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Characterization of *Beauveria bassiana* (Ascomycota: Hypocreales) isolates associated with *Agrilus planipennis* (Coleoptera: Buprestidae) populations in Michigan

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ABSTRACT

Earlier research in Michigan on fungal entomopathogens of the emerald ash borer (EAB), a major invasive pest of ash trees, resulted in the isolation of *Beauveria bassiana* from late-instar larvae and pre-pupae. In the present study, some of these isolates were characterized and compared to ash bark- and soil-derived isolates to determine their reservoir and means of infecting immature EAB. Genetic characterization using seven microsatellite markers showed that most of the EAB-derived strains clustered with bark- or soil-derived strains collected from the same site, indicating the indigenous nature of most strains isolated from EAB. More soil samples contained *B. bassiana* colony forming units than bark samples, suggesting that soil serves as the primary reservoir for fungal inocula. These inocula may be carried by rain splash and air current from the soil to the lower tree trunk where EAB may become infected. Additionally, inocula could come from infected EAB or other insects infesting ash trees. Bioassay of EAB adults, exposed by dipping in conidial suspensions (10^6 conidia/ml), showed all five representative strains with comparable virulence to the commercial strain GHA. These data demonstrate that indigenous strains of *B. bassiana* have potential for use as control agents against EAB and suggest that fungal inocula applied to ash trunks may prove viable for controlling EAB in the field.

1. Introduction

Agrilus planipennis Fairmaire (Coleoptera: Buprestidae), the emerald ash borer (EAB), is an invasive pest from northeastern Asia that causes mortality of ash trees (*Fraxinus* spp.) in North America. Adults feed on ash foliage and lay eggs in bark crevices or between bark layers. Eclosing larvae bore into the bark and feed on ash phloem, and tunneling by high numbers of larvae results in girdling and death of trees. Ash saplings can die after a year of infestation while large trees may die within 3–4 years (Poland, 2007). Since the discovery of EAB in 2002 near Detroit, Michigan, it has spread to contiguous areas of the United States and Canada. Due to the transport of infested ash firewood or nursery stock, however, infestations are now known as far east as Maryland and as far west as Minnesota. As of May 2007, infested areas in Michigan, Ohio, Indiana and Ontario exceeded 103,600 km^2, and over 30 million ash trees had been killed in Michigan alone (http://www.emeraldashborer.info).

The beetle is considered a minor and periodic pest of ash trees in most of its native range (Gao et al., 2004), likely due to the presence of natural enemies and ash species with higher resistance to the beetle (Liu et al., 2003). The potential of natural enemies for biological control of EAB in North America led to further research in China, within the beetle's native range, on the population biology of EAB and its parasitoids on different ash species (Bauer et al., 2005, 2006; Liu et al., 2007). Surveys of natural enemies attacking beetle populations in North America, primarily in Michigan where the beetle is most widespread, were also conducted (Bauer et al., 2004, 2005). The results of research conducted from 2002 to 2004 in southeastern Michigan revealed <1% of EAB were parasitized by braconids, a chalcid and a eupelmid (Bauer et al., 2005). Approximately 2% were infected with fungi, including isolates of *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium anisopliae* (Metschnikoff) Sorokin, *Isaria farinosa* (Holmsk.) Fries (formerly *Paecilomyces farinosus*), *I. fumosorosea* Wize (formerly *P. fumosoroseus*), and *Lecanicillium lecanii* (Zimmerman) Viegas (Bauer et al., 2004, 2005). Further sampling by L.S. Bauer and

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H. Liu in different areas of Michigan resulted in additional *B. bassiana* isolates from infected EAB (unpublished data). These isolates represent some of the presumably indigenous fungal populations pathogenic to the exotic EAB.

The objectives for our study were to: (1) determine the reservoir for *B. bassiana* by culturing fungi from soil and bark sampled from ash trees at Michigan field sites where infected beetles were previously collected; (2) characterize and compare the *B. bassiana* isolates obtained from infected EAB and the bark and soil samples; and (3) compare the virulence of representative *B. bassiana* isolates against adult EAB. These data will provide information on the potential of indigenous fungal strains for use in the microbial control of EAB and may demonstrate effective strategies for their deployment in the field.

2. Materials and methods

2.1. Sample collection and fungal isolation

Beauveria bassiana was isolated from EAB larvae and pre-pupae from infested ash trees felled in Michigan field sites from 2002 to 2006. Infected beetles were observed during dissection and rearing out of adults from infested logs in the laboratory. Some of the field sites sampled were part of earlier research on EAB natural enemies, initiated in 2002 (Bauer et al., 2004, 2005). Many of these fungal isolates were deposited in the USDA ARS Collection of Entomopathogenic Fungal Cultures (ARSEF; Ithaca, NY) (LSB and HL, unpublished results).

In our present study, we obtained 42 of the EAB-derived *B. bassiana* isolates from ARSEF, established monosporic isolates (Castrillo et al., 2004), and assessed their molecular diversity. To reveal possible reservoir of *B. bassiana* inocula, we returned to four of the previously-studied field sites in September 2007 and sampled soil and bark from some remaining stumps and dead trees, primarily green ash, *F. pennsylvanica* Marsh. There were numerous EAB exit holes and old galleries under the bark of these ash trees, indicating previous EAB infestation. The four sites were Hudson Mills Metropark in Dexter, Island Lake Recreation Area in Brighton, Western Golf and Country Club in Redford, and Willoughby City Park in Lansing. The number of bark and soil samples collected at each site are listed in Table 1. When these sites were sampled earlier, EAB densities were moderate (50–100 EAB/m^2) at Brighton, and high (>100 EAB/m^2) at Dexter, Redford, and Lansing (LSB and HL, unpublished data). *B. bassiana* was isolated from bark and from soil samples collected at the four sites and compared to EAB-derived isolates. These isolates and those sampled from infected beetles were identified as *B. bassiana* based on morphological characters (Humber, 1997).

