Enhanced detection of *Pityophthorus juglandis* – The insect vector of thousand cankers disease

I. Problem

Thousand cankers disease (TCD) is a pest complex formed by the association between the walnut twig beetle (WTB), *Pityophthorus juglandis* (Coleoptera: Curculionidae: Scolytinae), and fungal pathogen *Geosmithia morbida*. TCD has caused the widespread death of walnut trees throughout the West and has recently been introduced to the Midwestern and Eastern US, and threatens black walnut (*Juglans nigra*) within its native range. The disease has rapidly spread throughout the West and its further establishment within the native range of black walnut would have serious economic and environmental consequences. In an attempt to protect the black walnut—the most susceptible *Juglans* species to TCD (Utley et al. 2013)—many states in the North Central Hardwoods Region have issued an external quarantine prohibiting the interstate transportation of black walnut material (e.g., nuts, logs, and whole trees) from affected areas. There is approximately 3.4 billion cubic feet of black walnut growing on timber land in the Eastern United States, with an estimated value of over a half trillion dollars (USFS 2002). In Indiana alone, nearly 31.5 million walnuts provide ~17.7 million board feet of lumber and veneer each year at a value of $21.4 million (Indiana DNR 2010). Also, black walnut plays important ecological roles in the Eastern deciduous forest as wildlife feed upon the nutrient-rich nut meat of black walnuts and the polyphenol-rich leaves serve as a controlling ecological force within soil ecosystems. The progression of this disease would likely affect the natural regeneration of black walnut within the native range of the species.

The long term protection of the walnut resource at risk to TCD requires the integration of multiple tactics, including more efficient and cost effective monitoring techniques. Current monitoring efforts for WTB rely on visual surveys and the use of aggregation pheromone-baited multi-funnel traps but the pheromone lure is only effective from a limited distance (~20m). Adult beetles are also attracted to girdled trap trees, but the use of this technique for detection and monitoring is hampered by the labor and associated costs involved. However, the addition volatile organic compounds (VOCs) of walnut to the current lure for WTB may increase its range and efficacy. Preliminary evidence demonstrates that adult beetles are attracted to volatile monoterpenes emitted by black walnut and that this attraction is greater to VOCs emitted from black walnut limbs that are artificially wounded by girdling (Paschen et al. 2013). A critical need remains to identify and evaluate these walnut and *G. morbida* volatiles as potentially effective intervention agents against TCD, and the extent to which adult WTB are attracted to volatiles induced by host-fungal interactions. The overall objective of this proposal is to develop a synthetic kairomone-based detection tool for WTB to enhance surveys in the Midwestern and Eastern states.

This project is therefore significant because it will empirically field-test the attraction of WTB to volatiles of black walnut and *G. morbida* that mediate colonization behavior of the adult beetle. Compounds will be identified and their capacity to be used as a kairomone lure for the early detection of WTB will be evaluated. These results can likely be applied to both the native range of black walnut in the Eastern and North central US and the non-native range in the West. The rationale for this study is that the identification of kairomones used by WTB to locate suitable hosts will lead to the development of an enhanced tool to more effectively detect, delimit and monitor WTB.

II. Background

Natural history of WTB

The primary range of *P. juglandis* includes New Mexico, Arizona, and Chihuahua, Mexico and closely coincides with its original host Arizona walnut (*J. nigra*; Cranshaw and Tisserat 2008). In recent years, the WTB expanded its range to areas of Colorado, Utah, Idaho, Oregon, and Washington. From museum records, it appears that the beetle has been present in California for over twenty-five years, and it is currently widespread throughout the central and coastal areas of the state. In 2010, TCD was detected in Knoxville, Tennessee and has subsequently been detected in Virginia, Pennsylvania, North Carolina, and most recently Butler County, Ohio (Grant et al. 2011; Wiggins et al. 2014).

Adult WTB are very minute, yellowish-brown bark beetles about three times as long as wide, with length ranging from 1.5–1.9 mm (Blackman 1928; Wood 1982). The only known hosts of *P. juglandis* are walnut trees (*Juglans* spp.). It was not until WTB formed an association with the fungus *Geosmithia morbida* that symptoms of TCD began to appear (Tisserat et al. 2009). As beetles emerge from their larval host, the WTB carries *G. morbida* conidia on its elytra and introduces the fungus to walnut trees during colonization and gallery formation (Tisserat et al. 2009). Tissue of the walnut tree dies once it is infected with *G. morbida* which leads to a canker under the bark (Tisserat et al. 2009). As the disease progresses, outer leaves will yellow and coalescing cankers effectively girdle branches, causing large scaffold branches to die and the tree to eventually succumb to the disease (Seybold et al. 2012). Trees often succumb to the disease only after thousands of beetles have colonized them.

