.971804, −83.992083), and Warrior Trail (35.821485, −84.146238), with three to seven logs hung per tree in two to four trees at each site (total trees = 12; total logs = 16). In 2015, all 16 logs were hung in four trees at Warrior Trail. Logs were hung for approximately 7 ½ weeks beginning in early June 2014 and in late June 2015.

Newly infested logs were taken from the field and cut into two 30 cm long bolts for beetle rearing and collection. A screw hook was inserted into one end of each bolt before placing each in a rearing bucket described by Mayfield et al. (2014). The bolt was suspended inside the bucket by fitting the screw hook through an eye bolt mounted in the inside of the bucket lid. The bottom of the bucket was cut out and fitted with a 23-cm diameter 1-liter plastic funnel (LUBEQ Corp., Elgin, IL), leading into a 100 or 250 ml polypropylene straight sided jar with screw cap (Thermo Fisher Scientific Inc., Waltham, MA). Buckets were suspended by their handles on wooden racks and were illuminated continuously by an LED string light source (C7, 120 V warm light bulb, Sival Inc., Santa Clara, CA) beneath each collection jar (Supplementary Fig. 1). Rearing buckets were maintained at room temperature

(approximately 22–24 °C). Emergence of next generation WTB was monitored daily. Live beetles were placed in petri dishes lined with moistened filter paper and held at 10 °C until used in laboratory experiments within 7 days of collection. Prior to assays, beetles were examined under a dissecting microscope at 40 to 60X for identification and sorting by sex (Seybold et al., 2012b).

2.2. Fungal biological control agents

Cultures or spore powders of *B. bassiana* strain GHA and *Metarhizium brunneum* strain F52 (=ARSEF 5198) (Ascomycota: Hypocreales) were obtained from Laverlam International (Butte, MT) and the ARS Entomopathogenic Fungal Culture Collection (ARSEF, Ithaca, NY), respectively. Single spore isolates were established from pure cultures of each strain as described by Castrillo et al. (2004), and frozen stocks are maintained at the USDA-ARS Robert W. Holley Center, Ithaca, NY. Dried conidia were produced for both strains following protocols reported in Castrillo et al. (2008) and were stored at -20 °C until use. Viability of conidia was tested prior to bioassays by preparing suspensions of 10^3 to 10^2 conidia per ml of sterile 0.01% aqueous Tween 80 solution and spreading 200 ul aliquots on plates of ¼-strength Sabouraud dextrose with yeast extract (Difco Manual, 1977). There were three plates per dose per fungus. Culture plates were incubated at 25 °C in darkness for 18 to 24 h and examined under a compound microscope at 400X. The first 100 conidia encountered per plate during a scan were scored for germination. Viability of conidia in test suspensions ranged from 97% to 100%.

2.3. Laboratory bioassays: Fungal virulence to WTB adults

Stock suspensions of 25 mg fungal conidia in 15 ml of aqueous 0.01% Tween 80 with 1 g of 2 mm diameter glass beads (BioSpec Products, Bartlesville, OK) were prepared in 50-ml polypropylene centrifuge tubes and vortexed for 2 to 3 min. Concentrations of the resulting conidial suspensions were estimated using a Neubauer hemocytometer. In 2014, a series of dilutions was prepared for each strain to produce suspensions with an estimated target of 10^5 and 10^6 conidia per ml of 0.01% aqueous Tween 80 solution. WTB adults were dipped individually in 10 ml of conidial suspension for 30 s using a camel hair brush (size 1), with 10–15 females and 13–15 males per dosage per fungus per replicate. There were two replicates conducted on the same day using different beetle cohorts and different fungal suspensions. Dipped beetles were transferred to petri dishes lined with filter paper for 2 min to absorb excess moisture before placing them in individual Alcott shell vials (8 × 35 mm) with a small piece of moistened filter paper (15 mm diam.) lining the lower half of the inside. Tubes were capped with plastic lids punctured for ventilation. Tubes were held upright in polypropylene racks, incubated at 25 °C in darkness, and examined daily for 10 days for beetle mortality. Dead beetles were transferred to a semi-selective medium, wheat germ dodine agar (WGDA; Sneh, 1991), incubated at 25 °C in darkness, and examined for sporulation to verify fungus identity after 5–7 days. For controls, 11–15 females and 14–16 males were individually dipped in 0.01% aqueous Tween 80 solution and were handled as described above.

In 2015, the bioassay experiment was repeated using the same protocol as reported above, but with lower dosages of 10^3 and 10^4 conidia per ml of 0.01% aqueous Tween 80 solution because dosages used in 2014 resulted in very high mortality among treated beetles by day 4 or 5 for both fungi, and survival data were not amenable to computation of LD_{50} values. Results from the 2015 bioassays, however, showed high mortality among control insects, with 12.5 ± 7.2% mortality by day 1 and up to 25 ± 7.2% and 58 ± 4.0% mortality by day 4 and 5, respectively. Although the lower fungal dosages tested resulted in comparatively lower mortality among treated beetles compared to those observed in 2014 (data not shown), the relatively high control mortality prevented any further analysis of the bioassay data.

