Chapter 10
Use of Entomopathogens against Invasive Wood Boring Beetles in North America

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Abstract *Anoplophora glabripennis* and *Agrilus planipennis* are wood-boring beetles introduced from China to North America that are capable of killing healthy trees; *A. glabripennis* is polyphagous but attacks on maples (*Acer* spp.) are of major concern in North America, and *A. planipennis* attacks ash trees (*Fraxinus* spp.). Bioassays against *A. glabripennis* with entomopathogenic fungi identified that a strain of *Metarhizium anisopliae* (F 52) is virulent against adults. The primary deployment method investigated is propagation of the fungus within bands of non-woven fiber material. The fungal bands are then wrapped around tree trunks or branches, where wandering adults become contaminated with spores when walking across bands. Bands retain concentrations of viable conidia above the LC_{50} for >3 months. Infections also decrease reproduction before females die, resulting in fewer offspring. A strain of the entomopathogenic fungus *Beauveria bassiana* (GHA) sprayed on infested ash trees causes mortality of adult *A. planipennis* as they emerge from tree trunks. Cover sprays also result in fungal infections of *A. planipennis* larvae, pupae and adults that have not yet emerged, due to bark splits that form over the larval galleries providing points of entry for fungal inoculum under the tree bark. In addition, recent bioassays identified a strain of *Bacillus thuringiensis* virulent against *A. planipennis*. Development of this microbial control agent for aerial application is planned to target adult beetles that feed throughout their lives on ash foliage in the tree canopy.

10.1 Introduction

Twenty-five exotic species of bark- and wood-boring beetles were found to be established in the continental United States between 1985–2005, including two species of buprestids, five cerambycids, and 18 scolytids (Haack 2006). Invasive beetle species that bore in wood and are targets of microbial control agents belong to the families

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Cerambycidae (longhorned beetles) and Buprestidae (jewel beetles). It is thought these species were initially accidentally introduced when beetle-infested wood was moved to areas where these species are not native, most probably through shipping. Here we discuss microbial control of two invasive species of beetles in North America, the cerambycid *Anoplophora glabripennis* (Asian longhorned beetle) and the buprestid *Agrilus planipennis* (emerald ash borer). While many species in these beetle families are associated with dead or dying trees, both of these species are known to be able to kill healthy trees.

Wood-boring beetles are difficult to control because the larvae live under tree bark or within wood. These beetles develop slowly and larval stages, that may be present in trees for long periods, are usually very difficult to detect. Once adults emerge, they are also difficult to detect because pheromones and host attractants for these beetle groups are poorly understood, so allelochemical lures are inefficient or unavailable. Adults typically disperse high into tree canopies to feed, and are therefore also difficult to control. Adding to difficulties in control, adults of these species emerge from wood asynchronously, over many months, and can be long-lived.

One factor driving the development of microbial control agents for management of wood-boring beetles is the limited efficacy of conventional insecticides. It is difficult or impossible to reach all larval or adult beetle feeding and resting sites when cover sprays are applied to tree canopies. The favored application method for control of wood borers is systemic uptake of insecticides through either trunk or soil injections. Systemic uptake, however, can result in uneven distribution of low insecticide concentrations within trees (Poland *et al*. 2006a). Moreover, systemic insecticides also travel up the tree through the xylem, resulting in limited efficacy against phloem feeders such as *Agrilus* spp. and early instar *A. glabripennis*.

There are numerous advantages to using microbial agents for control, particularly for wood-boring beetles. Due to lack of optimal control methods for wood-boring beetles, infested trees, once detected, are often removed and destroyed either to eradicate an invasive pest or, if the pest is established, reduce the population and remove hazardous trees. When possible, the removal of valuable urban and suburban trees can be avoided by individual treatment and use of microbials; this is especially true for trees in urban/suburban areas. In forests, all infested trees cannot be removed necessitating development of alternate methods for controlling these destructive beetles.

The fungi constitute the only insect pathogen group that has been exploited against invasive wood-boring beetles. The fungal species used are anamorphs of Hypocreales, e.g. *Metarhizium anisopliae* and *Beauveria bassiana* (Hajek & Bauer 2007). Appropriate application methods have been adapted for the different behaviors of targeted species. As will be described below, the development of microbial control against *A. planipennis* has focused on use of conidial sprays. In contrast, for *A. glabripennis*, non-woven fiber bands impregnated with fungal cultures have been emphasized. The "fungal band" application technology was originally developed in Japan for control of native cerambycids attacking orchards (Higuchi *et al*. 1997). Fungal bands are now utilized for management of the cerambycid vector of the pinewood nematode, *Monochamus alternatus*, in Japan (Chapter 9), and fungal

bands in combination with allelochemicals are presently being developed against *M. alternatus* in China (Li *et al*. 2007).

The most widely used microbial control agents for all arthropods worldwide are formulated isolates of the insect-pathogenic bacterium *Bacillus thuringiensis* (*Bt*). Most registered products based on *Bt* are for control of lepidopteran, dipteran, or coleopteran pests (Glare & O'Callaghan 2000). A coleopteran-active *Bt* strain with high toxicity against *A. planipennis* adults is currently under development for possible use in an aerial spray program to suppress population densities of adults of this species, which feed in the ash canopy on foliage throughout their relatively long lives.

10.2 Development of Entomopathogenic Fungi for Control of *Anoplophora glabripennis*

Anoplophora glabripennis, the Asian longhorned beetle, is native to China and Korea but has been found in several areas of North America (Hajek 2007). It was first discovered in Brooklyn, New York, in 1996 and was subsequently also found in Chicago, New Jersey and Toronto. *A. glabripennis* can attack and kill relatively healthy trees of numerous hardwood species. This beetle species is a major killer of poplar (*Populus* spp.) and willow (*Salix* spp.) trees in China. In North America, maples (*Acer* spp.) are the hosts of greatest concern. Because this beetle has caused unparalleled tree mortality in China (Lingafelter & Hoebeke 2002), eradication is a priority in North America. Successful eradication seems possible because *A. glabripennis* is only present in a few areas of urban forest in North America, and is not known to occur in the native forest. Populations of *A. glabripennis* are relatively low, and the US and Canadian governments have been willing to support costly eradication campaigns. In the US alone, eradication programs cost $US 225 million from 1997–2006 (US GAO 2006) and >8,000 and >12,000 trees have been cut down in the US and Canada, respectively (see Poland *et al*. 2006a).

