1 Biological Control

2
3
4
5
6
7 **Competition between biological control fungi and fungal symbionts of ambrosia beetles**
8 ***Xylosandrus crassiusculus* and *X. germanus* (Coleoptera: Curculionidae): mycelial**
9 **interactions and impact on beetle brood production**

10
11 Louela A. Castrillo^{a,*}, Michael H. Griggs^b and John D. Vandenberg^b

12
13 ^a*Department of Entomology, Cornell University, Ithaca, NY 14853 and* ^b*USDA, Agricultural*
14 *Research Service, Robert W. Holley Center for Agriculture and Health, Ithaca, NY 14853*

15
16
17
18 Running title: Competition between fungal symbionts and biocontrol fungi

19
20 *Corresponding author: Louela A. Castrillo (lac48@cornell.edu)

ABSTRACT

Ambrosia beetles *Xylosandrus crassiusculus* and *X. germanus* are among the most important exotic pests of orchards and nurseries in the US and are difficult to control using conventional insecticides because of their cryptic habits. The use of biological control agents may prove effective by targeting both beetles and fungal symbionts inside tree galleries: entomopathogenic fungi could be used to target beetle foundresses and their brood, or mycoparasitic fungi, e.g., *Trichoderma harzianum*, could be used to target their associated fungal symbionts. We used a combination of *in vitro* assays and beetle bioassays to examine competition between symbionts and biological control fungi and the impact of biological control fungi on beetle brood production. The *in vitro* assays showed *T. harzianum* outcompeted different strains of *Ambrosiella roeperi* and *A. grosmanniae* associated with *X. crassiusculus* and *X. germanus*, respectively, whether in primary or secondary resource capture assays. In contrast, entomopathogenic fungi *Beauveria bassiana* and *Metarhizium brunneum* blocked the spread of symbionts only in primary competition assays. Complementary beetle bioassays showed that beetle galleries in *T. harzianum*-treated beech stems had sparse symbiont growth, many with no or only a small number of eggs present. Brood numbers produced by foundresses in *T. harzianum*-treated stems were comparable to those in stems treated with either entomopathogen at the higher dosages, in which brood reduction was likely due to foundress mortality prior to laying eggs or after laying only a small number of eggs. These results show the potential of using biological control fungi in targeting ambrosia beetle populations either directly by killing foundresses and reducing brood production or indirectly by suppressing symbiont growth in their galleries.

Key words: ambrosia fungi; *Ambrosiella* spp.; entomopathogenic fungi; *Beauveria bassiana*; *Metarhizium brunneum*; mycoparasitic fungi; *Trichoderma* spp.; microbial control

1. Introduction

Ambrosia beetles are wood boring insects associated with symbiotic fungi which they cultivate in their galleries as primary food source for the growing brood. Adult females, or foundresses, tunnel through sapwood to form galleries which they inoculate with the symbionts they carry in glandular, invaginated cuticular structures called mycangia (Francke-Grosmann, 1967). Foundresses generally do not lay eggs until establishment of the symbiont in their galleries (Biedermann et al., 2009; Kajimura and Hijii, 1992; Weber and McPherson, 1983a). A diverse fungal flora may be present in ambrosia beetle galleries and composition could vary over the course of brood development, but the dominant fungi in these galleries are the obligate symbionts of the beetles (Batra, 1963, 1967; Kajimura and Hijii, 1992). Generally only one or few fungal symbiont(s) have been found associated with a given ambrosia beetle species (Batra, 1963), but studies by Harrington et al. (2010, 2011) have shown multiple symbionts associated with the redbay ambrosia beetle and that a given symbiont could be associated with multiple ambrosia beetles. These fungal symbionts, commonly known as ambrosia fungi, are asexual and have modified hyphal tips suitable for grazing by ambrosia beetle larvae and adults (Batra, 1963, 1967; Beaver, 1989). Most of the ambrosia fungi so far described are in the genera *Ambrosiella* and *Raffaelea* (Ascomycota: Microascales) (Harrington et al., 2010; Mayers et al., 2015).

A number of exotic ambrosia beetles are now considered major pests in tree nurseries and orchards in the US (Haack and Rabaglia, 2013). *Xylosandrus crassiusculus* (Motschulsky) and *X. germanus* Blandford (Coleoptera: Curculionidae: Scolytinae) are two of the more important

invasive species, with a wide host range that includes woody ornamentals, fruit and nut trees (Schedl, 1963; Weber and McPherson, 1983b; Wood and Bright, 1992). Like other ambrosia beetles, both attack stressed, weakened or dying trees (Ranger et al., 2013; Weber and McPherson, 1983a). Studies by Ranger et al. (2010, 2015) have shown that both beetles are attracted to volatiles including ethanol produced by physiologically stressed trees, some of which may look apparently healthy. In orchards and nurseries where trees could be subject to a number of environmental factors or management practices that result in physiological stress (e.g., flooding, drought or high density planting), the affected trees produce higher concentrations of ethanol that act as the primary attractant to these beetles (Ranger et al., 2010, 2015). Improved growing conditions and cultural control methods have been recommended to reduce the number of trees attacked (Mizell and Riddle, 2004), but these strategies are not always feasible. Moreover, the use of conventional insecticides requires repeated applications timed to coincide with beetle attacks or spraying of insecticides with long residual effects because of the beetles' cryptic habits (Frank and Sadof, 2011; Hudson and Mizell, 1999). We are currently evaluating the use of biological control agents, specifically insect pathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium brunneum* Petch (Ascomycota: Hypocreales) against ambrosia beetles. Conidia from strains of these two fungi are active ingredients in EPA-registered, commercially available mycoinsecticides. Our studies have shown two commercial strains to be virulent against *X. crassiusculus* and *X. germanus* (Castrillo et al., 2011, 2013). Furthermore, we observed that the impact of these fungi is not limited to the foundresses; cadavers of infected adults produce fungal conidia that can infect their progeny, sometimes up to 100% of the brood in the gallery (Castrillo et al., 2011). Laboratory and field studies have also shown possible negative interactions between these entomopathogens and the symbionts in

beetle galleries (Castrillo et al., 2013; Vandenberg et al., unpublished). Any antagonistic interactions between *B. bassiana* or *M. brunneum* and the beetle symbionts will further enhance the impact of these control agents on ambrosia beetles populations since the beetles depend solely on their symbionts for food. Another approach to beetle management would be to target symbionts directly by use of mycoparasitic fungi, e.g., *Trichoderma* spp. (Ascomycota: Hypocreales), that are commercially available as biofungicides. Suppressing growth of the fungal symbionts will deny the developing brood nutrition for survival and limit beetle population increase.

