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Competition between biological control fungi and fungal symbionts of ambrosia beetles *Xylosandrus crassiusculus* and *X. germanus* (Coleoptera: Curculionidae): Mycelial interactions and impact on beetle brood production

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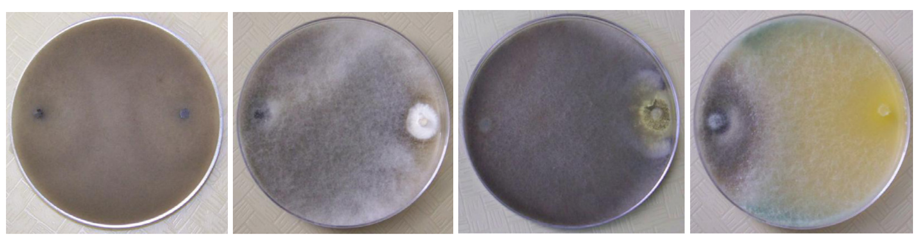
HIGHLIGHTS

- Ambrosia beetles cultivate fungal symbionts in their galleries for food.
- Biocontrol fungi were tested against symbionts of invasive ambrosia beetles.
- Symbionts defended acquired resources against entomopathogenic fungi.
- Mycoparasitic fungus *Trichoderma* outcompeted the fungal symbionts.
- Beetle galleries in *T. harzianum*-treated stems had sparse symbiont growth.
- Brood size was reduced in stems treated with either type of biocontrol fungi.

GRAPHICAL ABSTRACT



Ambrosiella grosmanniae | *Ambrosiella grosmanniae* + *Beauveria bassiana* GHA | *Ambrosiella grosmanniae* + *Metarhizium brunneum* F52 | *Ambrosiella grosmanniae* + *Trichoderma harzianum* T22

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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

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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.

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-Grossman, 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 grosmanniae* 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 ($\sim$0.5 cm^2) of 2-week old cultures were transferred to cryogenic vials with 10% glycerol and frozen at $-80\,^{\circ}C$ for long-term storage. All strains are maintained at