A. glabripennis generally has one generation per year in the US. Most adults emerge from trees from May–October and feed for 9–15 days before becoming sexually mature (see Hajek *et al*. 2008). Adults then mate, after which males guard females while egg niches are chewed by females in the bark and an egg is subsequently laid under the bark at the side of each niche. After eggs hatch, larvae tunnel in the sapwood under the bark for a few instars before moving into the heartwood. When initially attacking a tree, females often lay eggs near the tree crown base in the trunk and main branches but, with successive years of attack, eggs can be laid throughout much of the tree. Tunneling by larval *A. glabripennis* initially causes branch dieback and trees are eventually killed when there are high populations or when *A. glabripennis* reinfests the tree over successive years (Haack *et al*. 1997). Before tree death, larval tunneling weakens tree branches and trunks, which break more easily, leading to hazard trees.

The North American *A. glabripennis* eradication campaigns include detection, removal and then destruction of infested trees. Aside from removing infested trees, susceptible host trees occurring in a zone around the infestation are treated

with preventive systemic applications of the neonicotinoid insecticide imidacloprid. Imidacloprid applications principally target adult beetles that feed on midribs of leaves, leaf petioles and the bark of twigs. However, the distribution of imidacloprid within trees is variable (Poland *et al*. 2006a) so adults ingest unpredictable dosages. Applications could affect newly hatched larvae in the phloem-cambial region but it is thought that larger larvae in the xylem might not be exposed to lethal doses of insecticide (Poland *et al*. 2006a). The extent to which imidacloprid applications kill adults or larvae in North America is unknown (populations are very low) although it is known that sublethal doses could lead to increased adult dispersal because imidacloprid acts as an antifeedant (Poland *et al*. 2006b). Therefore, while systemic imidacloprid certainly impacts some *A. glabripennis*, there is a need for additional eradication methodologies.

Development of a microbial control strategy was based on a commercial product used against several cerambycid pests of Japanese orchards, including *Anoplophora chinensis* (= *A. malasiaca*) (Higuchi *et al*. 1997). In Japan, cultures of *Beauveria brongniartii* are grown in non-woven fiber bands, producing lawns of conidia on band surfaces. The bands are attached around branches and tree trunks when cerambycids reach adulthood and emerge from trees. Cerambycids commonly walk on tree trunks and branches and, as they walk across the bands, they contaminate themselves with conidia. Optimal exposure to bands occurs during the preovipositional period when infection can prevent or reduce oviposition. Contaminated beetles can also transmit infective spores when mating (Tsutsumi 1998). Bands of *B. brongniartii* can be stored at 5°C for >1 year and remain active in the field for at least one month (Higuchi *et al*. 1997). In addition, bands produced in Japan are made from wood pulp so they are biodegradable and therefore do not have to be removed from trees.

10.2.1 Laboratory Trials

Development of fungal bands for control of *A. glabripennis* began with bioassays comparing twenty-two isolates of four species of entomopathogenic Hypocrealean fungi [*Beauveria bassiana*, *Beauveria brongniartii*, *Isaria farinosa*, and *Metarhizium anisopliae*] against adults and larvae in China and in a quarantine in Ithaca, New York. Preliminary studies demonstrated lower virulence of *B. brongniartii* and *B. bassiana* isolates against larvae compared with adults so further bioassays focused only on adults (Dubois 2003). Survival times for 50% of the beetles tested (ST_{50}) ranged from 5.0 days (*M. anisopliae* ARSEF 7234 and *B. brongniartii* ARSEF 6827) to 24.5 days (*I. farinosa* ARSEF 8411) days (Dubois *et al*. 2007). Screening studies initially included strains of *B. brongniartii*, which is registered as a microbial control agent in Europe, Asia and South America but not in North America (Faria & Wraight 2007). At that time, we could not confirm that this fungal species is native to North America, which added uncertainty regarding future registration for pest control in the US. Further laboratory bioassays identified three isolates of *M. anisopliae* killing adults in 5–6 days, with no difference in time to death between males

and females. Therefore, our subsequent studies focused on one of these isolates (*M. anisopliae* F 52) that is already registered for control of various ticks and beetles, root weevils, flies, gnats and thrips in the US (US EPA 2003). Fungal bands were produced using similar procedures to those used in Japan (Shanley 2007). Adult beetles were exposed to squares of *M. anisopliae* F 52 fungal bands to establish the median lethal concentration of 6.8×10^6 conidia/cm^2 (Shanley 2007).

10.2.2 Field Trials with Fungal Bands

Field trials with *A. glabripennis* have been conducted in China because North American populations are very low and this species is targeted for eradication, which means that any infestations detected are quickly destroyed. Initial studies in 2000 used field-collected *A. glabripennis* adults caged within 1 m long window screen cages on tree trunks, with fungal bands encircling the trunks within some of the cages (Dubois *et al*. 2004a). Adults caged with *B. bassiana* and *B. brongniartii* bands died more quickly than controls and female oviposition decreased significantly in treated cages. For comparison, conidia were sprayed onto the surfaces of tree trunks within cages, and days to death for adults exposed to treated tree bark were the same as for adults in cages with fungal bands. However, 10 days after application, while conidial viability remained high on fungal bands, viability of conidia that had been sprayed onto tree trunks was drastically reduced. A similar study was conducted in 2001 but results were more variable. Researchers hypothesized that the variable results in 2001 were caused by high temperatures during the trial (range maxima: 30.2–38.5°C). This hypothesis was supported by loss of fungal band viability after 15 days on trees during 2001.

In 2001, uncaged trials compared areas with bands of two *B. brongniartii* isolates on each of 100 trees with nearby control areas without bands. Adult beetles were regularly collected and reared and oviposition was quantified (Dubois *et al*. 2004b). Some treatments resulted in decreased adult longevity compared with controls or decreased oviposition. In 2002, a similar study was conducted in a different site, large enough to allow additional replication and with higher populations of *A. glabripennis* (Hajek *et al*. 2006). Adults collected from fungal-treated plots 7–22 days after band placement died more quickly than controls, although results were more consistent for the *B. brongniartii* isolate tested than for the *M. anisopliae* isolate. Once again, oviposition was reduced in the treated plots compared with the control plots.