2.4. Small bolt laboratory assays

The impact of fungal treatment on beetle gallery establishment and brood production was determined by exposing untreated WTB adults to sprayed small walnut bolts (5–10 cm diameter, 13 cm length) that were cut from uninfested walnut trees in McDowell County, NC, and transported to Knox County, TN. The bolt ends were sealed with melted paraffin wax before spraying with either a mycoinsecticide (treatment) or distilled water (control). In 2014, two mycoinsecticides were tested: Botanigard® (a.i. *B. bassiana* strain GHA; Laverlam International) and Met52® (a.i. *M. brunneum* strain F52 [formerly identified as *M. anisopliae,* Bischoff et al., 2009]; Monsanto, St. Louis, MO). Each product was diluted in water (total volume of 600 ml) to produce a suspension of 11.5×10^8 conidia per ml and 7.8×10^8 conidia per ml for *B. bassiana* GHA and *M. brunneum* F52, respectively. A total of 28 small bolts was sprayed per treatment or control, and a small piece of bark (2.5 cm^2) was sampled from four representative bolts per treatment after spraying to quantify conidial deposition. Each peeled bark sample was placed in a 50 ml conical-polypropylene tube, washed with 5 ml of sterile 0.01% aqueous Tween 80 solution, and 200-µl aliquots of stock wash and 1:100 to 1:1000 dilutions spread on WGDA plates. Plates were incubated at 25 °C in the dark and colony forming units counted after 5 to 7 days. There were two replicate plates per dilution per sample. Conidial deposition on sprayed bark was approximately $8.2 \pm 9.9 \times 10^2$ and $3.3 \pm 4.7 \times 10^2$ colony forming units (CFU) per mm^2 for *B. bassiana* GHA and *M. brunneum* F52, respectively. Less than one fungal CFU per mm^2 of control bark was observed.

Each bolt was placed in a plastic container (1.9 L clear, square-grip, polyethylene terephthalate container [18.4 cm × 11.4 cm × 12.1 cm] with a 11 cm diameter circular opening). A 6 cm circular hole was cut in the center of the polypropylene screw-neck lid and covered with 200-µm mesh screen for ventilation. Two adult females and four males were introduced into each container. Assay bolts were incubated at 24 °C in the dark for 4 weeks. Afterwards, bolts were stored at 5 °C until processing. A longitudinal cut on the bark of each bolt was made using a band saw and the bark carefully removed using chisels. Each peeled bark was placed in a plastic bag to prevent moisture loss while small sections of it were examined under a dissecting microscope for beetle galleries. Galleries were evidenced by dark brown discoloration on the inner bark and were exposed using chisels and tweezers to remove strands of phloem tissue covering the galleries. Data were recorded on gallery length, status of adults (alive or dead/infected with fungus) and brood (stages present and number), if any.

In 2015, the experiment was repeated and modified using 100 small bolts, with 25 bolts per treatment and four mating pairs introduced into each holding container. In addition to treatments with *B. bassiana* GHA, *M. brunneum* F52, and water (control) as described above, a fourth set of bolts was sprayed with the biological fungicide Rootshield® WP (a.i. *T. harzianum* Rifai strain KRL-AG2; Bioworks, Victor, NY) to target the fungal pathogen *G. morbida*. Rootshield® WP was applied at the recommended rate of 85–141 g per gallon for outdoor nursery crops, with 1.51% active ingredient or 1×10^7 CFU per g. Conidial deposition on sprayed bark was approximately $9.1 \pm 5.4 \times 10^2$, $12 \pm 4.1 \times 10^2$ and $2.5 \pm 1.1 \times 10^1$ CFU per mm^2 for *B. bassiana* GHA, *M. brunneum* F52 and *T. harzianum* KRL-AG2, respectively. Less than one fungal CFU per mm^2 of control bark was observed.

Analysis of variance (ANOVA) was used to determine mycoinsecticide effects on mean gallery length, number of progeny and progeny per gallery length (mm) (JMP Pro® 12.2.0). Each scoring of an attack was based on evidence of beetle boring or tunneling on the small bolts, while the data on gallery length and number of progeny were based only on observed nuptial chambers with a female, with or without connecting egg or larval galleries, or chambers without a female but with adjacent egg or larval galleries. Means separation was conducted using Dunnett's test.

2.5. Large bolt field assays

To evaluate the effects of mycoinsecticide and biological fungicide treatments on WTB in a field setting, large walnut bolts (7–15 cm diameter, 0.6 m length) were obtained from uninfested *J. nigra* trees in McDowell County, NC, sprayed as described above, baited with WTB cap lure stapled at midpoint of each log, and hung in the crown of infested *J. nigra* trees in Knox County, TN, following procedures described in 2.1 (Supplementary Fig. 2). In 2014, thirteen bolts were assigned to each of three treatments (Botanigard® *B. bassiana* GHA, Met 52® *M. brunneum* F52, and water control) and sprayed as described for the small bolt laboratory assays (section 2.4) immediately prior to field deployment. Bark samples from four representative bolts were also collected prior to field deployment to determine conidial deposition. Estimates of conidial deposition on sprayed bark were $4.8 \pm 4.6 \times 10^2$ and $5.3 \pm 1.3 \times 10^2$ CFU per mm^2 for *B. bassiana* GHA and *M. brunneum* F52, respectively. From control bark samples, 2.6 ± 2.5 CFU per mm^2 was observed.