Host colonization and selection

Many wood- and fungus-feeding beetles, including WTB, first locate suitable host plants to colonize before they can reproduce, and preferentially attack stressed hosts (Ciesla 2011). Studies of the emerald ash borer (*Agrilus planipennis*) and other *Agrilus* spp., for example, have demonstrated that mechanically girdling potential hosts adequately stresses these trees and makes them highly attractive to adult beetles (Haack and Benjamin 1982; Poland et al. 2004, 2005; Francese et al. 2006; Fraser et al. 2006; McCullough et al. 2009). These girdled trap trees are effective for detecting low-density populations of the pest (see McCullough et al. 2009). Stress imposed by the girdle may not only elevate the nutrient content and/or reduce the chemical defenses of a tree (see Mattson and Haack 1987; Franco and Lüttge 2002), but also increases the release of VOCs that mediate host location and subsequent colonization by the beetle (Chen and Poland 2009). Nevertheless, it can be difficult to locate suitable trees to girdle and this monitoring technique is expensive and labor-intensive when used on a broad-scale. Once on a suitable host, pioneering male WTB release volatile aggregation pheromones that coordinate mass attack and mating (Seybold et al. 2013). The use of a plant kairomone lure could enhance the attraction of WTB to pheromone lures and increase the efficacy of monitoring efforts.

Association with Geosmithia morbida

Geosmithia morbida (Ascomycota: Hypocreales), the fungal symbiont of WTB, is a dry-spored anamorphic fungus and the first species in the *Geosmithia* genus documented as a plant pathogen (Kolarík et al. 2011). The complexity and high genetic diversity among *G. morbida* isolates from affected trees in the west suggests that this fungus is a long-established species (possibly on native Arizona walnut in the Southwest; Freeland et al. 2009), rather than a recently

introduced exotic. This weak plant pathogen is not systemic and it requires a vector to become established (Ploetz et al. 2013). In the early stages of the disease, small cankers develop around the galleries of colonizing beetles with the fungus often restricted to the cambium. As the disease progresses, cankers expand into the phloem and outer bark (Utley et al. 2009). In the more advanced stages, cankers become more diffuse, causing the tissues to become dark-colored and macerated.

Although adult ambrosia beetles farm fungus with which to feed their larvae, it is unclear if WTB gain advantages from the fungal associate, whether as a nutritional source or a microbial protectant (De Fine Licht and Biedermann 2012; Luna et al. 2014). Through choice test experiments, Luna et al. (2014) demonstrated that larvae are attracted to volatiles of common tree fungi, including *G. morbida* and *Fusarium solani*. There is no evidence of feeding by larvae on the *G. morbida* cankers but the attraction to fungi could suggest an advantageous relationship for both species; *G. morbida* is dependent on WTB for dispersal and areas where canker growth has weakened plant defenses may be a beneficial site for larval feeding (Bleiker and Six 2007; Luna et al. 2014).

III. Preliminary research

Attraction of WTB to volatiles of G. morbida

Olfactometry bioassays with G. morbida

In preliminary olfactometry bioassays (full procedure described below in Objective 1), it has been confirmed that adult beetles are attracted to volatiles emanating from three agar-plated isolates of *G. morbida* (i.e., isolates 12 and 17 from Tennessee and isolate UT-9602 from Utah; see figure 1; Ginzel unpub. data). This result is significant because it demonstrates that volatiles can be isolated from *G. morbida* and validates our olfactometry-based approach for testing the attraction of WTB to synthetic compounds.