10.2.3 Longevity of Activity of Fungal Bands

To evaluate the length of time that fungal bands maintain viability, during the summers of 2001–2004, bands impregnated with strains of *M. anisopliae*, *B. bassiana*, and *B. brongniartii* were attached to tree trunks in localized sites in Queens, New York City (AE Hajek unpublished data). Bands were removed at varying

intervals to quantify viable conidia and to conduct bioassays. Percent germination of conidia from bands did not decrease consistently with time in the field although total conidial density and density of viable conidia decreased with increasing time in the field (AE Hajek unpublished data).

For bioassays, death of quarantine-colony adults within 40 days of exposure to pieces of band from the field was used to calculate percent mortality. For bioassays of bands from 2001–2003, 100% mortality of treated beetles always occurred. In 2004, however, mortality dropped below 100% before the end of the trial with *M. anisopliae* F 52 (112 days after bands were placed in the field), although mortality still remained at $> 50\%$. Bioassays conducted using samples of *M. anisopliae* F 52 bands taken from the field identified an LC_{50} of 7.22×10^6 conidia/cm^2, which was very similar to laboratory results. Although densities of viable conidia on bands decreased over time in 2004, densities of viable conidia never decreased below the LC_{50}. These results demonstrated that *M. anisopliae* F 52 bands retain virulence in the field for at least 112 days (AE Hajek unpublished data).

10.2.4 Indirect Effects of Fungal Bands

Studies were also conducted to evaluate the indirect effects of fungal bands to address whether only adults walking across bands are affected by treatments. Field studies had demonstrated an impact of fungal treatment on female fitness so assays were conducted using a quarantine colony to explore this further. Bioassays tested the effects of *M. anisopliae* infection on reproduction by adult female *A. glabripennis* and progeny survival (Hajek *et al*. 2008). The effect of infection on fecundity was evaluated for females already laying eggs and for newly eclosed females using two isolates of *M. anisopliae*. Both longevity and oviposition were significantly lower for females that were already laying eggs when exposed to *M. anisopliae* ARSEF 7234, when compared with controls. Newly eclosed females exposed to *M. anisopliae* ARSEF 7711 also displayed shortened longevity compared with controls (10.0 ± 0.7 days vs 74.3 ± 6.8 days for controls) and decreased oviposition (1.3 ± 0.7 eggs per ARSEF 7711-exposed female vs 97.2 ± 13.7 eggs per female for controls). Percentages of eggs that did not hatch were greater than controls for both age groups of fungal-treated females and unhatched eggs frequently displayed signs of fungal infection. The percentage of larvae dying within 9 weeks of oviposition was higher for progeny from sexually mature females exposed to ARSEF 7234 compared with controls, and dead larvae usually displayed signs of fungal infection. Thus, for both ages of females and both fungal isolates, fewer surviving larvae were produced after fungal inoculation of females, compared with controls. Infection with *M. anisopliae* affects female fitness by decreasing female longevity and fecundity, and through horizontal transmission of *M. anisopliae* to some offspring.

Japanese studies have shown that when adult yellow-spotted longicorn beetles (*Psacothea hilaris*) were exposed to a *B. brongniartii* band and then introduced to potential mates, horizontal transmission could occur (Tsutsumi 1998). During initial studies, female *A. glabripennis* were exposed to *M. anisopliae* bands and then caged

with males. All exposed males died more quickly than controls but they did not die as quickly as the exposed females, which is consistent with receiving a lower dose (AE Hajek unpublished data).

In a related study, Shanley & Hajek (2008) investigated whether conidia from *M. anisopliae* bands could be dispersed to other parts of the environment by *A. glabripennis* adults that had contacted bands and whether *A. glabripennis* would become infected with exposure to tree bark contaminated with conidia in this way. One or five adult *A. glabripennis* were used to contaminate tree bark with conidia. All adults subsequently exposed to contaminated environments were killed by *M. anisopliae* infections. Beetles exposed to environments contaminated by five beetles died more quickly than beetles exposed to environments contaminated by one beetle. Beetles at both density treatments died in fewer days than beetles exposed to environments without *M. anisopliae* conidia.

A follow-up field study examined whether conidia from *M. anisopliae* bands spread naturally to other parts of the environment, and if these conidia are infective to *A. glabripennis*. In the field, bands containing *M. anisopliae* were hung on tree trunks at 3 m height (Shanley & Hajek 2008). Bark samples were taken 10–30 cm above and 10–60 cm below bands up to 9 days after band placement to quantify densities of viable conidia. More viable conidia were detected in samples below bands compared with samples above bands. A significant positive correlation was found between rainfall and the occurrence of conidia on any of the bark samples. However, the concentrations of viable conidia were lower than LC_{50} estimates, suggesting that *A. glabripennis* adults may not become infected based on environmental contamination resulting from natural conidial dispersal from fungal bands.

10.3 Development of Entomopathogens for Control of *Agrilus planipennis*

Agrilus planipennis, the emerald ash borer, is a periodic pest of ash trees (*Fraxinus* spp.) in northeast Asia (Yu 1992). In 2002, this buprestid was identified as the causal agent of ash tree mortality in southern Michigan and Ontario (Haack *et al*. 2002). *A. planipennis* was likely introduced from China during the 1990s to Lower Michigan in infested wooden packing materials or manufactured goods and became established in the abundant ash resources throughout urban, forested, and riparian ecosystems (Poland & McCullough 2006). Infestations of *A. planipennis* have since been detected in the Upper Peninsula of Michigan, Ohio, Indiana, Illinois, Maryland, Pennsylvania, and West Virginia.