Ash bark (~3.6 cm^2) was sampled from four to five remaining ash trees or stumps in each site by use of a wood chisel (1.9 cm) that was disinfected with 10% bleach between samples. Each sample was collected in a sterile 50-ml conical polypropylene tube, transported in a cooler and stored at 4 °C until processing within a few days. Soil samples were collected from the upper 8 cm of soil surface beneath each sample tree using a soil auger (1.6 cm diam.) (Oakfield Apparatus Company, Oakfield, WI, USA) that was also disinfected with 10% bleach between samples. Additional soil samples were collected in Redford from mounds where infested ash trees were felled. Each soil sample was stored in a sterile sampling bag and handled as the bark samples. The bark samples were vortexed in 10 ml of autoclaved 0.2% aqueous Tween 80 (Fisher Scientific, Suwanee, GA), a 10-fold dilution was prepared and 200-μl aliquots plated on wheat germ dodine agar (WGDA; Sneh, 1991), a semi-selective medium. For soil samples, 10 g subsamples were transferred to sterile 50-ml conical polypropylene tubes and vortexed in 9 ml of 0.2% aqueous Tween 80. The soil suspension was held stationary for 5–10 min to allow large particles to settle before the supernatant was subjected to a series of three 10-fold serial dilutions, after which 200-μl aliquots were plated on WGDA. We used three replicate plates per stock wash and dilutions per bark or soil sample. Plates were incubated at 24 °C with 16:8 L:D and examined after 5-7 days.

Representative colonies with *Beauveria* sp. morphology were transferred to Sabouraud dextrose agar plates supplemented with 1% yeast extract (SDAY; Difco Manual) and incubated under the same conditions used for WGDA plates. Monosporic isolates were established from *B. bassiana* colonies representative of the different morphotypes detected. A total of 21 soil-derived and 15 bark-derived isolates were included in this study. We included three additional soil-derived isolates collected in 2006 at Gee Farms Nursery, Stockbridge, Michigan, where we evaluated the field efficacy of the *B. bassiana* commercial strain GHA. These three represented the more common genotypes collected from this site. We also included the commercial strain GHA, which we isolated from a technical grade spore product (lot 980528) provided by Mycotech (now Laverlam International Corp., Butte, MT). Throughout this paper, we will refer to each fungal collection as an isolate, whereas isolates will be referred to as strains if they have been characterized by molecular assays in other studies or later in this study (Carlile et al., 2001). Following molecular analysis, we deposited representative strains derived from the bark and soil samples to ARSEF. The fungal strains used in this study are listed in Fig. 1.

2.2. Molecular characterization of fungal isolates

Fungal DNA was extracted from blastospores, grown as described by Pfeifer and Khachatourians (1993), using the DNeasy Plant Mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol with modifications. Blastospore pellets from 2 ml of 4-day-old cultures were homogenized with 0.5 g of 0.5 mm zirconia/silica beads (BioSpec Products, Bartlesville, OK) in 400 μl of lysing buffer (Qiagen) using a Mini Bead Beater (BioSpec Products) for 30 s at 4200 rpm. DNA was eluted once with 100 μl elution buffer (Qiagen) and stored at −20 °C until used.

Seven microsatellite markers developed by Rehner and Buckley (2003) for *B. bassiana* were used to assess genetic diversity among the beetle-, bark-, and soil-derived isolates. The primers (GenBank accession No.) used were: Ba01 (AY212020), Ba02 (AY212021), Ba03 (AY212022), Ba06 (AY212024), Ba08 (AY212025), Ba12 (AY212026), and Ba13 (AY212027). PCR assay conditions were as reported in Castrillo et al. (2008). Assays were repeated at least twice for each primer-isolate pair. PCR products were visualized in 3% (wt/vol) Metaphor gel (Lonza, Rockland, ME) in TBE buffer (90 mM Tris-borate, 2.0 mM EDTA), stained with ethidium bromide. Gels were analyzed using GeneGenius gel documentation and imaging system (Syngene, Frederick, MD).

The molecular size of each PCR product generated by each microsatellite primer pair was calculated using Gene Tools (Syngene).

Table 1
Beauveria bassiana colony forming units (CFU) obtained from ash bark and soil samples from different sites in Michigan.

Collection site	Mean *B. bassiana* CFU ± SE (samples with CFU/total collected)	
	per 3.6 cm^2 bark sample	per 10 g soil sample
Brighton	0 (0/5)	0 (0/5)
Dexter	$1.9 \pm 2.78 \times 10^2$ (2/5)	189 ± 135 (4/5)
Lansing	0 (0/5)	$7.85 \pm 9.89 \times 10^3$ (5/5)
Redford	$3.25 \pm 5.74 \times 10^3$ (3/4)	20 ± 40 (2/8) [a]

[a] Four of the eight soil samples from Redford, MI, were collected from mounds where infested ash trees were felled. None of these samples yielded any *B. bassiana* CFU. The other four samples were collected at the base of ash trees.