In competitive fungal interactions in which one fungus gains nutrients from another, the relationship is referred to as mycoparasitism (Jeffries, 1995). These antagonistic interactions often result in the death of the host, with the mycoparasite utilizing the host as a nutrient source (Jeffries, 1995). Mycoparasitic fungi include members of the genus *Trichoderma*, with species and strains that are used as biological control agents against a wide range of plant pathogenic fungi (Woo et al., 2014), including pathogens of forest trees and wood decay fungi (e.g., Schubert et al., 2008). We showed that the use of *T. harzianum* (commercial strain T-22; KRL-AG2) against *X. crassiusculus* resulted in reduced brood production among foundresses exposed to beech stems treated with it (Castrillo et al., 2013). Examination of beetle galleries in the treated stems showed sparse symbiont growth versus those in untreated stems. In some cases long tunnels were observed with no symbiont or brood, suggesting that the suppression of symbiont growth prevented the foundress from laying eggs. To further examine the impact of these interspecific fungal interactions on symbiont growth in beetle galleries and, consequently, their impact on brood production, we carried out 1) *in vitro* competition assays between fungal symbionts associated with *X. crassiusculus* and *X. germanus* versus biological control fungi

Beauveria bassiana, *Metarhizium brunneum*, and *Trichoderma harzianum* and 2)
complementary beetle bioassays using treated beech stems treated with biological control fungi.

2. Materials and Methods

2.1 Fungal symbionts from ambrosia beetles

Fungal symbionts *Ambrosiella grosmanii* Mayers, McNew & Harrington and *A. roeperi* Harrington & McNew from *X. germanus* and *X. crassiusculus*, respectively (Harrington et al., 2014; Mayers et al., 2015), were isolated from beetles collected from NY, OH, TN, and VA, as part of a separate study on the genetic diversity among the symbionts associated with these beetles species in eastern US. Preliminary data on different genotypes present (Castrillo, unpublished), based on multilocus sequencing, were used to select strains for use in the *in vitro* competition assays. All symbionts were grown on potato dextrose agar (PDA) or 2% malt extract agar (MEA). Blocks (~ 0.5 cm²) of 2-week old cultures were transferred to cryogenic vials with 10% glycerol and frozen at -80 °C for long-term storage. All strains are maintained at the USDA ARS, Robert W. Holley Center for Agriculture & Health in Ithaca, NY.

2.2 Biological control agents

Entomopathogenic fungi *B. bassiana* strain GHA and *M. brunneum* strain F52 (= ARSEF 5198; previously identified as *M. anisopliae*) and mycoparasitic fungus *T. harzianum* strain T-22 were used for both *in vitro* competition assays and beetle bioassays. Spore powder and culture of *B. bassiana* GHA and *M. brunneum* F52 were obtained from Laverlam International (Butte, Montana) and the USDA ARS Entomopathogenic culture collection (ARSEF, Ithaca, NY), respectively. *T. harzianum* T-22-based products were obtained from (BioWorks, Inc., Victor,

NY). Single spore isolates were established for each strain (Castrillo et al., 2004) and blocks of 2-wk old sporulating cultures were frozen as described above for long- term storage.

Conidia of *B. bassiana* GHA and *M. brunneum* F52 were mass produced as described in Castrillo et al. (2008) for use in beetle bioassays and for inocula in starting mycelial cultures for *in vitro* competition assays. Harvested conidia were dried overnight in a glass desiccator with anhydrous indicating CaSO₄ granules (W. A. Hammond Co., Xenia, OH) at room temperature and then stored at -20°C until use.

2.3 Fungi competition assays

Initial studies were conducted using *in vitro* assays on possible competition between fungal symbionts associated with the ambrosia beetles and fungal biological control agents using symbionts *A. grosmanii* XgNY1 and *A. roeperi* XcOH2 versus the control fungi listed above. Tests were conducted to examine different types of competition: 1) primary resource capture (interacting fungi compete to acquire uncolonized resource or space; competing fungi were inoculated at the same time); 2) secondary resource capture (interacting fungi compete to defend acquired resource or gain colonized resource; one fungus was allowed to grow prior to the introduction of the competing fungus); and 3) differential competition (varying proportion of inocula of the competing fungi were inoculated into the plate) (Boddy, 2000; Klepzig, 1998). Results showed that mycelial interactions could vary with species of the biological control agents and that the presence of conidia from entomopathogenic fungi could complicate the scoring of results. Conidia dislodged from culture plugs inoculated onto assay plates resulted in multiple colony forming units (CFU) that prevented accurate measurement of the symbiont colony growth. Further experiments were based on dual culture tests - primary resource capture and

secondary resource capture - since biological control fungi are more likely to be introduced by a single source, an infected foundress after exposure to treated beech stems.

Fungal symbionts *A. grosmanniae* strains XgNY1, XgOH7, XgOH11, and XgVA6 from *X. germanus* and *A. roeperi* strains XcNY1 and C2451 from *X. crassiusculus* were tested. All cultures were grown on PDA to produce mycelial inocula for competition plates. For each fungal symbiont, source plates were first grown starting with plug inocula and incubated for 1 to 2 wk at 25 °C with 0:24 L:D. The mycoparasite T-22 inocula were grown similarly except for only one week, as the fungus grows at a faster rate than the symbionts. The entomopathogenic fungi were grown by spreading a 200- μ l suspension of 10^5 conidia/ml in aqueous 0.01% Tween 80 on PDA plates incubated as described for symbionts, but only for 48 hr. This was to ensure that culture plugs of either *B. bassiana* or *M. brunneum* contain only mycelia and not newly-produced conidia.

For the primary competition assay, PDA plates (100 $\times$ 15 mm, d $\times$ h) were inoculated with a mycelial plug (0.5 cm) of a symbiont cut from the actively growing margin and a biological control fungus. Each plug was placed upside down approximately 1.0 cm from the edge diametrically opposite each other. Plugs were cut using autoclaved 1 ml pipet tips with ends trimmed to a diameter of 0.5 cm. For controls, PDA plates were inoculated with mycelial plugs of a given symbiont at both edges or a plug at one edge only. Plates were sealed with parafilm, incubated at 25 °C with 0:24 L:D and examined after 1 and 2 wks. Symbiont growth was measured from the center of each mycelial plug to the colony edge opposite that of the competing fungus. Results of primary competition plates with T-22 were recorded after 1 wk, while those with entomopathogenic fungi were recorded after 2 wk. A longer incubation time

was necessary for the latter to allow the two competing fungi to grow and spread before the colonies met.