Agrilus planipennis threatens the 16 species of *Fraxinus* native to North America (USDA NRCS 2008), which include abundant ash species used for lumber and wood products. Forest inventories report almost 8 billion ash trees on US timberlands at a compensatory value of $US 282.25 billion (USDA FS 2008). *Fraxinus* species are also the most common trees used to replace landscape plantings of American elms (*Ulmus americana*), decimated by Dutch elm disease in much of North America. The costs for removal and replacement of ash trees killed by *A. planipennis* to

communities and smaller landholders are also high, e.g. the expense for ash removal and replacement in six infested southeastern Michigan Counties was estimated at $US 11.7 billion (US Federal Register 2003).

All *Fraxinus* species endemic to the northeastern states and provinces of North America are susceptible to mortality from *A. planipennis*. These include white ash (*F. americana*), green ash (*F. pennsylvanica*), and black ash (*F. nigra*) trees, major components of forests, and the less common blue ash (*F. quadrangulata*) and pumpkin ash (*F. profunda*). Each *Fraxinus* species is adapted to slightly different habitats within forest ecosystems. Several species are tolerant of poorly-drained sites and wet soils, protecting environmentally-sensitive riparian areas, e.g. pure stands of black ash grow in bogs and swamps in northern areas where they provide browse for various wildlife species, thermal cover and protection for ungulates such as deer and moose. In agricultural and shelterbelt areas, ash trees are one of the more prevalent tree species, and protect fragile riparian zones, prevent erosion, and provide shelter for livestock. In forested areas, the bark of young ash trees is a favored food of mammals including beaver, rabbit, and porcupines, whereas older trees provide habitat for cavity-nesting birds such as wood ducks, woodpeckers, chickadees and nuthatches, and seeds are consumed by ducks, song and game birds, small mammals and insects.

In an effort to contain the spread of *A. planipennis* in North America, US and Canadian regulatory agencies imposed quarantines and developed eradication programs. In the US, the eradication program involved *A. planipennis* survey and detection, followed by cutting and chipping of all ash trees in a 0.8 km (1/2-mile) zone around known infestations. In Canada, the effort to contain *A. planipennis* included cutting a 10 × 30 km ash-free zone across the Windsor Peninsula, i.e. from Lake Ontario to Lake Erie (CFIA 2008). These efforts were largely unsuccessful due to limited knowledge about *A. planipennis* biology and dispersal potential, lack of detection and control methods, difficulties with quarantine compliance and enforcement, the prevalence of ash, and the sheer size of the *A. planipennis* infestation, which was first discovered ca 10 years after initial introduction. Moreover, regulatory agencies soon learned that humans are responsible for the long-range spread of *A. planipennis* through illegal transport of infested ash nursery stock, firewood, manufactured goods, and timber. In both the US and Canada, eradication strategies are being replaced by management approaches. Researchers are optimistic that improved detection will result in earlier discovery of *A. planipennis* infestations, and various management tools will suppress *A. planipennis* populations below lethal thresholds for North American *Fraxinus* species.

Although the biology of *A. planipennis* was virtually unknown at the time of its discovery in North America, we now have a better understanding of its life cycle (Liu *et al*. 2003, Cappaert *et al*. 2005, Poland & McCullough 2006). *A. planipennis* completes its life cycle in one or two years, depending on the age of the infestation, tree health, and other biotic and abiotic factors. In Michigan, emergence of adults begins in mid to late May and peaks during June. Adults chew and emerge through D-shaped exit holes in the tree bark, begin maturation feeding on ash foliage followed by mating, and after about three weeks, females start to oviposit in bark

crevices and between bark layers. Although egg-laying peaks in July, eggs are laid throughout the summer and into early fall due to asynchronous adult emergence and long-lived adults. After egg hatch, neonates tunnel through the tree bark until reaching the phloem where they continue feeding through four larval stages. If mature by fall, larvae chew pupation cells in the outer sapwood or bark, overwinter as mature larvae and pupation occurs during the spring or summer. Early in an infestation, *A. planipennis* oviposit in the upper crown of large ash trees, and as populations increase, the trees become weaker. Tree mortality is caused by larval girdling of the main trunk, when *A. planipennis* populations reach lethal density thresholds for ash. This occurs over a period of several years depending on initial tree health, species, site, rainfall and other factors.

Research on control of *A. planipennis* has focused on the use of protective cover sprays and systemic insecticides, mainly neonicotinoids such as imidacloprid (Poland & McCullough 2006). Efficacy of these products, however, is variable and dependent on infestation level and tree condition when insecticide treatments are initiated, timing, frequency, and method of application, product concentration and formulation, weather, etc. (USDA FS FHTET 2008). *A. planipennis* regulatory activities are limited to quarantine and tree removal. In addition, most communities and homeowners have opted to remove their infested ash trees rather than apply annual insecticide treatments due to the expense, environmental and health risks, and uncertain efficacy.

Research on the development of a microbial control strategy for *A. planipennis* using entomopathogenic fungi was initiated following research on its natural enemies in Michigan field populations from 2002 to 2004 (Bauer *et al*. 2004b, 2005). About 2% of larvae removed from infested ash trees and cultured for entomopathogenic fungi, were infected with strains of *Beauveria bassiana, Isaria farinosa, Isaria fumosorosea, Lecanicillium lecanii*, or *Metarhizium anisopliae*. The successful use of entomopathogenic fungi for insect management (Feng *et al*. 1994, Higuchi *et al*. 1997, Jaronski & Goettel 1997), including trunk sprays of *M. anisopliae* for control of *A. auriventris* (Coleoptera: Buprestidae) on citrus trees in China (Fan *et al*. 1990), led to expanded laboratory, greenhouse and field studies for possible development of entomopathogenic fungi as a management tool for *A. planipennis* on ash trees in North America.

10.3.1 Laboratory and Field Investigations with Entomopathogenic Fungi

10.3.1.1 Laboratory Trials

Preliminary laboratory screening of *A. planipennis* with five isolates of two fungal species (*B. bassiana* and *M. anisopliae*) resulted in higher virulence against adults compared with larvae. All subsequent laboratory studies, therefore, focused on the effects of fungal isolates on *A. planipennis* adults (Liu & Bauer 2006). Adult *A. planipennis*, reared from infested ash trees felled in Michigan, were inoculated

by direct immersion in conidial suspensions at two concentrations to determine the time-mortality responses for each fungal isolate. The majority of adult mortality occurred within 4–6 days for all isolates, and for most isolates, higher conidial concentrations resulted in shorter days to death. The cumulative percent mortality 6 days after fungal exposures ranged from 80–97.5 and 97.5–100% for 10^6 and 10^7 conidia per ml, respectively. Within the same time period, only 12.5% of adults died in the control groups. At both concentrations, *B. bassiana* strain GHA treatments resulted in faster mortality for *A. planipennis* adults than the other isolates.