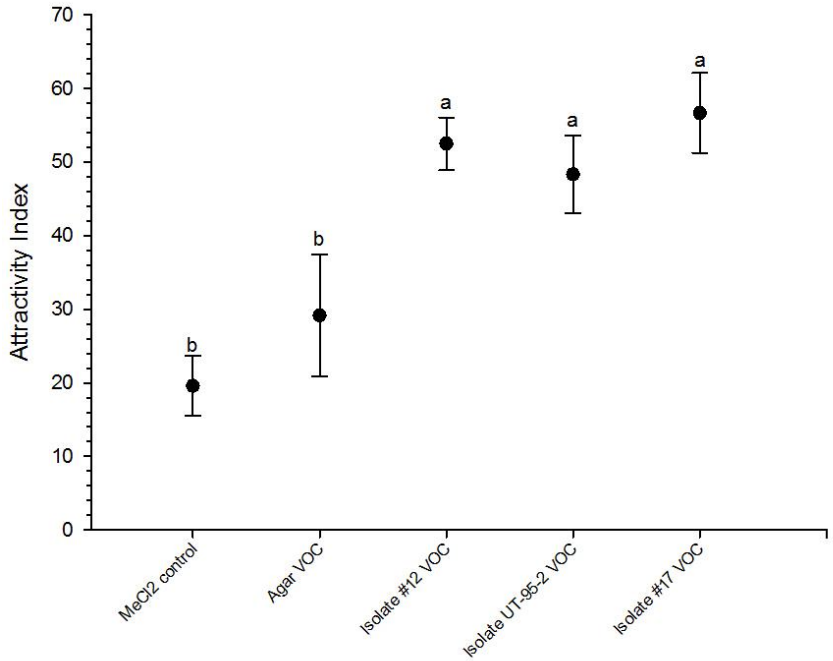

Figure 1. Attraction of adult WTB to volatiles of *G. morbida*, uncolonized agar, and a blank control in a glass-tube olfactometer (ANOVA $F_{4, 41} = 8.8$, $P < 0.0001$). Bars marked with the same letter are not significantly different (Duncan's test, $P < 0.05$). Sex of the beetles was neither a significant main effect nor interaction term, therefore data for males and females were combined. Error bars display standard error.

Field trial with G. morbida inoculated walnut bolts

During the summer of 2014, we evaluated the extent to which black walnut bolts inoculated with *G. morbida* would increase the attraction of WTB to pheromone baited traps at five sites in Tennessee. Of five sites, there was a significant attraction from WTB to cut walnut bolts inoculated with *G. morbida* (Gm) isolate 10 and isolate 17 (figure 2) at Sandy Springs Park. This result is significant because it demonstrates the probability that unique compounds are produced by the interaction between black walnut and the fungus. This also shows promise for further testing to create a fungal kairomone lure.

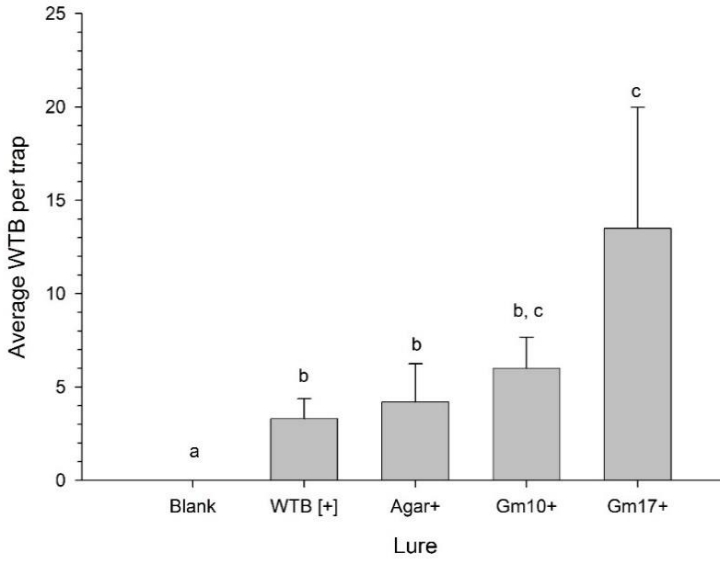

Figure 2. Average adult WTB trap capture with walnut bolts inoculated with *G. morbida* or an agar control, paired with a WTB pheromone lure [+], and a blank control (ANOVA $F_{4, 45} = 8.13$, $p < 0.001$). Bars marked with the same letter are not significantly different (Fisher's LSD test, $p < 0.05$). Error bars display standard error.

Volatile profile of G. morbida

To identify *G. morbida* specific VOCs, we compared the volatile profiles of colonized agar plates to those of uncolonized control plates. Interestingly, one compound, 5-ethyl-4-methyl-3-heptanone, was present in the headspace samples of *G. morbida* and not present in the PDA agar control (see figure 2, * on the top chromatogram). Preliminary data shows that WTB are repelled by ethanol which is present in both samples but more highly abundant in the PDA control (see figure 3, bottom asterisk; Seybold unpub. data). This preliminary result is significant because a VOC specific to *G. morbida* has been identified and validates our experimental approach.