For the secondary competition assay, PDA plates were first inoculated with a mycelial plug of either symbiont approximately 1.0 cm from the edge of the plate and allowed to grow for 1 wk before introduction of the biological control fungus on the opposite edge of the plate. Culture plates were incubated as described for primary competition assays. Results were recorded after 1 and 2 wk. There were three plates per replicate and two replicates per symbiont-biological control fungus combination for either primary or secondary fungi competition assays. Analysis of variance (ANOVA) (JMP Pro 11; SAS Institute, 2015) was done on mean colony radius after 1 or 2 wk of growth. Means separation tests were done using Dunnett's test or Tukey-Kramer HSD test.

2.4 Beetle bioassays

To examine the impact of these biological control fungi on beetle gallery establishment, symbiont growth and brood production, complementary bioassays were conducted using treated beech stems to which laboratory-reared ambrosia beetles were introduced. Both *X. crassiusculus* and *X. germanus* were reared on beech sawdust-based artificial diet as described in Castrillo et al. (2012, 2013). Next generation adult females, emerged from galleries inside rearing tubes, were collected from 5 to 6 wk old and 6 to 7 wk old broods of *X. germanus* and *X. crassiusculus*, respectively, and held in Petri dishes lined with moist filter paper at room temperature until use on the same day.

A fungal stock suspension was prepared for *B. bassiana* GHA and for *M. brunneum* F52, with 0.34 g conidia in 400 ml of 0.01% aqueous Tween 80 solution. Aliquot dilutions were

prepared from each stock and fungal conidia counted using a Neubauer hemocytometer to determine concentration. Suspensions were adjusted to produce stock suspensions of $\sim 1 \times 10^6$ conidia per ml. Two 10-fold serial dilutions were prepared in 300 ml of 0.01% aqueous Tween 80 to generate the three dosages of each strain for testing (Table 1). For the mycoparasitic fungus, a commercial formulation of *T. harzianum* strain T-22 (RootShield® WP, with 1.5% active ingredient or 1×10^7 CFU per gram and a recommended rate of 85-141 g per gallon for outdoor nursery crops) was used. A stock suspension of 15 g commercial spore product in 400 ml of 0.01% aqueous Tween 80 solution was prepared and used to prepare 300 ml of two 10-fold serial dilutions.

Beech stems (~ 1.5 cm diameter) were collected from Tompkins County, NY, and cut to ~ 10 cm length. Stems were soaked in 10% ethanol for 2 h to make them attractive to ambrosia beetles (Castrillo et al., 2013) and then allowed to air dry for at least 30 min. Cut stems were fully immersed and swirled in 300 ml of fungal suspension for approximately 30 sec, allowed to air dry for at least 30 min and then transferred to individual 50-ml conical polypropylene tubes. Control stems were dipped in 0.01% aqueous Tween 80 solution and handled similarly as the treated stems. Four foundresses were released into each tube. Assay tubes were placed horizontally in plastic bins ($11 \times 15.5 \times 28$ cm, h $\times$ w $\times$ l) lined with moist paper towels, fitted with a plastic lid and incubated at room temperature (22 ± 5 °C) for 10 days. This incubation time allowed gallery establishment (beetle tunneling and symbiont growth) and egg laying after symbiont is established; in untreated stems, beetles build galleries and start laying eggs after ~ 5 d, and progeny will be mostly eggs after another 5 d (Castrillo et al., 2012, 2013). This minimized presence of the larval stages to permit better examination of the growth of fungal symbiont in galleries. After the 10-day incubation period all holding bins were stored at 4 °C for

examination within 2 wk. Each stem was examined for foundress survival, including location especially if dead, tunneling, symbiont growth, and brood production. To examine each stem, gallery entrances (evidenced by entrance hole and extruded sawdust) were marked and the stem was cut within a few mm of either end of each tunnel entrance using a 6-inch band saw. Each stem section, representing one gallery, was split into two or more segments using a 1-inch chisel and examined under a dissecting microscope.

There were twelve stems per control (untreated) or treatment (each biological control fungus- dosage combination) per replicates. The study was replicated on three separate days. Ambrosia beetle survival, tunneling activity, symbiont growth and egg production were scored as either alive/present (1) or dead/absent (0) for each foundress and the percentage of foundresses surviving or showing these activities were calculated. Observations were also made on the condition of the symbiont in galleries in treated versus untreated stems. The total number of eggs laid (brood size) was counted and infection noted, if any. ANOVA was done on arcsine square root-transformed proportions in response to treatment (JMP Pro 11). Analysis of data on brood size was done on mean number of eggs per foundress per treatment. Means separation tests were done using Dunnett's test or Tukey-Kramer HSD test.

3. Results

3.1 Fungi competition assays

Strains of fungal symbionts *A. grosmanniae* and *A. roeperi* inoculated onto PDA plates both grew from the plug inoculum, with mycelia spreading to completely cover the surface of each plate by 2 wk regardless of whether a single plug or two plugs at opposite sides were introduced (Fig. 1A). In competition plates, growth of each symbiont was observed until contact with the

competing fungus. Hyphal contact was observed in all symbiont-biological control fungus combination. The interface between the competing fungi was often marked by dark pigmentation along the edge of symbiont colony that is visible on the underside of PDA plates and sometimes on the surface as well (Fig. 1B, black arrow). This was observed in primary resource capture and in initial studies of differential competition assays (Supplementary Fig. 1). In the latter, colonies of biological control fungi are surrounded by a dark ring at the interface with symbiont mycelia. Pigmentation produced in contact with *B. bassiana* was darker than those observed in *M. brunneum* co-inoculated plates and none was observed in *T. harzianum* co-inoculated plates.

In primary resource capture assays with the faster growing *T. harzianum* T-22, growth of either symbiont species covered less than half the diameter of the plates where the two colonies met after a week of incubation (Figs. 1D and 1E). For the biological control fungi *B. bassiana* GHA and *M. brunneum* F52, slower growth of these two required another week of incubation before the two fungal colonies met, with the symbiont covering more surface area (Figs 1B and 1C). Compared to control plates, growth of the different symbiont strains was affected by the presence of any of the biological control fungi tested (Dunnett's $t = 2.54$, $P < 0.05$, $df = 3$). The impact of each biological control fungus was comparable among the different symbiont species and strains for *B. bassiana* GHA ($F_{5,30} = 1.46$, $P = 0.23$) and *M. brunneum* F52 ($F_{5,30} = 0.68$, $P = 0.64$), but not for *T. harzianum* T-22 ($F_{5,30} = 3.81$, $P < 0.05$). Strains XgOH7 and XgNY1 had significantly smaller radial growth (2.1 cm and 1.9 cm, respectively) compared to strain XgVA6, which had the largest radial growth of 2.6 cm in competition with T-22.