Bolts were hung at the same four sites reported in section 2.1, in two to four trees at each site, with one replicate of the three treatments per tree (total trees = 13). Bolts were spaced as widely and as evenly within the crown of each tree as possible to minimize the risk of cross contamination between treatments. Mycoinsecticide-treated and control bolts were deployed for 8 days (14–22 Aug 2014) and then transferred to 32-gallon ventilated plastic garbage bins incubated outdoors in the shade for 3 weeks to allow for brood development. Afterwards, bolts were held at 5 °C until processing to delay adult emergence. When processed, bolts were cut in half, and one half of each was carefully peeled as described in section 2.4 to determine the number of attacks and expose galleries present. Gallery length, status of adults (alive or dead/infected with fungus) and brood (stages present and number), were recorded when present. Empty short tunnels from exploratory attacks (usually a WTB body length or ≤2 mm length and without brood) or male-only chambers were omitted from analysis of gallery lengths because these were not directly indicative of the potential numbers of beetles in the next generation. The other half of each bolt was placed in a rearing container (described in 2.1) for monitoring WTB adult emergence. Beetles emerging from these bolts were collected in 100 or 250-ml polypropylene screw cap jars with antifreeze to preserve samples between collection. Samples were collected every 2 to 4 weeks until April of the following year. Numbers of males and females emerged per bolt were counted separately but pooled for analysis, and emergence was standardized by log surface area (beetles per 500 cm^2).

In 2015, the experiment was repeated with modifications. In addition to treatments with *B. bassiana* GHA, *M. brunneum* F52, and untreated controls, a fourth treatment was added using the biological fungicide Rootshield® WP, applied as described in the small bolt assays. Estimates of conidial deposition on sprayed bark were $6.5 \pm 4.5 \times 10^2$, $16.3 \pm 4.6 \times 10^2$ and 4.1 ± 2.9 CFU per mm^2 for *B. bassiana* GHA, *M. brunneum* F52, and *T. harzianum* KRL-AG2, respectively. From control bark samples less than one CFU was observed per mm^2. Twelve large bolts were sprayed with either a mycoinsecticide/biological fungicide or water (for control) (total bolts = 48) and were hung in the crown of infested trees for 10 days (26 Aug – 4 Sep 2015). There were one to four trees at each of the same four sites used in 2014, with one replicate of each treatment per tree. Afterwards, newly infested bolts were handled as described for 2014 experiment, with modification. One half of each bolt was placed into emergence containers for monitoring WTB adult emergence, whereas the other half was not peeled but used for a separate study (not reported here). Adult emergence in 2015–2016 was monitored every 2–4 weeks through April 2016 for all treatments, and through February 2017 for the control treatment only. Emergence was standardized as numbers of beetles per 500 cm^2 of bark surface area.

ANOVA was used to evaluate treatment effects on mean number of WTB attacks, gallery length, number of progeny and progeny per gallery length (JMP Pro® 12.2.0) on the large walnut bolts peeled in 2014. Consistent with the small bolt experiment, the number of attacks on large bolts was based on all observed beetle chambers or galleries (with and without females or brood), whereas the data on gallery length and number of progeny were based only on observed nuptial chambers and brood galleries.

In 2015–2016, peak emergence from control bolts in January 2016 was followed by steadily increasing emergence from all treatments by March and April 2016 (see Results). This pattern strongly suggested that beetles were re-colonizing bolts within the container and producing additional generations that were no longer affected by the initial mycoinsecticide or biological fungicide treatments. Therefore, for statistical analyses in both 2014–2015 and 2015–2016, emergence data were summarized by month and analyzed for the period October through February only. ANOVA was used to evaluate the effects of treatment, sampling month, and the treatment × month interaction. Means separation was performed using Dunnett's test and Tukey-Kramer HSD.

3. Results

3.1. Laboratory dip assays: Fungal virulence to WTB adults

WTB adults were susceptible to both entomopathogenic fungi *B. bassiana* strain GHA and *M. brunneum* strain F52. Exposure of male and female adults to suspensions of these fungi at dosages of 10^5 and 10^6 conidia per ml by dipping resulted in 63.8 ± 4.6% and 96.3 ± 2.2% (for GHA) and 76.1 ± 3.1% and 90.6 ± 7.3% (for F52) uncorrected combined mortality for the two sexes, respectively, by day 4 (Fig. 1). Control mortality was 17% ± 4.5% on the same day. By day 5, beetle mortality from treatments increased, but so did control mortality to 33.5 ± 5.0%, which was too high to continue with the bioassay (S. Wraight, personal communication). Plating of treated cadavers on WGDA resulted in fungal growth and spore production identifiable as either *B. bassiana* of *M. brunneum* on most cadavers (Supplementary Fig. 3). By contrast, control cadavers had saprophytic fungal or bacterial growth.