At the time of these studies, two species of entomopathogenic fungi were registered as bioinsecticides in the United States: (1) *B. bassiana* strain GHA, registered in 1995, formulated as BotaniGard ES (petroleum formulation) and Mycotrol O (organic vegetable oil-based) and (2) *M. anisopliae* strain F 52, registered in 2005, formulated as TAE-001 Granular. To compare the concentration-mortality responses of *A. planipennis* adults exposed to each product, a swinging boom spray cabinet with a flat-fan nozzle was used to apply serial dilutions of conidial suspensions to the upper surfaces of leaf rectangles (2×4 cm) cut from fresh, greenhouse-grown ash leaves. Adult beetles were exposed to the treated leaf rectangles for 24 hours, then placed on fresh leaves and daily mortality was determined for 10 days. Each bioassay was replicated twice. At the lowest concentration, adult mortality ranged from 0–35% and at the highest concentration mortality ranged from 95–100%, while average control mortality ranged from 10–20%. The median lethal concentrations (LC_{50}s) were similar for BotaniGard, Mycotrol, and *M. anisopliae* F 52, ranging from 114.5–309.6, 18.4–797.3, and 345.3–362.0 conidia/cm^2, respectively. Subsequent greenhouse and field trials, designed to evaluate the efficacy of cover sprays for *A. planipennis* control, focused on *B. bassiana* GHA-based products because these are (1) registered for use against insects including borers in the US, (2) formulated for aerial application, and (3) a history of use in the US has provided data for a body of literature on environmental persistence and risks to non-target organisms. Fungal bands, containing live cultures of *B. bassiana* strain GHA, were also field tested against *A. planipennis* due to the promise of this deployment method for control of cerambycids (Hajek & Bauer 2007).

10.3.1.2 Greenhouse Pre-Emergent Spray Trials

The efficacy of *B. bassiana* GHA, formulated as BotaniGard, was evaluated as a pre-emergent trunk spray on ash logs infested with *A. planipennis* (Liu & Bauer 2008). It was presumed that ash bark, with its irregular surface, might provide both a large surface area for entrapping sprayed conidia and protection from UV degradation. Moreover, *A. planipennis* adults must chew through the bark of ash trees to emerge from larval phloem-feeding sites; thus, the chance of adults becoming infected was presumably higher following trunk sprays with *B. bassiana* GHA. The latter hypothesis was tested by felling *A. planipennis*-infested green ash trees in southeastern Michigan in March 2003. The trees were cut into 60 cm long logs,

refrigerated until July, and then placed in an incubator for 4 weeks at 24°C, 16:8 (L:D) h, and 50–60% RH. Groups of infested ash logs were subsequently sprayed at three rates with BotaniGard ES or BotaniGard ES blank (no fungus) formulation (for the control) using a swinging-boom spray cabinet with a flat fan nozzle. The treated logs were placed individually in aluminum cages and maintained in a greenhouse at ambient conditions ranging from 20–26°C, 20–40% RH, and natural lighting. Each cage was provisioned with a potted evergreen ash tree (*Fraxinus uhdei*) to provide food for emerging *A. planipennis* adults and an uninfested green ash log was placed in each cage for ovipositing females. Adult emergence was monitored daily by marking each new adult emergence hole. Dead adults were removed daily and incubated at 24°C individually in a moist chamber consisting of a 60 mm plastic Petri dish lined with moist sterile filter paper. Fungal infection was confirmed by the presence of mycosis on cadavers 7 days after death. At the end of the study, *A. planipennis* eggs were counted on each oviposition log.

Adult *A. planipennis* mortality, resulting from fungal infection, averaged 33% for beetles emerging from the BotaniGard-treated logs (Liu & Bauer 2008). No fungal infection was detected among adults emerging from the control logs. Interestingly, adult longevity and fecundity was reduced by almost half for the *A. planipennis* adults surviving BotaniGard treatments compared to adults emerging from control logs.

10.3.1.3 Caged Field Trials

The promising results from laboratory and greenhouse studies of fungal control of *A. planipennis* led to expanded field trials using caged sections of ash tree trunks in the field (Liu & Bauer in press). In one caged field trial, BotaniGard ES was sprayed on infested ash trunks in the spring, before adult emergence. The trees selected for this study were green ash trees growing in a small nursery. They were moderately infested with *A. planipennis* and averaged 12 cm in diameter and 9 m in height. A section of tree trunk 50 cm above the ground and 180 cm long was delineated on each tree, and all *A. planipennis* adult emergence holes from previous years were marked and counted. A few days before emergence of adult *A. planipennis*, the trunk sections were sprayed with BotaniGard ES at two application rates with a calibrated professional sprayer fitted with a flat fan nozzle; the control trees were left unsprayed. After treatment, the trunk sections were caged to contain the *A. planipennis* emerging as adults. The cages consisted of aluminum screening stapled around the tree trunks to form a cylinder. To provide food for the emerging adults, an ash branch with several healthy leaves was enclosed inside each cage. The cages were disassembled after 6 weeks and dead adults within cages were cultured for fungal infection. In early fall, the trees were felled and treatment and control trunk sections were dissected for quantification of *A. planipennis* larval and adult densities and percent infection. Percent infection for *A. planipennis* adults emerging from the sprayed trunks averaged 59% at lower and 83% at higher concentrations of BotaniGard. Infected adults

that died before or during the emergence process were not found until the trees were felled and dissected in the fall. Larval densities in the treated trees were reduced by more than half compared to control trees. In addition, the larvae dissected from fungal-treated trees were consistently younger than those from control trees.