In secondary resource capture assays, where each symbiont was allowed to grow for a week before introduction of the competing fungus, spread of symbiont mycelia was also affected by the presence of a biological control fungus. But among the biological control agents tested,

only *T. harzianum* T-22 halted their growth (Dunnett's $t = 2.54$, $P < 0.05$, $df = 3$). Neither *B. bassiana* GHA nor *M. brunneum* F52 affected the spread of symbiont mycelia. The symbiont grew over the slower growing entomopathogens and covered the whole surface of assay plates. Comparison of *T. harzianum* T-22 secondary versus primary competition plates both showed blocking of symbiont mycelial spread, but with greater area of symbiont growth in the latter (Figs. 1D versus Fig. 1E). The advantage provided to symbiont fungi by growing them first, however, did not last. Extending incubation time to another week for *T. harzianum* T-22 co-inoculated plates, whether primary or secondary, showed *T. harzianum* T-22 growing over the established symbiont colony (Figs. 1F and 1H), followed by its production of conidia (Fig. 1H, black arrows).

In plates co-inoculated with *B. bassiana* GHA, we observed that extending incubation time for another 1 to 2 wk, thus allowing conidial production, resulted in the spread and growth of *B. bassiana* GHA over a few strains of symbiont fungi (C2451 and XcNY1) (Fig. 1I). The same was not observed in *M. brunneum* F52 co-inoculated plates for any of the symbiont strains tested.

3.2 Beetle bioassays

Both *X. crassiusculus* and *X. germanus* foundresses released into tubes with either untreated (control) or treated beech stems were observed to initiate attacks within 24 hours. Within 48 hours most had produced tunnels, as evidence by the frass / sawdust extruded as beetles bore into the stems. Among *X. crassiusculus* attacking untreated stems, 100% of the foundresses were found alive after 10 days, with approximately 96% producing galleries and 79% producing brood (Table 1). For *X. germanus* controls, 100% were also found still alive, with 97% producing

galleries and approximately 63% with brood (Table 1). The mean control brood size per foundress was 3.97 ± 0.22 (range 0 to 8) and 2.63 ± 0.22 (range 0 to 10) for *X. crassiusculus* and *X. germanus*, respectively.

Compared to the controls, percentage of foundresses surviving and with galleries and brood among those exposed to biological control fungi varied depending on beetle species and treatments (fungus-dosage combination). In *X. crassiusculus*, survival was affected by treatment, with exposure to *M. brunneum* F52 at dosages 10^6 and 10^5 conidia/ml resulting in significantly reduced survival (Dunnett's $t = 2.94$, $P < 0.05$, $df = 9$) (Table 1). No significant differences were observed in percentage of foundresses producing galleries ($F_{9, 20} = 1.32$, $P = 0.28$) and in percentages forming galleries with symbiont ($F_{9, 20} = 1.22$, $P = 0.34$). Similarly, percentage of foundresses producing brood was not affected ($F_{9, 20} = 2.22$, $P = 0.08$), but all treatments, except for *B. bassiana* GHA at the lowest dosage, resulted in reduced brood size ($F_{9, 1421} = 14.45$, $P < 0.0001$) (Table 1, Fig. 3).

For *X. germanus*, percentage survival was also affected by treatments compared to controls. Exposure of foundresses to *B. bassiana* GHA and *M. brunneum* F52 at the highest dosage resulted in significant reduction in foundress mortality compared to controls (Dunnett's $t = 2.94$, $P < 0.05$, $df = 9$). Significant differences were also observed in percentage of foundresses producing galleries, with those exposed to *B. bassiana* GHA at 10^6 and 10^5 conidia/ml and to *M. brunneum* F52 at 10^6 conidia/ml resulting in fewer galleries compared to controls (Dunnett's $t = 2.94$, $P < 0.05$, $df = 9$). Percentages forming galleries with symbiont ($F_{9, 20} = 2.07$, $P = 0.08$) and producing brood ($F_{9, 20} = 1.93$, $P = 0.11$) were not affected. But all treatments, except for *B. bassiana* GHA at the lowest dosage, resulted in reduced brood size ($F_{9, 1429} = 13.74$, $P < 0.0001$) (Table 1, Fig. 3).

Among beetles of both species that did not produce galleries, some were found outside the stem or, more often, at the entrance of a short tunnel (length of 2 to 4 mm). In those exposed to either *B. bassiana* GHA or *M. brunneum* F52, primarily at the higher two dosages, many were found dead and already producing mycelia (Supplementary Fig. 2). None of the eggs produced in these treated stems showed discoloration or evidence of fungal infection.

Examination of symbiont growing in beetle galleries in control stems showed galleries with thick mycelial growth with cluster of eggs (Supplementary Fig. 2). This is accompanied by dark stain on the wood beneath the growing symbiont. In treated stems, mostly in *T. harzianum* T-22-treated stems, many galleries had very sparse mycelial growth, with the dark pigmentation not present (Supplementary Fig. 2).

4. Discussion

Ambrosia beetles *X. crassiusculus* and *X. germanus* are difficult to control because of their cryptic habits. Most often, only the adult females are found outside of their host trees, during dispersal and host selection. Once females bore into host trees and produce galleries, they and their brood are protected from conventional insecticides (Hudson and Mizell, 1999). The use of biological control agents, specifically entomopathogenic and mycoparasitic fungi, offers a means of managing these beetles by targeting not only the foundresses, but also the symbiotic fungi and the developing brood inside. Using entomopathogenic fungi, foundresses could be killed in time (< 5 d post exposure) to prevent brood production. And those killed later still generate fungal inocula, from their cadavers, that could kill their brood, thus effectively reducing viable progeny (Castrillo et al., 2011, 2013). Using mycoparasitic fungi, symbiont growth could be suppressed to reduce brood production. Although mycoparasitic fungi do not kill foundresses, our results

showed that *T. harzianum* T-22 treatments reduced *X. crassiusculus* and *X. germanus* brood size to numbers comparable to those from foundresses exposed to entomopathogens *B. bassiana* GHA and *M. brunneum* F52.