3.2. Small bolt laboratory assays

Galleries formed by the WTB in the small walnut bolts consisted of a mix of nuptial chambers with or without a female adult, and with or without connecting brood galleries. Brood was observed in galleries

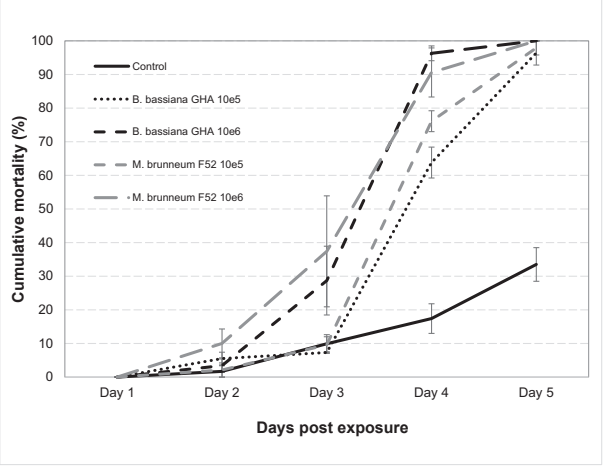

Fig. 1. Cumulative mortality (mean ± standard error) of combined female and male *Pityophthorus juglandis* adults after exposure to two dosages of entomopathogenic fungi *Beauveria bassiana* strain GHA and *Metarhizium brunneum* strain F52. Adults were dipped in 0.01% aqueous Tween solution with or without fungal conidia (control) for 30 s before transfer to individual Alcott vials for daily observations.

that ranged from 7 to 166 mm, with the shortest gallery containing 3 larvae in an untreated bolt. Most of the progeny observed were eggs and larvae, with a few pupae observed only in the 2015 experiment.

The number of beetle attacks and nuptial chambers/brood galleries was variable among treated and untreated small bolts (Table 1). In 2014, 86% (24 of 28) of untreated bolts had evidence of beetle attacks, while only 36% and 25% of the *B. bassiana* GHA- and *M. brunneum* F52-treated bolts, respectively, exhibited attacks. In 2015, 56% of the untreated bolts were attacked, whereas 32%, 24%, and 60% of the *B. bassiana* GHA-, *M. brunneum* F52- and *T. harzianum*-treated bolts, respectively, were attacked. Not all attacks resulted in galleries with brood; some attacks resulted in only very short (≤2 mm length) and empty tunnels or chambers containing only an adult male.

Of the nuptial chambers and brood galleries examined in 2014, no significant differences were observed between untreated and treated bolts in mean gallery length ($F_{2,71} = 1.29$, $P = 0.28$), mean progeny per gallery ($F_{2,71} = 1.79$, $P = 0.17$), and the progeny produced per gallery length ($F_{2,71} = 2.90$, $P = 0.06$) (Table 1). Similarly, in 2015 no significant differences were observed between untreated and treated bolts in mean gallery length, mean progeny per gallery and in the progeny produced per gallery length (Table 1). Comparisons among the three fungal treatments, however, showed significant differences in gallery length ($F_{3,60} = 4.49$, $P = 0.007$) and in progeny per gallery ($F_{3,60} = 6.04$, $P = 0.001$), with higher numbers in *T. harzianum*-treated bolts compared to those sprayed with *B. bassiana* GHA. Progeny produced per gallery length was comparable among all fungal treatments ($F_{3,60} = 0.60$, $P = 0.61$).

In the untreated small bolts that lacked galleries, dead adults were observed outside of the bolt in the container or in nuptial chambers. None of these cadavers showed evidence of fungal infection. By contrast, several dead adults examined in chambers in both the *B. bassiana* GHA- and *M. brunneum* F52-treated bolts were covered with fungal conidia. Only a few of the progeny found associated with these infected adults showed signs of fungal infection, however.

3.3. Large bolt field assays

In 2014, all of the untreated and most (92%) of the treated large walnut bolts hung in the crowns of infested *J. nigra* trees were attacked by WTB (Table 2). The number of attacks was significantly higher in untreated bolts compared to those treated with either fungus (Dunnett's /d/ = 2.25, P < 0.0001, df = 2) (Table 2). As in the small bolt assays, not all of these attacks resulted in nuptial chambers with females and brood galleries. In untreated bolts, 61% (73 of 119) of the attacks included nuptial chambers/brood galleries, whereas in *B. bassiana* GHA- and *M. brunneum* F52-treated bolts, 46% and 45% of the attacks, respectively, resulted in nuptial chambers/brood galleries. Among these galleries, no significant differences were observed in gallery length ($F_{2,104} = 0.87$, $P = 0.41$) or brood size per gallery length ($F_{2,104}$ 1.11, $P = 0.33$) between untreated and treated bolts (Table 2). A significant difference in brood size, however, was observed between untreated and treated bolts ($F_{2,104} = 4.55$, $P = 0.01$), with fewer brood in both fungal treatments (Table 2).