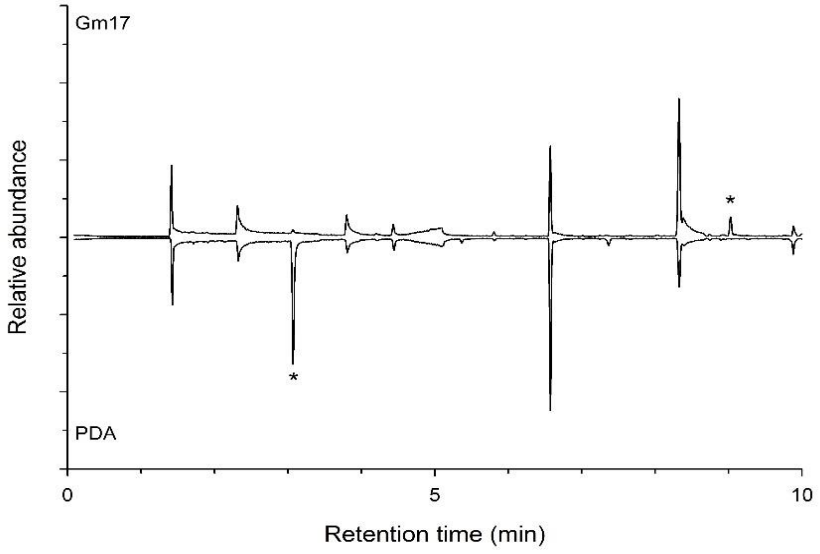

Figure 3. Representative total ion chromatograms from solid phase microextractions of VOCs from plated *G. morbida* (top) compared to a PDA control (bottom).

Attraction of WTB to walnut volatiles

Olfactometry bioassays with girdled walnut branches

Through olfactometry bioassays we have established that adult WTB are strongly attracted to girdled branches of black walnut (figure 4; Ginzel unpub. data). This preliminary evidence demonstrates that adult beetles are attracted to volatile monoterpenes emitted by black walnut and that this attraction is greater to VOCs emitted from black walnut limbs that have been artificially wounded by girdling to simulate ecological susceptibility (Paschen et al. 2013). By comparing the volatile profiles of intact and girdled branches, four compounds (i.e., α-pinene, β-pinene, camphene, and cymene) were identified that are more highly represented in the volatile collections from girdled black walnut. This result is significant because it suggests these compounds may comprise an effective kairomone lure for WTB.

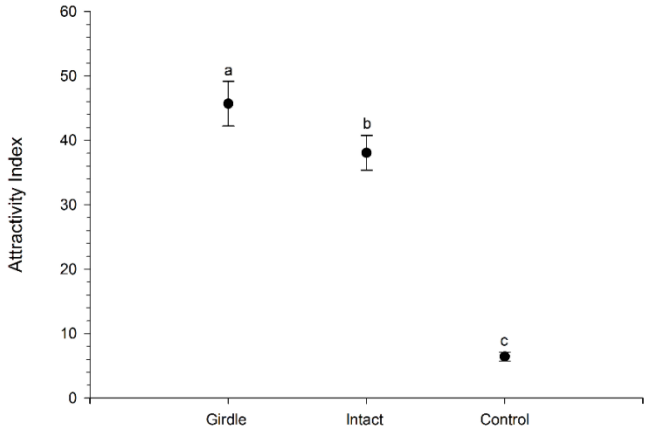

Figure 4. Attraction of adult WTB to volatiles of girdled and intact branches and a blank control (ANOVA $F_{2, 111} = 75.75$, $p < 0.001$). Bars marked with the same letter are not significantly different (Duncan's test, $p < 0.05$). Error bars display standard error.

Olfactometry bioassays with synthetic walnut volatiles

We then tested the attraction of adult WTB to these fours compounds in the a straight-tube olfactometer, and found that males and females were attracted to both camphene and cymene. However, neither sex responded to either α-pinene or β-pinene more strongly than to the blank

control (figure 5). However, the attraction of adult WTB was not inhibited by these two compounds when they were presented as part of a blend of all four volatiles. These preliminary results hold promise for the creation of a synthetic tree kairomone lure.

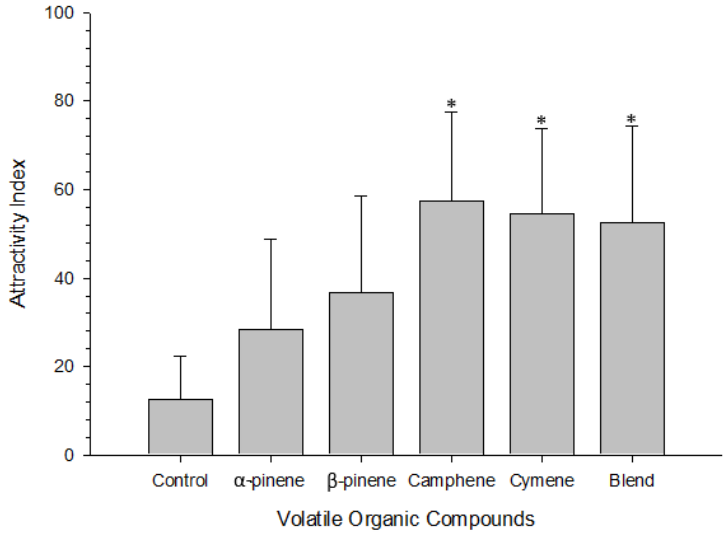

Figure 5. Attraction of adult WTB to walnut VOCs and a blank control (Kruskal-Wallis $H_{5, 54} = 30.82$, $p < 0.001$). * indicates significance ($p < 0.05$), error bars represent standard error.