The potential of *Trichoderma* spp. as biological control agents is based on their ability to compete for nutrients, produce cell wall degrading enzymes and exert antibiosis against other fungi (Benitez et al., 1998). Our in vitro assays showed *T. harzianum* T-22 able to outcompete different strains of *Ambrosiella* spp. associated with *X. crassiusculus* and *X. germanus*. In primary resource capture assays *T. harzianum* T-22 showed faster mycelial extension, which resulted in control over more space and resources, and then grew over (and presumably consumed) the symbionts. In secondary resource capture assays the symbiont advantage from priority effects (timing of colonization or inoculation) was temporary: the symbiont was unable to defend captured resources or space, and *T. harzianum* T-22 grew over it. *Trichoderma* spp. produce extracellular chitinases that dissolve host cells (Brunner et al., 2003), which in turn trigger more enzyme production and physiological changes that result in rapid and directed growth towards host cells (Zeilinger et al., 1999). Although we observed the mycoparasite to grow faster than the symbiont in primary competition plates, we did not compare its growth rate with or without a symbiont (host) to determine whether the presence of fungal symbionts resulted in more rapid growth of *T. harzianum* T-22.

In competition assays between symbionts and entomopathogenic fungi, priority effects were significant. Symbionts that were established for a week grew over the entomopathogens and covered the whole plate. In contrast, in primary assays, the entomopathogen became established and grew until surrounded by symbiont mycelia, resulting in a deadlock between the competing fungi. Colonies of either *B. bassiana* GHA or *M. brunneum* F52 adjacent to symbiont

mycelia, however, were often found surrounded by dark pigment produced by the symbiont. These dark pigments, also observed in interspecific interactions between wood decay fungi (Boddy, 2000), are produced at the interface between competing fungi and are associated with areas of intense enzyme activity (i.e., phenoloxidase and peroxidases) producing melanin that could function as a barrier to enzymes produced by competing fungi (White and Boddy, 1992). This defense mechanism, however, may not be sufficient in primary resource capture competitions between symbionts and *B. bassiana* GHA, since the latter has been observed to start growing over a few symbiont strains after longer incubation period. In this case, *B. bassiana* GHA was observed to grow and produce conidia over *A. roeperi* strains. This specificity, however, may be an artifact of the *in vitro* assay since we have observed in our field studies galleries of *X. germanus* lined with *B. bassiana* GHA or *M. brunneum* F52 mycelia and conidia from infected foundresses (Castrillo et al., unpublished). We have also observed the same phenomenon following attack of *B. bassiana* GHA-treated beech logs by another ambrosia beetle, *Cnestus mutilatus* (Blanford) (Coleoptera: Curculionidae: Scolytinae) (Castrillo et al., unpublished). *Beauveria bassiana* GHA or other strains of this entomopathogen may be able to outcompete other ambrosia fungi, but this would require additional testing.

The complementary beetle bioassays were critical in determining the impact of fungal competition on beetle brood production, more so for *T. harzianum* T-22 than the entomopathogenic fungi. Effects of the latter on beetle brood productions are determined primarily by the death of the foundress and its timing in relation to egg laying and the production of fungal inocula by the infected cadaver. Any negative interactions between symbiont and conidia of *B. bassiana* GHA, though, could further impact brood numbers. However, since our beetle bioassays were conducted over a relatively short time period, we did not observe

sporulating cadavers that could result in continued growth within galleries or infect beetle brood. An earlier study has shown progeny of all stages, including eggs, susceptible to fungal infection from inocula produced by dead foundresses (Castrillo et al., 2011). We also did not observe *T. harzianum* T-22 mycelia overtaking symbiont growth, as in *in vitro* assays, or filling up beetle galleries, but the sparse and patchy growth of symbionts and the smaller brood produced in these galleries provide evidence of the indirect negative impact of this mycoparasite on beetle populations. Fungal *in vitro* competition assays may yield results of limited use because the outcome could vary based on the nutritional content of the artificial medium (e.g., Schubert et al., 2008) or the life stage of the competing fungi (i.e., spore versus mycelia; e.g. Kennedy et al., 2011, 2007). Results do not always agree with those from tests done on natural substrates (e.g., Klepzig, 1998), and thus could be poor predictors of biological control potential. These plate assays, however, provide initial data for evaluating negative interactions possible between fungal species or strains of interest. In our study the plate competition assays, in combination with beetle bioassays, showed that *in vitro* assays could be used for screening mycoparasitic fungi and predicting impact on beetle brood production.

The shorter incubation time of beetle bioassays allowed better comparison of symbiont growth among treatments but limited our observations on how changes in symbiont growth could affect foundress behavior. In our initial study on *T. harzianum* against *X. crassiusculus* (Castrillo et al., 2013), in which stems were incubated for 14 d, we observed longer tunnels with no symbiont growth. Although the foundresses were still alive, no eggs were present. These tunnels were often readily distinguishable externally by more sawdust produced by the boring foundresses. Since foundresses lay their eggs only after establishment of their fungal symbiont, the suppression of symbiont growth by *T. harzianum* could lead to longer tunnels and possibly

even foundresses leaving the gallery for another site. We have often seen foundress vacate existing galleries in logs or stems held in conditions that do not favor symbiont growth (e.g., lack of adequate moisture) (Castrillo et al., unpublished). In tree nurseries where cosmetic damages can result in unmarketable trees, multiple holes from repeated beetle attempts to tunnel and build galleries can make the use of mycoparasitic fungi a less attractive option. These fungi, however, offer the benefit of controlling plant pathogenic fungi that may be accidentally vectored by ambrosia beetles (e.g., Agnello et al., 2015; Beaver, 1989; Harrington, 2009). Abandoned beetle galleries could also be vulnerable to pathogenic or wood decay fungi (Hijii et al., 1999). *Trichoderma* spp. have been applied as control agents against several plant diseases, potentially including important fungal pathogens of trees (Woo et al, 2014; Schubert et al., 2008), but this secondary benefit needs to be weighed in consideration of other economic factors.

The aggressiveness of *T. harzianum* T-22 against other fungi extends to the entomopathogens *B. bassiana* GHA and *M. brunneum* F52. Preliminary competition studies (Castrillo et al., unpublished) showed the mycoparasite growing over the slower growing entomopathogens. This means that pursuit of a combined strategy to kill both foundresses and symbionts would require further study. Nevertheless, either entomopathogenic fungi or mycoparasitic fungi offer the potential of limiting beetle population build up in the field directly or indirectly, respectively, if applied to target the emerging first generation foundresses in late spring when colonization activity is highest (Oliver and Mannion, 2001; Reding et al., 2013). Moreover, possible negative interactions between entomopathogenic fungi and the fungal symbionts could compound the former's impact on beetle brood production. Additional studies on the field efficacy of these biological control fungi are currently underway in efforts to develop

application strategies for management of *Xylosandrus* spp. and other ambrosia beetles attacking trees in orchards and nurseries.