In the first six months following field exposure in late August-early September, WTB emergence from containerized walnut bolts was highest from December through February in both the 2014–2015 experiment and the 2015–2016 experiment (Table 3). Comparison of the two experiments, however, indicated much higher emergence in 2015–2016, with up to 424 beetles per 500 cm^2 in December, compared to 2014–2015, with up to only 61 beetles per 500 cm^2 in January. One untreated bolt in the 2015–2016 experiment produced a total of 1,248 beetles from November into February, or an average of 312 beetles per month (or 133 beetles per 500 cm^2 per month).

Comparison of early beetle emergence from untreated bolts with those treated with biological control fungi revealed significant effects of treatment ($F_{2,765} = 10.98$, $P = < 0.0001$), sampling month ($F_{4,765} = 8.09$, $P = < 0.0001$), and interaction between treatment and sampling month ($F_{8,765} = 3.58$, $P = 0.0004$) in 2014–2015 (Table 3). Similar results were observed in 2015–2016, with significant effects of treatment ($F_{3,412} = 16.19$, $P = < 0.0001$), sampling month ($F_{4,412} = 6.7$, $P = < 0.0001$) and interaction between treatment and sampling month ($F_{2,412} = 2.9$, $P = 0.0007$). In 2014–2015, adult emergence from untreated bolts was higher than from *B. bassiana* GHA- and/or *M. brunneum* F52-treated bolts in January 2015 (Dunnett's /d/ = 2.23, P < 0.0001, df = 2) and February 2015 (Dunnett's /d/ = 2.37, P < 0.0001, df = 2) only. In 2015–2016, adult emergence was higher from untreated bolts versus *B. bassiana* GHA- and/or *M. brunneum* F52-treatments during all the sampling months: October (Dunnett's /d/ = 2.39, P 0.04, df = 3), November (Dunnett's /d/ = 2.37, P 0.04, df = 3) and December (Dunnett's /d/ = 2.39, P 0.04, df = 3) 2015; and January (Dunnett's /d/ = 2.43, P 0.03, df = 3) and February (Dunnett's /d/ = 2.67, P 0.02, df = 3) 2016. Mean adult emergence from untreated controls was at least 25 times higher than emergence from *B. bassiana* GHA- and *M. brunneum* F52-treated bolts in January 2016. By contrast, *Trichoderma harzianum* KRL-AG2 did not reduce emergence during any sampling month relative to untreated controls (Table 3).

Extended monitoring of WTB emergence in March and April 2016 revealed a similar pattern of increasing emergence in all treatments (Supplementary Fig. 4), suggesting the production of another

Table 1
Pityophthorus juglandis attacks, gallery establishment and brood production in small walnut bolts untreated or treated with biological control fungi *Beauveria bassiana* strain GHA, *Metarhizium brunneum* strain F52 or *Trichoderma harzianum* strain KRL-AG2.[a]

Year/ Treatment	Total No. bolts (w/ attacks; w/ brood)	Total No. attacks (w/ females/brood)[b]	Gallery length (mm) mean ± SE (range)[c]	Brood size mean ± SE (range)[c]	Brood/gallery (mm) mean ± SE[c]
2014					
Untreated (Control)	28 (24; 19)	47 (46)	37.25 ± 6.47 (3–204)	5.80 ± 1.32 (0−37)	0.22 ± 0.05
B. bassiana GHA	28 (10; 6)	25 (17)	25.15 ± 4.7 (5−69)	7.53 ± 2.18 (0−30)	0.34 ± 0.11
M. brunneum F52	28 (7; 7)	24 (11)	21.08 ± 2.14 (5−31)	11.27 ± 2.10 (0−22)	0.51 ± 0.10
2015					
Untreated (Control)	25 (14; 10)	29 (24)	34.95 ± 4.18 (5−77)	19.42 ± 3.21(0−50)	0.56 ± 0.10
B. bassiana GHA	25 (8; 6)	16 (15)	23.27 ± 2.83 (4−39)	12.33 ± 2.30 (0−44)	0.55 ± 0.13
M. brunneum F52	25 (6; 5)	8 (5)	39.80 ± 16.87 (11–105)	20.80 ± 4.89 (4−32)	0.68 ± 0.22
T. harzianum	25 (15; 15)	27 (20)	49.50 ± 5.16 (12–108)	34.6 ± 4.47 (0−65)	0.73 ± 0.11

No significant differences were observed between control (untreated) and treated bolts (Dunnett's Method, alpha = 0.05).

[a] Commercially available mycoinsecticides Botanigard® (a.i. *B. bassiana* strain GHA) and Met52® (a.i. *M. brunneum* strain F52) and biological fungicide Rootshield® (a.i. *T. harzianum* strain KRL-AG2) were sprayed on walnut bolts (5–10 cm diameter, 13 cm length) before exposing *P. juglandis* mating pairs into individual containers holding these bolts. Rootshield® was tested only in 2015.