Field trial with synthetic walnut volatiles

During the fall of 2014, we conducted a field experiment in Tennessee to evaluate the capacity of these four compounds to increase the attraction of adult WTB to pheromone-baited traps in the field. We found a significant attraction of adult WTB to the kairomone lures when paired with the commercially available pheromone lure at Duck Pond, in Knoxville, Tennessee. There was significant attraction to a WTB pheromone lure alone, and α-pinene, camphene, and cymene when paired with a WTB pheromone lure (Duncan test, $p < 0.05$; figure 6). These results coupled with the olfactometry bioassay results indicates that β-pinene could repel WTB. The experimental tree lure to be tested will be α-pinene, camphene, and cymene because these demonstrated the most synergistic attraction with the WTB pheromone lure.

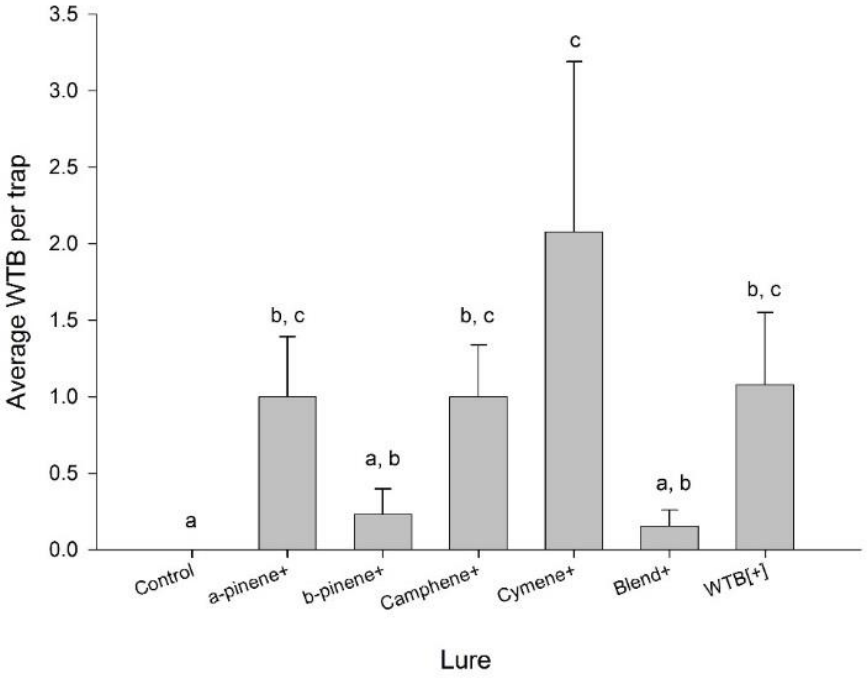

Figure 6. Average adult WTB trap capture with walnut bolts inoculated with *G. morbida* or an agar control, paired with a WTB pheromone lure [+], and a blank control (ANOVA $F_{6, 78} = 4.00$, $p = 0.0015$). Bars marked with the same letter are not significantly different (Duncan test, $p < 0.05$). Error bars display standard error.

IV. Objectives

Collectively, the preliminary results suggest that an effective kairomone lure for the WTB can be developed by characterizing the VOCs from black walnut and *G. morbida.* My objectives in this proposal are to identify volatile compounds specific to black walnut and *G. morbida* that mediate host location by WTB and determine the extent to which these volatile host compounds will synergize the attractivity of a commercially available pheromone lure. The rationale for these studies is that the identification of kairomones used by WTB to locate suitable hosts will lead to the development of an effective lure that may synergize the attractivity of the current pheromone lure used to monitor for WTB. These studies are innovative and timely because a kairomone lure for the WTB would provide for more effective monitoring and control methods. This work will be divided into two primary objectives:

Objective 1: Test the capacity of *Geosmithia morbida* to increase the attraction of WTB to bolts of black walnut in the field and identify *G. morbida*-related VOCs.

Objective 2: Test the efficacy of synthetic black walnut and *G. morbida* VOCs in the field and determine the extent to which they synergize a commercially available pheromone lure.