Acknowledgements

We thank Richard Humber (USDA ARSEF Culture Collection, Ithaca, NY) for providing strain ARSEF 5198 (= F52), Laverlam (Butte, MT) for providing strain GHA and Matthew Krause (BioWorks, Inc., Victor, NY) for providing RootShield® WP. This work was funded in part by grants from the Horticultural Research Institute (Project nos. 476 and 593) and through an agreement between the USDA ARS and Cornell University (A. E. Hajek, cooperator).

This paper reports the results of research only. Mention of a proprietary product does not constitute a recommendation or endorsement for its use by Cornell University or the U. S. Department of Agriculture.

References

Agnello, A., Breth, D., Tee, E., Cox, K., Warren, H.R., 2015. Ambrosia beetle - An emergent apple pest. New York Fruit Quarterly 23, 25-28.

Atkinson, T.H., Foltz, J.L., Wilkinson, R.C., Mizell, R.F., 2011. Granulate ambrosia beetle, *Xylosandrus crassiusculus* (Motschulsky) (Insecta: Coleoptera: Curculionidae: Scolytinae). University of Florida IFAS Extension Publication EENY131. (<http://edis.ifas.ufl.edu/in288>)

Batra, L.R., 1967. Ambrosia fungi: A taxonomic revision, and nutritional studies on some species. Mycologia 59, 976-1017.

459

460 Batra, L.R., 1963. Ecology of ambrosia fungi and their dissemination by beetles. Transactions of
 461 the Kansas Academy of Science. 66, 213-236.

462

463 Beaver, R.A., 1989. Insect-fungus relationship in the bark and ambrosia beetles, in: Wilding, N.,
 464 Collins, N.M., Hammond, P.M., Webber, J.F. (Eds.), Insect –Fungus Interactions. Academic
 465 Press, NY, pp. 121-143.

466

467 Benitez, T., Delgado-Jarana, J., Rincon, A.M., Rey, M., Limon, C.M., 1998. Biofungicides:
 468 *Trichoderma* as biocontrol agents against phytopathogenic fungi. Recent Research
 469 Developments in Microbiology 2, 129-150.

470

471 Biedermann, H.W., Klepzig, K.D., Taborsky, M., 2009. Fungus cultivation by ambrosia beetles:
 472 behavior and laboratory breeding success in three Xyleborine species. Environmental
 473 Entomology 38, 1096-1105.

474

475 Boddy, L., 2000. Interspecific combative interactions between wood decaying Basidiomycetes: a
 476 review. FEMS Microbiology Ecology 31, 185-194.

477

478 Brunner, K., Peterbauer, C.K., Mach, R.L., Lorito, M., Zeilinger, S., Kubicek, C.P., 2003. The
 479 Nag1- N-acetylglucosaminidase of *Trichoderma atroviridae* is essential for chitinase induction
 480 by chitin and of major relevance to biocontrol. Current Genetics 43, 289-295.

481

482 Castrillo, L. A., Griggs, M. H., and Vandenberg, J. D. 2013. Granulate ambrosia beetle,
483 *Xylosandrus crassiusculus* (Coleoptera: Curculionidae), survival and brood production following
484 exposure to entomopathogenic and mycoparasitic fungi. *Biological Control* 67, 220-226.
485

486 Castrillo, L.A., Griggs, M.H., Vandenberg, J.D., 2011. Virulence of commercial strains of
487 *Beauveria bassiana* and *Metarhizium brunneum* (Ascomycota: Hypocreales) against adult
488 *Xylosandrus germanus* (Coleoptera: Curculionidae) and impact on brood. *Biological Control* 58,
489 121-126.
490

491 Castrillo, L.A., Ugine, T.A., Filotas, M.J., Sanderson, J.P., Vandenberg, J.D., Wraight, S.P.,
492 2008. Molecular characterization and comparative virulence of *Beauveria bassiana* isolates
493 (Ascomycota: Hypocreales) associated with the greenhouse shorefly, *Scatella tenuicosta*
494 (Diptera: Ephydrididae). *Biological Control* 45, 154-162.
495

496 Castrillo, L.A., Griggs, M.H., Vandenberg, J.D., 2004. Vegetative compatibility groups in
497 indigenous and mass-released strains of the entomopathogenic fungus *Beauveria bassiana*:
498 Likelihood of recombination in the field. *Journal of Invertebrate Pathology* 86, 26-37.
499

500 Francke-Grossman, H. 1967. Ectosymbiosis in wood-inhabiting insects, in: M Henry (Ed.),
501 *Symbiosis*, Vol. 2. Academic Press, NY, pp. 141-205S.
502

503 Frank, S. D., and C. S. Sadof. 2011. Reducing insecticide volume and nontarget effects of
504 ambrosia beetle management in nurseries. *Journal of Economic Entomology*. 104, 1960-1968.

505

506 Haack, R.A., Rabaglia, R.J., 2013. Exotic bark and ambrosia beetles in the USA: Potential and
 507 current invaders, in: Pena, J. (Ed), Potential and Invasive Pests of Agricultural Crops. CAB
 508 International, Cambridge, MA, pp. 48 -74.

509

510 Harrington, T.C., 2009. The genus *Ceratocystis*. Where does the oak wilt fungus fit?, in: Billings
 511 RF, Appel, D.N. (Eds.), Proceedings of the 2nd National Oak Wilt Symposium. Texas Forest
 512 Service Publication 166, Austin, TX, pp. 21-35.

513

514 Harrington, T.C., Aghayeva, D.N., Fraedrich, S.W., 2010. New combinations in *Raffaelea*,
 515 *Ambrosiella*, and *Hyalorhinocleriella*, and four new species from the redbay ambrosia beetle,
 516 *Xyleborus grabratus*. Mycotaxon 111, 337-361.

517

518 Harrington, T.C., Yun, H.Y., Lu, S-S., Goto, H., Aghayeva, D. N., and Fraedrich, E. W., 2011.
 519 Isolations from the redbay ambrosia beetle, *Xyleborus glabratus*, confirm that the laurel wilt
 520 pathogen, *Raffaelea lauricola*, originated in Asia. Mycologia 103, 1028-1036.

521

522 Harrington, T. C, McNew, D., Mayers, C., Fraedrich, S. W., Reed, S. E., 2014. *Ambrosiella*
 523 *roeperi* sp. nov. is the mycangial symbiont of the granulate ambrosia beetle, *Xylosandrus*
 524 *crassiusculus*. Mycologia 106, 835-845.