[b] Beetle attacks included short (≤2 mm) empty tunnels, chambers with only an adult male, and chambers with adult female and brood galleries.

[c] Only chambers with females and brood galleries were included in the means and analysis of gallery length and brood size.

Table 2
Pityophthorus juglandis attacks, gallery establishment and brood production in large walnut bolts untreated or treated with biological control fungi *Beauveria bassiana* strain GHA or *Metarhizium brunneum* strain F52 in 2014[a].

Year/ Treatment	Total No. bolts (w/ attacks)	Total No. attacks (mean ± SE) (range)[b]	Gallery length (mm) mean ± SE (range)[c]	Brood size mean ± SE (range)[c]	Brood/gallery length (mm) mean ± SE[c]
Untreated (Control)	13 (13)	119 (7.9 ± 0.6) (1–27)	20.88 ± 2.11 (2–85)	12.47 ± 1.59 (0–70)	0.69 ± 0.19
B. bassiana GHA	13 (12)	35 (2.9 ± 0.3)* (1–8)	20.51 ± 4.09 (2–54)	6.19 ± 1.3* (0–15)	0.33 ± 0.08
M. brunneum F52	13 (12)	40 (2.9 ± 0.3)* (1–7)	14.94 ± 3.31 (4–59)	4.22 ± 1.69* (0–21)	0.21 ± 0.06

[a] Commercially available mycoinsecticides Botanigard® (a.i. *B. bassiana* strain GHA) and Met52® (a.i. *M. brunneum* strain F52) were sprayed on uninfested walnut bolts (7–17 cm diameter, 0.6 m length) that were then hung in the crown of *P. juglandis*-infested *Juglans nigra* trees in Knox County, TN, in 2014. There was one set of treated and untreated bolts per tree, with two to four trees at four sites (total = 13 trees).

[b] One half of each newly infested bolt was peeled and examined for beetle attacks. Beetle hits observed include short empty tunnels, chambers with only a male adult, chambers with female adults and brood galleries. Total hits reflect cumulative total for all infested bolts per treatment. Means followed by an asterisk are significantly different from control (untreated) (Dunnett's Method, alpha = 0.05).

[c] Only chambers with females and brood galleries were included in the analysis; beetle attacks that resulted in a very short (usually ≤2 mm) and empty tunnel or chambers with only male adults were omitted. Mean (± SE) gallery length and brood size values were based on these chambers/galleries (control n = 73; *B. bassiana* GHA n = 16; and *M. brunneum* F52n = 18). Means within a column followed by an asterisk are significantly different from control (untreated) (Dunnett's Method, alpha = 0.05).

Table 3
Emergence of adult *Pityophthorus juglandis* from large walnut bolts untreated or treated with biological control fungus *Beauveria bassiana* strain GHA, *Metarhizium brunneum* strain F52 or *Trichoderma harzianum* strain KRL-AG2[a].

Year/Treatment	Beetle emergence per month[b]					
	Bolts (n)	October (mean ± SE)	November (mean ± SE)	December (mean ± SE)	January (mean ± SE)	February (mean ± SE)
2014–2015						
Untreated (Control)	13	0.12 ± 0.04	0.35 ± 0.11	1.03 ± 0.21	1.18 ± 0.27	0.72 ± 0.18
B. bassiana GHA	13	0.05 ± 0.02	1.04 ± 0.37	1.45 ± 0.49	0.32 ± 0.1*	0.15 ± 0.05*
M. brunneum F52	13	0.05 ± 0.02	0.08 ± 0.03	0.35 ± 0.23	0.18 ± 0.08*	0.04 ± 0.02*
2015–2016						
Untreated (Control)	12	0.59 ± 0.19	1.12 ± 0.43	17.67 ± 8.36	25.52 ± 13.36	21.85 ± 9.37
B. bassiana GHA	12	0.39 ± 0.18	0.28 ± 0.13*	2.11 ± 0.94*	0.61 ± 0.39*	2.71 ± 1.37*
M. brunneum F52	12	0.09 ± 0.04*	0.34 ± 0.15	2.80 ± 1.20*	0.94 ± 0.44*	2.61 ± 1.17*
T. harzianum KRL-AG2	12	0.15 ± 0.05	0.32 ± 0.12	5.0 ± 1.62	3.09 ± 1.71	6.88 ± 2.27

[a] Commercially available mycoinsecticides Botanigard® (a.i. *B. bassiana* strain GHA), Met52® (a.i. *M. brunneum* strain F52) and biological fungicide Rootshield® (a.i. *T. harzianum* strain KRL-AG2) were sprayed on uninfested walnut bolts that were then hung in the crown of *P. juglandis*-infested *Juglans nigra* trees in Knox County, TN, for 8–10 days. One half of each newly infested bolt was placed in individual rearing containers and monitored for beetle emergence. There was one set of treated and untreated bolts per tree, with one to four trees at four sites (2014 total trees = 13; 2015 total trees = 12). Rootshield® was tested only in 2015.