In another caged field trial, 5 cm wide fungal bands, made by the Hajek laboratory from non-woven polyester fiber bands covered with conidia produced by the culture of *B. bassiana* GHA growing within the band (Higuchi *et al*. 1997; Dubois *et al*. 2004a,b), were stapled around 40 uninfested green ash trees in July. As described above, a cylindrical aluminum screen cage (76.2 cm × 110 cm) was constructed around a section of trunk containing a fungal band. A small ash branch with foliage was enclosed in each cage as adult food. Field-collected *A. planipennis* adults were added at the rate of 10 adults per cage. After 4 weeks, *A. planipennis* adult mortality was determined and each cadaver was cultured to detect mycosis. The fungal bands caused 32% mortality due to fungal infection among *A. planipennis* adults compared to 1% for control adults (H Liu & L Bauer unpublished data).

10.3.1.4 Larval Field Trial

Relatively healthy ash trees respond to *A. planipennis* attack by the formation of callous tissue around larval galleries during the summer. By fall, the growing ridge of callous causes longitudinal splits to develop in the bark over many of the larval galleries, exposing larvae to the external environment. To explore the efficacy of *B. bassiana* GHA sprays for *A. planipennis* larval control, infested white ash trees with bark splits, were sprayed with BotaniGard ES during late fall (Liu & Bauer 2008). These were shade trees in a parking lot and were about 15 years old, 7–10 cm diameter, and 5–6 m tall. The lower 180 cm trunk section of 13 trees was sprayed with BotaniGard at a single concentration using a hand atomizer, and the immediate upper 180 cm trunk section was used as the untreated control. The following winter, the trees were felled, cut into 60 cm logs, dissected in the laboratory, and all *A. planipennis* were screened for fungal infection with mycosis confirmed after 14 days under moist conditions.

The infection rate of the *A. planipennis* larvae was 7.9% in the sprayed trunk sections, significantly higher than the 1.6% infection found in the control trunk sections. The prevalence of fungal infection was positively correlated with larval density in the trunk sections. Once again, larval development was delayed in the fungal-treated ash trunks compared to the controls.

10.3.1.5 Cover-Spray Field Trials

Annual insecticide treatments to preserve high value ash trees must begin before symptoms of *A. planipennis* infestation are visible (Rebek & Smitley 2007). In large ash trees, *A. planipennis* initiates attack in the upper canopy, so early infestation is difficult to detect. One sign of early infestation is upper tree limbs with wood

pecks, which are made as woodpeckers remove patches of outer bark while searching beneath for larvae. Another early sign is yellow flagging in the crown, which is caused by yellowing leaves on infested branches. Attack by *A. planipennis* on the main trunk results in small branches, or epicormic shoots, sprouting along the trunk, dead branches and limbs, bark splits, adult emergence holes, and general crown dieback.

To slow the rate of *A. planipennis* colonization of ash trees, topical sprays of *B. bassiana* GHA formulated as BotaniGard ES were tested on 6 year old uninfested white ash trees transplanted to the site the previous year. BotaniGard ES was applied to the foliage and trunks of each of 14 white ash trees four times at 2 week intervals between 25 June and 5 August 2004, with a CO_2 backpack sprayer equipped with a flat fan nozzle. Thirteen white ash were left as untreated controls. In October, the trees were felled, cut into logs, dissected in the laboratory, and all *A. planipennis* were placed in saturated conditions to determine the presence of fungal infection. New colonization of fungal-treated ash trees by *A. planipennis*, as determined by the number of young larvae present in each tree, was reduced by 40.7% compared to control trees. During the course of this study, older larvae were also found in these trees, confirming a 2 year life cycle for some *A. planipennis*. These larvae began developing during the previous year from eggs laid late in the season. In the sprayed trees, fewer young larvae were found but fungal infections were not detected among this younger larval cohort. It is hypothesized that many more young larvae had been present but had died of fungal infection and had decomposed during or soon after egg hatch while tunneling through the fungal-contaminated bark to reach the phloem. This would make finding, collecting, and culturing these small larval cadavers for inclusion in the data set virtually impossible. However, 19.6% of older larvae, developing in the trees since the year before, were infected with *B. bassiana*; none of the large larvae from control trees were infected. These findings support the hypothesis that fungal conidia infiltrate bark splits that form over *A. planipennis* galleries, resulting from the growth of callous around *A. planipennis* feeding damage within the cambial region (Liu & Bauer in press).

The efficacy of BotaniGard ES foliar and trunk cover sprays against well-established *A. planipennis* populations was evaluated in a small nursery of infested green ash trees. The plantation was divided into two plots, each with 25 trees. After leaf flush, crown condition was rated for each tree on a scale of one to three, before fungal application in 2004 and in 2005. Pre-treatment larval densities were estimated in each plot by sampling 50 cm logs, cut at 200–250 cm in height from the main trunks of two randomly selected trees. These logs were dissected and the number of *A. planipennis* was determined on the basis of log surface area. In the treatment plot, BotaniGard ES was sprayed on the trunk and crown of each tree individually using a truck-mounted hydraulic sprayer. The trees were sprayed every two weeks from 23 June to 3 August 2004, a total of four times. Trees in the control plot were not treated. During the following winter, all 50 trees were felled, cut into logs, and transported to the laboratory. A portion of each tree was dissected and *A. planipennis* larvae were used to determine the prevalence of

fungal infections. The remaining logs were stored at 4°C until the following summer (2005) when logs were incubated in individual cardboard tubes to collect emerging adults (Liu & Bauer 2006). Adults were collected daily and incubated to determine fungal infection prevalence. Overall, fungus-treated trees contained 46.7% fewer larvae and produced 63.3% fewer adults for the next generation when compared to the controls. The treatment trees sustained 41.5% less crown dieback than did the control trees. The prevalence of fungal infection was positively correlated with larval densities, which likely resulted from increasing horizontal transmission of fungal infection due to increasing numbers of overlapping larval galleries as larval densities increased (Liu & Bauer in press).