525

526 Hijii, N., Kajimura, H., Nishibe, Y., 1991. A note on the discoloration and fungal infiltration
 527 processes on wood tissues surrounding the gallery systems of scolytid beetles. Bulletin of the
 528 Nagoya University Forest 11, 31-38.
 529
 530 Hudson, W., Mizell, R., 1999. Management of Asian ambrosia beetle, *Xylosandrus*
 531 *crassiusculus*, in nurseries, in: James, B.L. (Ed.), Proceedings of the 44th Southern Nursery
 532 Association Research Conference, McMinnville, TN, pp. 198-201.
 533
 534 Jeffries, P., 1995. Biology and ecology of mycoparasitism. Canadian Journal of Botany 73
 535 (Supplement 1), S1284-S1290.
 536
 537 Kajimura H, Hijii, N., 1992. Dynamics of the fungal symbiont in the gallery system and the
 538 mycangia of the ambrosia beetle, *Xylosandrus mutilatus* (Blanford) (Coleoptera: Scolytidae) in
 539 relation to its life history. Ecological Research 7, 107-117.
 540
 541 Kennedy, P.G., Higgins, L.M., Rogers, R.H., Weber, M.G., 2011. Colonization-competition
 542 tradeoffs as a mechanism driving successional dynamics in ectomycorrhizal fungal communities.
 543 PLoS ONE 6, e25126.
 544
 545 Kennedy, P., Hortal, S., Bergemann, S., Bruns, T., 2007. Competitive interactions among three
 546 ectomycorrhizal fungi and their relation to host plant performance. Journal of Ecology 95, 1338-
 547 1345.
 548

549 Klepzig, K.D., 1998. Competition between a biological control fungus, *Ophiostoma piliferum*,
550 and symbionts of the pine beetle. *Mycologia* 90, 69-75.
551

552 Mayers, C.G., McNew, D.L., Harrington, T.C., Roeper, R.A., Fraedrich, S. W., Biedermann,
553 P.H.W., Castrillo, L.A., Reed, S., 2015. Three genera in the Ceratocystidaceae are the respective
554 symbionts of three independent lineages of ambrosia beetles with large, complex mycangia.
555 *Fungal Biology* 119, 1075-1092.
556

557 Mizell, R.F., Riddle, T.C., 2004. Evaluation of insecticides to control the Asian ambrosia beetle,
558 *Xylosandrus crassiusculus*, in: James, B.L. (Ed.), *Proceedings of the 49th Southern Nursery*
559 *Association Research Conference*, McMinnville, TN, pp. 152-155.
560

561 Oliver, J.B., Mannion, C.M., 2001. Ambrosia beetle (Coleoptera: Scolytidae) species attacking
562 chestnut and captured in ethanol-baited traps in middle Tennessee. *Environmental Entomology*
563 30, 909-918.
564

565 Peer, K., Taborsky, M., 2004. Female ambrosia beetles adjust their offspring sex ratio according
566 to outbreeding opportunities for their sons. *Journal of Evolutionary Biology* 17, 257-264.
567

568 Ranger, C.M., Schultz, P.B., Frank, S.D., Chong, J.C., Reding, M.E., 2015. Non-native ambrosia
569 beetles as opportunistic exploiters of living but weakened trees. *PLoS ONE* 10, e0131496.
570

571 Ranger, C.M., Reding, M.E., Schultz, P.B., Oliver, J.B., 2013. Influence of flood-stress on
 572 ambrosia beetle host selection and implications for their management in a changing climate.
 573 Agricultural and Forest Entomology 15, 56-64.
 574

575 Ranger, C.M., Reding, M.E., Persad, A.B., Herms, D.A., 2010. Ability of stress-related volatiles
 576 to attract and induce attacks by *Xylosandrus germanus* and other ambrosia beetles. Agricultural
 577 and Forest Entomology 12, 177-185.
 578

579 Reding, M.E., Ranger, C.M., Oliver, J.B., Schultz, P.B., 2013. Monitoring attack and flight
 580 activity of *Xylosandrus* spp. (Coleoptera: Curculionidae: Scolytinae): The influence of
 581 temperature on activity. Journal of Economic Entomology 106, 1780-1787.
 582

583 Schedl, K.E., 1963. Scolytidae und Platipodidae Afrikas, Band II. Revista de Entomologia de
 584 Mocambique 5, 1-594.
 585

586 Schubert, M., Fink, S., Schwarze, F.W.M.R., 2008. Evaluation of *Trichoderma* spp. as a
 587 biocontrol agent against wood decay fungi in urban trees. Biological Control 45, 111-123.
 588

589 Weber, B.C., McPherson, J.E., 1983a. Life history of the ambrosia beetle *Xylosandrus germanus*
 590 (Coleoptera: Scolytidae). Annals of the Entomological Society of America 76, 455-462.
 591

592 Weber, B.C., McPherson, J.E., 1983b. World list of host plants of *Xylosandrus germanus*
 593 (Blandford) (Coleoptera: Scolytidae). The Coleopterists Bulletin 37, 114-134.

594

595 White, N.A., Boddy, L., 1992. Extracellular enzyme localization during interspecific fungal
596 interactions. FEMS Microbiology Letters 98, 75-80.

597

598 Woo, S.L., Ruocco, M., Vinale, F., Negro, M., Marra, R., Lombardi, N., Pascale, A., Lanzuise,
599 S., Manganiello, G., Lorito, M., 2014. *Trichoderma*-based products and their widespread use in
600 agriculture. The Open Mycology Journal 8, 71-126.

601

602 Wood, S.L., Bright, D.E., 1992. A catalog of Scolytidae and Platipodidae (Coleoptera), Part 2:
603 Taxonomic Index Volume A. Great Basin Naturalist Memoirs 13, 1-833.

604

605 Zeilinger, S., Galhaup, C., Payer, K., Woo, S.L., Mach, R.L., Fekete, C., Lorito, M., Kubicek,
606 C.P., 1999. Chitinase gene expression during mycoparasitic interaction of *Trichoderma*
607 *harzianum* with its host. Fungal Genetics and Biology 26, 131-140.