[b] Number of female and male adults were combined per log and values divided by bolt surface area (500 cm^2). Means within a column per experimental period followed by an asterisk are significantly different from control (untreated) (Dunnett's Method, alpha = 0.05).

generation of beetles that was not affected by the initial mycoinsecticide sprays; thus monitoring of emergence from sprayed bolts was discontinued. To determine how long beetles would continue to utilize the same piece of containerized wood, however, emergence from the control treatment was monitored until February 2017. Overall, beetles emerged from control bolts continuously for over 14 months (early October 2015 to mid-December 2016) (Supplementary Fig. 4).

4. Discussion

In the epidemiology of TCD, *G. morbida* does not produce a systemic infection; rather walnut tree mortality ensues from numerous beetle attacks (and attendant infections), often from multiple generations of beetles (Graves et al., 2009). Each beetle attack results in a localized canker and the coalescence of numerous cankers produces a girdling effect (Graves et al., 2009; Utley et al., 2013). Although the fungus produces a large number of conidia, cankers are observed only at beetle feeding sites or in and around their galleries (Utley et al., 2013). This epidemiology suggests opportunities to develop strategies that protect lightly-infested trees by reducing beetle populations below damaging thresholds without completely eliminating infestations. The use of biological control agents that reduce succeeding beetle generations but not affect the nut crop, may be particularly advantageous with black walnut. Our studies with entomopathogenic fungi, specifically *B. bassiana* and *M. brunneum*, demonstrate that these fungi are virulent against WTB adults and that applications of commercially available mycoinsecticides based on these fungi to walnut bark can reduce the numbers of emerging next generation adults.

Bioassays of adults of both sexes showed comparable susceptibility to both fungi, with mortality from infection occurring as early as three to four days after exposure. This is important because death of attacking adults within a short period following exposure to fungus on sprayed bark would be necessary to prevent progeny production. Unfortunately, we were not able to establish a lethal dosage for either fungus to kill 50% of the beetles (LD_{50}), nor establish the time to kill 50% of the beetles at a given dosage for each fungus (LT_{50}), due to insufficient numbers of beetles available for multiple assays. Ideally, bioassays designed to determine LD_{50} and LT_{50} require numerous insects of about the same age at the same time, which is difficult to obtain when an artificial rearing system is not available. To obtain enough WTB to test two dosages at a time for our assays, emerged adults collected over a few days were held at 10 °C. This protocol and the holding of dipped beetles in glass vials with no food (i.e., small piece of fresh bark) likely contributed to high control mortality by day 4 or 5 after exposure. This prevented any statistical analysis of available data or additional tests of multiple lower dosages of each fungus. For insect bioassays, it is ideal to test four to five fungal dosages to establish an LD_{50} for given fungal isolate (e.g., Castrillo et al., 2011; Castrillo et al., 2013). Neither the exposure method itself nor the immediate handling procedure caused beetle mortality, which is evident in zero beetle mortality observed the day after dipping the beetles in 0.01% Tween, with or without fungal conidia in bioassays conducted in 2014. This exposure method has been used for other beetles such as the emerald ash borer collected from infested ash logs (e.g., Liu and Bauer, 2006; Castrillo et al., 2010). For

this beetle, however, ash foliage was provided during the assay period of 6 days. In bioassays of the ambrosia beetles *Xylosandrus germanus* and *X. crassiusculus,* with fungal suspension applied using a spray tower, no food was provided, however, and control adults remained alive for ≥7–9 days, respectively (Castrillo et al., 2011; Castrillo et al., 2013). These results show that ideal insect holding conditions to minimize control mortality during a bioassay vary among different insect species.

The complimentary walnut bolt assays provided additional evidence on the impact of these fungi on beetle survival and brood production. Although the small walnut bolt assays did not reveal any significant differences in gallery length, brood size, or brood per gallery between untreated and mycoinsecticide-treated bolts, attacks were initiated less frequently on small bolts treated with *B. bassiana* GHA or *M. brunneum* F52 compared to untreated bolts (Table 1). In field studies, treatment of large walnut bolts with *B. bassiana* GHA and *M. brunneum* reduced the mean number of attacks per bolt by at least 66% and the observed mean brood size by at least 50% relative to control logs (Table 2). With the large bolts hung in the crown of infested *J. nigra*, the numerous beetles present and attacking these logs in the field provided the beetle numbers necessary to show treatment effects. Furthermore, treatment with either *B. bassiana* GHA or *M. brunneum* F52 greatly reduced the number of WTB adults that emerged from bolts from December through February, following 8–10 d of exposure to field populations of WTB in late August or early September (Table 3). Given the relatively low emergence of WTB adults in October and November (which may have included emergence of the parental adults that attacked the bolt in the field), the increased emergence from December through February likely represented emergence of the next WTB generation. The contrast between untreated and mycoinsecticide-treated bolts was most evident during this period of peak emergence observed in the months of December to February from the incubated bolts. This reduced emergence may have been due to reduced number of beetle attacks, mortality of adults exposed to fungus as they tunneled into treated bolts, or a combination of both.