Details for each objective

Objective 1: Test the capacity of *Geosmithia morbida* to increase the attraction of WTB to bolts of black walnut in the field and identify *G. morbida*-related VOCs. My *working hypothesis* is that adult WTB will be more attracted to bolts of black walnut inoculated with *G. morbida* than black walnut bolts alone and that *G. morbida* produces distinctive VOCs.

Introduction

This objective represents a critical first step in accomplishing the overall project goal of developing a kairomone lure for the walnut twig beetle. Specifically, this experimental approach

will allow us to identify volatiles specific to *G. morbida* and determine if unique compounds are produced by the interaction of black walnut and the fungus.

Attraction of WTB to fungal volatiles in the field

Adult WTB are attracted to cut bolts of black walnut in the field (Bray et al. 2012). However, the extent to which fungal volatiles may enhance this attraction is unclear. In the first year of the project, I will test the attraction of adult WTB to bolts of black walnut inoculated with *G. morbida* at locations in Tennessee and Butler Co., Ohio with active WTB populations. While adult WTB are attracted to cut bolts of black walnut, the presence of competing walnut VOCs in the field environment may limit trap capture of WTB in this experiment. To overcome this gap, WTB aggregation lures (Contech Enterprises, Inc., Victoria, BC Canada, prod. no. 300000736) will be included in the experimental design. Specifically, four-unit Lindgren multi-funnel traps (Contech Enterprises, Inc.) will be baited individually with one of the following seven treatments:

1. Bolt of black walnut (c.a. 30 cm length x 7 cm dia.) inoculated with an agar slurry plus the commercially available WTB pheromone lure;
2. Bolt of black walnut (sized as above) inoculated with one isolate of *G. morbida* plus pheromone lure;
3. Bolt of black walnut inoculated with a different isolate of *G. morbida* plus pheromone lure;
4. Bolt of non-host wood (e.g., oak) inoculated with an agar slurry plus pheromone lure;
5. Bolt of black walnut inoculated with the fungus *Fusarium solani* plus pheromone lure;
6. Pheromone lure alone; and
7. An unbaited trap with no pheromone lure (blank control).

Walnut bolts will be placed in fine-mesh fabric bags (c.a. 35 cm x 10 cm) and fine-mesh screen bags (c.a. 40 cm x 15 cm) to prevent new beetles from infesting the bolts. Cut ends of the bolts will be sealed with a thin layer of paraffin wax to minimize the amount of moisture lost while in the field. All traps will be coated with Fluon PTFE (AGC Chemicals Americas, Exton, PA, USA) to enhance trapping efficiency (see Graham et al. 2010), and collection cups will be filled with RV antifreeze to kill and preserve captured insects. Traps will be suspended from three meter frames constructed of aluminum conduit pipe along linear transects at the leading edge of the WTB infestation in counties adjacent to the Great Smoky Mountains National Park (e.g., Knox, Blount, Sevier, Anderson, Morgan, and Cocke Co., TN) and Butler Co., OH in three blocks that each contain one trap of each treatment (10 m apart, position assigned randomly), with blocks separated by at least 20 m. Traps will be checked for beetles every two weeks, at which time the position of treatments will be rotated within transects. Lures will be examined when the traps are checked and replenished when visibly depleted. All captured beetles will be returned to the laboratory for identification. The experiment will be conducted during the active flight period of adult WTB.

Methods for inoculation

The fungus-treated walnut bolts will be prepared four weeks prior to deployment of the baits. Two *G. morbida* isolates from Hamilton, OH, and two from Knoxville, TN, will be grown for 7 days on ½ strength potato dextrose agar in 100 mm dia. Petri dishes. Resulting colonies with underlying agar will be macerated in a small amount of sterile, distilled water to make an

"inoculum slurry". A grid of 1 hole/60 cm^2 bark surface on laboratory film will be lightly pressed around the bolt and result in approximately 12 points to inoculate. A small hole will be drilled (3 mm dia. bit) just to the cambium and a small mass of inoculum transferred to the hole using a sterile toothpick. Each treated hole will be sealed with petroleum jelly. The inoculated bolts will be stored in plastic bags for four weeks prior to field deployment. Bolts inoculated with appropriate isolates will be used at the corresponding field trial site. Bolts inoculated with sterile agar slurry will serve as controls. At the end of the deployment period in Tennessee, the outer bark on all walnut bolts will be peeled with a small drawknife to expose any bark cankers. Numbers of cankers found on each bolt will be recorded. Isolation for *G. morbida* will be attempted from the margins of cankers found on each bolt.