In 2004, the persistence of *B. bassiana* GHA conidia was evaluated using treated and control leaves harvested 0, 4, 7, and 11 days after fungal spray. After harvest, leaves were sealed individually in plastic zip-lock bags and transported to the laboratory. One- to six-day old *A. planipennis* adults were exposed to leaves in groups of five for 48 hours. The adults were then transferred to fresh ash leaves in clean dishes, mortality was monitored daily for 14 days and cadavers were placed under high humidity to determine the prevalence of fungal infection. Adult mortality ranged from 100% for those exposed to foliage collected directly after fungal application to 78% for adults exposed to foliage collected 11 days after application, with no statistically significant difference in adult mortality among leaves collected on different days post-application. Control mortality ranged from 20–46%, with no significant difference among leaves collected on different days after application. *B. bassiana* was the primary cause of *A. planipennis* adult mortality in both treatment and controls. Due to the high infectivity of *B. bassiana* strain GHA in *A. planipennis* (Liu & Bauer 2006) and the close proximity of ash trees in the stand, fungal infection from the control leaves likely resulted from drift during application. Time to death for *A. planipennis* adults ranged from 4–7 days after exposure to fungus-sprayed leaves. Longevity was significantly less for adults exposed to fungal-treated leaves compared to control leaves (Liu & Bauer in press).

In summary, ground-based foliar and trunk applications of BotaniGard ES reduced the number of *A. planipennis* feeding in and emerging from infested ash trees, reduced crown dieback in infested ash trees, and reduced new infestation in healthy ash trees (Liu & Bauer in press). These findings, and those from previous studies (Liu & Bauer 2006, 2008), support a role for *B. bassiana* GHA in the management of *A. planipennis* in the field. The use of insect pathogenic fungi for controlling destructive wood-boring insects is not without precedent. Many of these fungal pathogens exhibit high infectivity and virulence, and, given the moist habitat inside trees that provides an excellent environment for fungi, these agents have great potential as important biological control agents. Continued research is needed to reduce the application frequency and area sprayed with *B. bassiana* GHA in order to reduce costs and possible non-target effects. BotaniGard should also be tested as a cover spray for ash trees outside the ash-free zones during eradication to eliminate outlier infestations; at present, all studies have been conducted in the generally infested areas (Liu & Bauer in press).

10.3.2 Investigations of Bacillus thuringiensis *for* A. planipennis *Control*

Strains of *Bt* are found naturally in soil, on leaves, and in other places where insects are abundant, and these strains have restricted host ranges (Crickmore *et al*. 1998). During sporulation, *Bt* produces insecticidal crystal proteins, also known as Cry toxins. *Bt* must be ingested by the insect host because the action of these Cry toxins begins in the midgut; if sufficient toxin is consumed, the result is death by septicemia. *Bt*-based microbial insecticides are the primary tools used to manage forest insect pests due to their limited host ranges, good safety records in human health and the environment, public acceptance, and compatibility with other management strategies such as use of insect biological control agents (Glare & O'Callaghan 2000). Insect larvae are the primary target of *Bt* cover sprays, so larvae living in cryptic environments, such as wood borers, are inaccessible to conventional *Bt* application methods. However, adult insects are also susceptible to *Bt*, including some adult coleopterans, and many beetles, including wood borers, feed on foliage at some point in their life cycles. Thus a *Bt* strain with sufficient virulence could provide adequate control when targeting this life stage.

The first *Bt* strain identified as pathogenic to a coleopteran was *Bt tenebrionis*, which is active against some species of Tenebrionidae and Chrysomelidae (Krieg *et al*. 1983). This discovery stimulated worldwide interest in searching for novel *Bt* strains, and thousands of *Bt* strains are now characterized. At least 30 strains are known to be toxic to various coleopteran pests including *Bt japonensis* (Ohba *et al*. 1992) and *Bt galleriae* SDS-502 (Asano *et al*. 2003) with pathogenicity to certain species of Scarabaeidae. Activity of *Bt* has also been discovered for several wood-boring insects including isolates of *Bt tenebrionis* pathogenic to some species of Bostrichidae, Curculionidae, and Scolytinae (Cane *et al*. 1995, Beegle 1996, Weathersbee *et al*. 2002); *Bt darmstadiensis* with activity against a species of Bostrichidae; *Bt israelensis* with activity against Cerambycidae and Scolytinae (Alfazairy 1986, Méndez-López *et al*. 2003); and *Bt thuringiensis, Bt entomocidus* and *Bt morrisoni* with activity against Scolytinae (Jassim *et al*. 1990, de la Rosa *et al*. 2005). The toxicity spectrum of *Bt* continues to expand as more species are screened, including *Bt* 866 with toxicity against two cerambycids: the Asian longhorned beetle (*A. glabripennis*) and the mulberry longicorn beetle (*Apriona germari*) (Chen *et al*. 2005). For most wood-boring beetles, however, the most effective deployment of *Bt* may require expression of their *cry* toxin genes in transgenic trees.

With international trade, movement of wood-boring beetles that attack and kill live trees has escalated, and there is increasing need to develop environmentally-sensitive control strategies for managing these beetles in natural ecosystems such as forests and riparian areas. Other chapters in this book illustrate how *Bt*-based aerial sprays are used worldwide to suppress or eradicate populations of invasive and native forest insects. Although *Bt* cover sprays have targeted defoliating insect larvae, adult insects can also be susceptible to *Bt*. Moreover, *A. planipennis* adults are defoliators of ash leaves throughout their lives, thus aerial control is feasible. In addition, *A. planipennis* females require a 3 week maturation-feeding period before

beginning to lay eggs, providing land managers a window of opportunity for initiating aerial sprays before oviposition begins. Aerial application of a *Bt* strain with high toxicity against *A. planipennis* adults has the potential to reduce the high adult populations, thereby reducing numbers of larvae below a lethal density threshold for North American ash species.

10.3.2.1 Bt *Adulticide Laboratory Bioassays*

Following the discovery of *A. planipennis* in North America in 2002, regulatory agencies and land managers were interested in a registered bioinsecticide for use in the eradication program. Four *Bt*-based products were sprayed on ash leaves in a spray tower and bioassayed against *A. planipennis* adults reared from infested ash logs in the laboratory. The active ingredients of these bioinsecticides are *Bt* strains toxic to certain species of Coleoptera (Novodor®), Lepidoptera (Foray 48B®, Xentari®), or both (Raven®). The products showed some activity against *A. planipennis*, but 4–12 times maximum labeled rates were needed to achieve 66–98% mortality after 6 days of exposure (Bauer *et al*. 2004a). Further bioassays demonstrated *A. planipennis* adults were not susceptible to the Cry toxins from the *Bt* strains used in these products, although a crude extract of zwittermicin A, another compound produced by *Bt* during fermentation, was toxic (Bauer *et al*. 2006).