608

609

Figure captions

Fig. 1. *In vitro* competition assays between fungal symbionts *Ambrosiella grosmanii* and *A. roeperi* versus biological control fungi *Beauveria bassiana* GHA, *Metarhizium brunneum* F52 and *Trichoderma harzianum* T-22. *A. grosmanii* XgVA6 control plate (A) grew to cover the whole surface of potato dextrose agar (PDA) plate, while spread of those co-inoculated at the same time (primary resource capture) with *B. bassiana* (B), *M. brunneum* (C), or *T. harzianum* (D) were blocked by the competing fungus. At the interface with competing *B. bassiana*, *A. grosmanii* was observed to produce dark pigments (B, arrow), which was more easily detected on the underside of PDA plates. Area of symbiont growth was also affected by the timing of addition of the competing fungus, especially for *T. harzianum*. Symbionts allowed to grow for a week prior to addition of biological control fungus (secondary resource capture) covered more area (E) than those co-inoculated at the same time (D). In the case of *T. harzianum*, however, the mycoparasitic fungus grew over the symbiont *A. roeperi* XgNY1 after another week (F), whether competition was primary or secondary. Similar results were observed in *A. grosmanii* XcNY1 assays (G, primary competition after one week; H, secondary competition two weeks after *T. harzianum* inoculation, with *T. harzianum* also producing conidia [arrows]). Additionally, longer incubation time of *B. bassiana* GHA co-inoculated PDA plates also allowed the biological control fungus to sporulate and spread over the symbiont *A. roeperi* C2451 (I).

Fig. 2. Radial growth of fungal symbionts *Ambrosiella grosmanii* and *A. roeperi* co-inoculated (primary resource capture) with biological control fungus, *Beauveria bassiana* GHA, *Metarhizium brunneum* F52 or *Trichoderma harzianum* T-22, on potato dextrose agar plates. Four strains of *A. grosmanii* (XgNY1, XgHO7, XgOH11, and XgVA6) and two of *A. roeperi*

(XcNY1 and C2451, representing different haplotypes for each species, were tested. Symbiont growth was measured from the center of each mycelial plug to the colony edge opposite the growing edge of the competing fungus. Means ($\pm$ SE) significantly different from control are marked with an asterisk (α 0.05; Dunnett's test).

Fig. 3. Brood production by ambrosia beetles in galleries in beech stems either untreated (control) or treated with biological control fungus, *Beauveria bassiana* GHA, *Metarhizium brunneum* F52 or *Trichoderma harzianum* T-22, at three different dosages. There were 48 foundresses per replicate, with three replicates per control or treatment-dosage combination per beetle species. Means not connected by the same letter are significantly different (α 0.05; Tukey-Kramer HSD).

Table 1. *Xylosandrus crassiusculus* and *X. germanus* survival, gallery establishment and brood production after exposure to beech stems either untreated (control) or treated with biological control fungi *Beauveria bassiana*, *Metarhizium brunneum* or *Trichoderma harzianum* at different dosages^a.

Treatment	dosage (conidia/ml)	% alive (mean $\pm$ SE)	% with gallery (mean $\pm$ SE)	% with symbiont (mean $\pm$ SE)	% with brood (mean $\pm$ SE)
<i>X. crassiusculus</i>					
Control		100 $\pm$ 0	95.7 $\pm$ 3.1	95.0 $\pm$ 3.8	79.1 $\pm$ 8.2
<i>B. bassiana</i> GHA	1 x 10 ⁶	80.4 $\pm$ 5.7	82.6 $\pm$ 6.0	79.8 $\pm$ 5.6	48.2 $\pm$ 4.9
	1 x 10 ⁵	87.1 $\pm$ 3.6	71.7 $\pm$ 8.2	66.0 $\pm$ 10.8	53.4 $\pm$ 9.8
	1 x 10 ⁴	94.5 $\pm$ 2.8	86.1 $\pm$ 7.0	82.0 $\pm$ 3.9	69.4 $\pm$ 8.4
<i>M. brunneum</i> F52	1 x 10 ⁶	49.3 $\pm$ 12.1*	63.2 $\pm$ 7.7	61.8 $\pm$ 7.0	37.5 $\pm$ 8.3
	1 x 10 ⁵	68.0 $\pm$ 11.1*	80.4 $\pm$ 1.8	78.3 $\pm$ 1.8	47.7 $\pm$ 9.4
	1 x 10 ⁴	76.2 $\pm$ 3.0	82.6 $\pm$ 14.3	67.8 $\pm$ 15.5	39.8 $\pm$ 7.2
<i>T. harzianum</i> T22	3.7 x 10 ⁵	94.9 $\pm$ 1.6	79.8 $\pm$ 8.8	78.4 $\pm$ 10.2	41.2 $\pm$ 14.8
	3.7 x 10 ⁴	88.9 $\pm$ 8.2	74.8 $\pm$ 6.3	72.0 $\pm$ 6.1	30.7 $\pm$ 7.7
	3.7 x 10 ³	84.7 $\pm$ 7.3	79.4 $\pm$ 7.2	77.1 $\pm$ 8.4	40.2 $\pm$ 14.9
<i>X. germanus</i>					
Control		100 $\pm$ 0	97.1 $\pm$ 1.5	97.0 $\pm$ 1.5	62.6 $\pm$ 5.7
<i>B. bassiana</i> GHA	1 x 10 ⁶	64.8 $\pm$ 2.7*	69.0 $\pm$ 4.9*	69.0 $\pm$ 4.9	20.4 $\pm$ 9.9
	1 x 10 ⁵	87.9 $\pm$ 1.7	65.3 $\pm$ 9.9*	65.3 $\pm$ 9.9	35.8 $\pm$ 6.3
	1 x 10 ⁴	84.6 $\pm$ 8.8	76.7 $\pm$ 7.0	76.7 $\pm$ 7.0	41.0 $\pm$ 8.5

<i>M. brunneum</i> F52	1 x 10 ⁶	46.9 ± 15.9*	68.1 ± 7.5*	68.1 ± 7.5	16.4 ± 6.1
	1 x 10 ⁵	78.1 ± 9.3	72.2 ± 4.0	72.4 ± 4.0	22.1 ± 0.5
	1 x 10 ⁴	86.8 ± 5.8	78 ± 7.7	76.6 ± 8.9	21.3 ± 6.5
<i>T. harzianum</i> T22	3.7 x 10 ⁵	95.6 ± 2.3	86.3 ± 2.5	80.4 ± 7.2	23.3 ± 10.2
	3.7 x 10 ⁴	99.3 ± 0.7	88.7 ± 4.7	85.6 ± 4.2	30.6 ± 13.9
	3.7 x 10 ³	96.5 ± 2.6	85.3 ± 5.5	82.5 ± 3.0	39.0 ± 12.4

^a Means followed by an asterisk within a column for each beetle species is significantly different from control (alpha = 0.05; Dunnett's method).

There were total of 144 beetles per species for three replicates per control or treatment-dosage combination.

Graphical Abstract

Ambrosiella grosmanniae

Ambrosiella grosmanniae +
Beauveria bassiana GHA

Ambrosiella grosmanniae +
Metarhizium brunneum F52

Ambrosiella grosmanniae +
Trichoderma harzianum T22