Statistical analysis

Differences between trap treatments in the number of beetles captured per trap will be tested for WTB by ANOVA followed by a post-hoc Dunnett test or LSD test (StatSoft 2005), blocked by sample day and treatment block. In the event that assumptions of ANOVA are violated by heteroscedasticity, differences will be evaluated between trap treatments in the number of beetles captured per trap with the nonparametric Kruskal-Wallis test (StatSoft 2005).

Collection and identification of G. morbida volatiles

By comparing the volatile profiles of uncolonized agar plates with those colonized with *G. morbida,* VOCs specific to the fungus will be identified. Methodology of Kuhns et al. (2014) will be followed to collect and analyze *G. morbida* volatiles with a triphase 50/30 μm DVB/Carboxen/PDMS StableFlex™ solid phase microextraction (SPME) fiber for volatiles and semivolatiles with molecular weight between 40 and 275 (Supelco, Bellefonte, Pennsylvania). SPME sampling will be used to capture the headspace volatiles from agar plates that have been colonized by *G. morbida* and agar plates alone. Actively growing cultures of *G. morbida* (AZ, IN, OH, and TN isolates) will be prepared by transferring colonized agar plugs of each to the center of freshly-made ½ strength PDA Petri dishes and incubated for 3 weeks at ambient temperature (~24°C) in the dark until approximately 75–100% of the surface area is covered with fungus.

Volatiles emanating from colonized and uncolonized Petri dishes were captured by capping the plates with aluminum foil for 24 hours. To sample the headspace volatiles of *G. morbida,* the SPME fiber will be inserted through a tin foil lid and exposed to the fungal odors for five minutes. The SPME fiber will then be desorbed for five minutes at 240°C and analyzed by coupled gas chromatography-mass spectrometry (GC-MS) with electron impact ionization (EI, 70 eV) using a Agilent 6890N GC (Agilent Technologies, Santa Clara, CA) equipped with a HP-Innowax capillary column (30 m x 0.25 mm x 0.25 μm film; J&W Scientific, Folsom, CA) in splitless mode, interfaced to an Agilent 5975B mass selective detector, with helium carrier gas. The oven temperature will be programmed from 40°C/5 min, ramped at 7°C/min to 240°C, and held for 5 min at 240°C. Injector temperature will be 240°C and transfer line temperature held at 280°C. Analytes will be identified by mass spectral analysis after comparing their retention times to those of authentic standards. If structures cannot be determined from GC-MS, larger samples will be collected and purified by HPLC and/or preparative GC for NMR analysis to be performed at the Purdue University Spectrometry Center. If *G. morbida*-related volatile compounds are not commercially available, Synergy Semiochemicals Corp., Burnaby, BC, Canada will synthesize them.

Bioassay of synthetic G. morbida VOCs

Insects used in bioassays will be reared from infested material collected near the epicenter of the TCD outbreak in TN. Beetles will be reared in a greenhouse at the University of Tennessee, Knoxville according to Browne (1972) and emerged adults will be separated by sex and stored at 4°C until being used in experiments. The bioactivity of synthetic compounds will be tested using a glass straight tube olfactometer. This technique is often more fruitful than gas chromatography-electroantennogram detection (GC-EAD) for identifying plant/fungal kairomones, because GC-EAD responses to such compounds can be quite weak and the antennae often only respond to multiple compounds in specific ratios. Olfactometry bioassays are more beneficial for identifying blends of bioactive compounds that elicit specific behavioral responses. In the assay, the walking response of beetles to synthetic *G. morbida* volatiles in a glass straight tube olfactometer (30 cm x 3 cm) will be measured (Peña et al. 1992; Szauman-Szumski et al. 1998). One end of the olfactometer will be connected to a small glass chamber containing the odor source (filter paper treated with 150 µl of synthetic compound approximating the concentration found in the crude extract or 150 µl of $MeCl_2$ as a control), and air will be pulled through the system by a laboratory vacuum supply (~1 L/min). Newly emerged beetles (n = 5) will be placed at the downwind end of the olfactometer and allowed to respond to the odor source. In response to an attractive odor, beetles move upwind in the olfactometer. The position of each beetle within the tube will be recorded after 30 minutes and an attractivity index of the odor will be calculated per Peña et al. (1992). Males and females will be tested separately, individuals will be used only once, and each bioassay will be replicated ten times (five reps for each sex). This work will take place in the laboratory of Dr. Bill Klingeman at the University of Tennessee, Knoxville.

Statistical analysis

The response of male and female beetles will be compare to that of the control by ANOVA followed by Duncan's post-hoc test or the nonparametric Kruskal-Wallis test. (StatSoft 2005).