After reviewing the literature and searching patent databases, 18 narrow host-spectrum coleopteran-active *Bt* strains were acquired from culture collections, grown in liquid shake culture, and crystal/spore mixtures were purified. The Cry toxin concentration was estimated for each strain by measuring the intensity of the protein band from SDS-PAGE gels using a densitometer, and comparing the reading to a standard curve prepared from known concentrations of BSA. After standardizing the amount of Cry toxin to a similar concentration for each strain, *A. planipennis* adults were inoculated with a *Bt* crystal/spore mixture using a droplet imbibement bioassay method in which the beetles readily ingest a 0.5 μL droplet containing a known amount of Cry toxin. Using this method, one of the scarab-active strains, *Bt galleriae* (*Btg*) SDS-502 and its Cry8Da toxin (Asano *et al*. 2003), demonstrated high toxicity against *A. planipennis* adults (LS Bauer unpublished data). The median lethal dose of the *Btg* SDS-502 crystal/spore mixture ranged from 0.16–0.35 μg Cry8Da toxin per beetle and time-to-death ranged from 24–96 hours. Toxicities were similar for solubilized Cry8Da protoxin (130 kDa) and activated toxin (65 kDa); however, the toxicity of the crystal/spore mixture was about 10-fold lower, suggesting somewhat reduced crystal solubilization in the midgut of *A. planipennis*. To evaluate the potential efficacy of *Btg* SDS-502 for aerial application, ten 0.02 μL droplets of crystal/spore mixture, suspended in a 10% sucrose solution, were dispensed onto 1 cm^2 pieces of ash leaf. Each droplet contained ca 0.2 μg Cry toxin; the sucrose served to help the toxin droplets adhere to the ash leaves and to overcome feeding inhibition, which occurs during intoxication of insects by *Bt*. One-week-old adult *A. planipennis* were exposed individually to a treated or control (sucrose only) leaf. After 72 hours, 90% of adults feeding on *Bt*-treated leaves died vs 10% control mortality. In the future, we plan to use the leaf droplet bioassay to optimize formulation

ingredients. Once *Btg* SDS-502 is formulated, droplet analyses will be conducted using a rotary atomizer to apply *Btg* and achieve droplet sizes and densities similar to those that would be delivered onto leaves during aerial application in the field. If successful, a new microbial insecticide will be registered for control of *A. planipennis* in ash forests and along riparian areas, thus conserving ecological resources in North America.

10.4 Prospects for Use of Entomopathogens for Control of Invasive Wood Boring Beetles in North America

Methods for microbial control of *A. glabripennis* and *A. planipennis* are under development. In both instances, strategies are constrained by the characteristic difficulties of controlling wood-boring beetles. However, in both cases, the levels of control provided by pathogens (often not 100% immediate mortality) would be appropriate because it takes many beetles to kill individual trees, often over numerous years. Programs for controlling these hosts are quite different due to the different host biologies. Only adults of *A. glabripennis* are directly targeted by fungal bands, although reproduction by females also decreases once infected and conidia can be horizontally transmitted to eggs and larvae, so there are also indirect effects. However, the fungal band strategy still requires that adults contact conidia by walking across a band. The next step in development of fungal bands is to add an attractant so that wandering beetles will be attracted to bands (unfortunately, to date identification of an efficient attractant has been elusive although there now appears to be some progress (S Teale & A Zhang personal communication). Once an attractant can be placed in trees along with fungal bands, the issue of how many fungal bands should be applied to one tree or an area will still need to be addressed but certainly fewer bands will be needed than without attractants. At present, *A. glabripennis* only occurs in urban/suburban areas in North America and its slow spread has helped with a successful eradication strategy. Fungal bands would be highly appropriate to use for protection of high value urban/suburban trees, in conjunction with the eradication program, which relies in part on conventional systemic insecticides. While the applicability of the fungal band approach in forests is not necessary to address at present, fungal bands plus attractants are being very successfully used on a large scale in China against another cerambycid, *Monochamus alternatus* (Li *et al*. 2007).

In contrast, microbial control strategies being investigated against *A. planipennis* have targeted both adults and larvae. Adult *A. planipennis* prefer to navigate a tree by wing rather than by walking, and considerable time is spent feeding on leaves in the canopy, reducing the efficacy of fungal bands for this beetle. However, fungal cover sprays applied to trunks and upper limbs before and after adult emergence, have allowed for conidial contact and infection of *A. planipennis* adults as they (1) chew through the bark to emerge from the tree and (2) during the oviposition period, when adult females search for oviposition sites between layers of bark and in bark crevices. The complex structure and surface area of tree bark serves to conserve

fungal inoculum after cover sprays by providing niches where spores are protected from UV. In addition, splits that form in the bark of relatively healthy ash trees directly over *A. planipennis* larval galleries due to callous formation, allow fungal cover sprays to enter, contact, and infect *A. planipennis* larvae in their galleries. Aerial application of a *Bt* cover spray would target adults during the maturation feeding period, and a second application would target late-emerging adults that result from asynchronous development.

For both of these systems, invasive tree-killing pests are of great concern; while eradication may be possible for *A. glabripennis*, eradication programs for widespread and fast-moving *A. planipennis* have been abandoned by regulatory agencies. Without microbial and biological controls (Bauer *et al*. 2004a, 2005, Liu *et al*. 2007), that are generally accepted for use in forested ecosystems, native ash trees will be extirpated from North America. Microbial controls and use of parasitoids can provide the management tools needed by forest managers to suppress *A. planipennis* population densities below the tolerance threshold for ash trees in forests and riparian areas. These methods are also more acceptable to the public and reduced pest populations in forested areas may facilitate survival of ash trees planted throughout urban/suburban landscapes of North America.

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