The pattern of sharply increasing emergence after February 2016 indicated that walnut twig beetles were re-attacking the bolts within the container and producing additional brood generations. Because this increase was observed in all treatments, it suggests that the entomopathogenic fungal treatments were no longer effective, likely due to reduced conidial viability, on the bark surface. The decline in viability of fungal conidia on these logs is likely slower compared to logs continuously exposed to field conditions of UV light and rainfall (Braga et al., 2002; Castrillo et al., 2010; James et al., 1995; Moore et al., 1993). With limited residence time of viable entomopathogen spores on walnut logs, it is therefore expected that treatment effects would not persist beyond the first brood generation of beetles that emerged following field exposure. These results support the idea that multiple treatments in a single season, timed to coincide with peak WTB flights and attacks, would likely be more efficacious at reducing WTB populations than a single treatment. This strategy concurs with the recommended re-treatment interval for these commercial products that varies with the pest management program being applied and requires pest scouting to determine optimal application frequency.

Examination of nuptial chambers with female adults in treated bolts showed sporulating cadavers evident of fungal infection, but infection was observed only in a few immatures in their galleries. This is in contrast to similar studies on ambrosia beetle where cadavers of foundresses infected with either *B. bassiana* or *M. brunneum* produce conidia that could also infect her brood, sometimes killing up to 100% in the gallery (Castrillo et al., 2011; Castrillo et al., 2013). Unlike ambrosia beetles with a single gallery housing mixed brood stages along with the foundress or the foundress blocking entrance to the gallery, WTB larvae are found in separate tunnels branching perpendicular from the initial egg chamber. This separation from an infected parent minimizes the chances of exposure to inocula and could reduce the likelihood of infection in developing brood.

The reduction in beetle brood size in galleries and subsequent population build up in mycoinsecticide-treated bolts result in part from lower number of beetle attacks. This decrease may be due to the repellency of the mycoinsecticides to the beetles, which could be due to the fungus/active ingredient, the formulation, or a combination of both. Other studies have shown repellency of *M. anisopliae* conidia and mycelia to some insects, including bruchids (*Caryedon serratus*; Ekesi et al., 2001), scarabs (*Popillia japonica*; Villani et al., 1994), and termites (*Coptotermes formosanus* and *Macrotermes michealseni*; Sun et al., 2008; Mburu et al., 2009, respectively). Adults of the flower bug (*Anthocoris nemorum*) and ladybirds (*Coccinella septempunctata*) have been observed to also avoid *B. bassiana* conidia (Meyling and Pell, 2006; Ormond et al., 2011). Similarly, field studies by Castrillo et al. (unpublished) on the ambrosia beetle *X. germanus* have also shown reduced number of attacks on beech bolts sprayed with either Botanigard® or Met52®. The lowest number of attacks per fungus was observed on bolts sprayed with the highest concentration of each mycoinsecticide, and dilution of the products by 1:10 and 1:100 significantly reduced repellency (Castrillo et al., unpublished). This repellent effect of entomopathogenic fungi could be an important component of insect control, especially against burrowing beetles, and the determination of an economically practical rate that can significantly reduce attacks and kill those that still tunneled would be helpful in the development of a feasible control strategy.

In contrast to the mycoinsecticides tested, the biological fungicide did not reduce beetle brood production, indicating that either this fungus had no impact on *G. morbida* or that any negative interaction between the mycoparasite and the symbiont did not impact beetle gallery establishment or reproduction. Since WTB feeds on the phloem and not the symbiont, any negative interaction between these fungi may not be detrimental to the beetle. It may still be worth examining possible competition between *T. harzianum* or another biological fungicide and *G. morbida*, as this could help control TCD. Preliminary in vitro competition studies between isolates of *G. morbida* from Knox County, TN, and *T. harzianum* strain KRL-AG2 showed negative interactions, with the mycoparasite growing over the fungal pathogen (Castrillo, unpublished data). These are in vitro studies, however, and additional tests should be conducted on wood material to examine interspecies interactions under more natural conditions. Should *T. harzianum* prove to be an effective antagonist against *G. morbida*, a two-pronged approach to control TCD by combining an entomopathogenic fungus and *T. harzianum*, however, would require interaction assays between these biological control fungi to determine their compatibility.

A combination of laboratory and field studies to evaluate entomopathogenic fungi provided evidence of the potential of these fungi as control agents against TCD. Although the use of beetles collected from infested logs in laboratory assays proved challenging, the current rearing protocol using infested logs still provided enough beetle to show comparable susceptibility of both sexes to both *B. bassiana* GHA or *M. brunneum* F52. Given the effective reduction of next-generation adults from sprayed bolts, additional field studies to determine effective and economical application strategies (i.e., optimal spray rates, frequency, and timing) on branches and stems of live trees is warranted, and may lead to an environmentally acceptable strategy for reducing the impact of TCD on individual walnut trees.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocontrol.2017.08.007.

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