Objective 2: Test the efficacy of synthetic black walnut and *G. morbida* VOCs in the field and determine the extent to which they synergize a commercially available pheromone lure. My *working hypothesis* is that select polyketides and short-chain alcohols produced by *G. morbida* fungal mycelia will be attractive to adult WTB and increase the response to synthetic walnut volatiles.

Introduction

This objective will be accomplished by conducting a field experiment in Tennessee and Butler Co., Ohio where there are active populations of WTB. This aim is based on the *working hypothesis* that the walnut and *G. morbida*-derived kairomone lures will attract adult WTB in the field and synergize the attractiveness of the commercially available pheromone lure. The goal in taking this approach is to validate the efficacy of these kairomone lures as an enhanced tool for detecting, delimiting, and monitoring for WTB.

Experimental design

A field experiment will be conducted to test the capacity of synthetic walnut and *G. morbida* VOCs to attract beetles and synergize the commercially available pheromone lure. Specifically, four-unit Lindgren multi-funnel traps will be baited with one of the following treatments:

1. A lure comprised of a combination of α-pinene, camphene, and cymene from the preliminary field evaluation (i.e., tree lure);
2. Synthetic *G. morbida* volatiles (i.e., fungal lure);
3. Commercially-available WTB pheromone lure;
4. Tree lure along with the WTB pheromone lure;
5. Fungal lure along with the WTB pheromone lure;
6. Both the tree lure and fungal lure with the WTB pheromone; or
7. A control containing the solvent carrier used in treatments above;

All lures, other than the WTB pheromone lure, will consist of compounds loaded into a polyethylene sachet (press-seal bags, Bagette model 14770, 5.1 by 7.6 cm, 0.05-mm wall thickness, Cousin Corp., Largo, FL). In preliminary experiments with the tree lure, the release rate of compounds was ~100 mg/day. A fungal lure created for the Redbay ambrosia beetle (*Xyleborus glabratus*) showed that a higher release rate (~220–520 mg/day) has better trap capture (Kuhns et al. 2014). Traps will be deployed at locations in TN and Butler Co., OH as described above and lures will be examined when the traps are checked and replenished when visibly depleted. All captured beetles will be returned to the laboratory for identification. This experiment will be conducted during the active flight period of adult WTB in the second year of the project.

Statistical analysis

Differences between trap treatments in the number of beetles captured per trap will be tested separately for WTB and the most abundant species of other ambrosia and bark beetles by ANOVA (StatSoft 2005), blocked by sample day and treatment block. Differences between preplanned pairs of treatment means by using orthogonal contrasts will be tested (StatSoft 2005). Thus, we will compare 1) the solvent control with each kairomone lure (to determine the extent to which they attract WTB); 2) the combined tree and fungal lure to each individually; 3) the pheromone lure to its carrier (to confirm that the pheromone lure is attractive); 4) the tree or fungal lure with pheromone lure (treatment 4 or 5) to the pheromone lure alone; and 5) the combined kairomone (tree and fungal) and pheromone lure (treatment 6) to the pheromone lure alone to determine the extent to which the kairomones together increase the efficacy of the pheromone. Differences in the sex ratio of responding beetles will also be tested between treatments with χ^2 goodness-of-fit test (StatSoft 2005).

V. Conclusion

These objectives will advance the long-term goal to create effective early detection tools to monitor for WTB. If action is not taken, the sustainability of black walnut trees as a wildlife and timber resource may become unsupportable within its native range. At the completion of this study, I expect to have identified and field-tested VOCs of black walnut and *G. morbida* that could potentially serve as a synthetic kairomone lure for WTB. I expect this experimental approach to also demonstrate the efficacy of a synergistic kairomone lure that could be used by state first responders and forest managers to detect, delimit, and monitor incipient WTB populations and thus minimize costs associated with pest detection for the WTB and other bark beetle pests of hardwoods.

VI. Timeline

Milestone	Year 1 (1st month of quarter)				Year 2 (1st month of quarter)			
	Jul	Oct	Jan	Apr	Jul	Oct	Jan	Apr
Objective 1								
Inoculate bolts with *Geosmithia morbida*								
Perform field experiment with inoculated bolts								
Collect and identify *G. morbida* VOCs								
Rear WTB for use in bioassays								
Olfactometry bioassays with synthetic *G. morbida* VOCs								
Data analysis								
Objective 2								
Identify sites in Butler Co., OH and TN for study								
Formulate and/or prepare tree and fungal lures								
Perform field experiment with tree, fungal, and pheromone lures								
Data analysis								
Prepare manuscripts								

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