MS Research Proposal- Spring 2013

Towards developing ash varieties resistant to emerald ash borer and increasing the efficacy of its biological control agents

I. Introduction

A. Overview and Objectives

The emerald ash borer (EAB) is among the most destructive insect pests threatening North American forests. Since its arrival in 2002, EAB has been found in 15 states and two Canadian provinces and its range is expected to continue spreading (MSU 2013). EAB is particularly difficult to control because larvae develop concealed beneath the bark of the host tree, feeding on the vascular tissue and disrupting nutrient flow. Due to the cryptic nature of EAB, infestations often go undetected until the trees begin to die. There are at least 16 endemic *Fraxinus* species at risk in North America and millions of trees have already been lost (USDA 2010). All North American species are vulnerable to EAB but susceptibility varies between species (Cappaert et al. 2005, Poland and McCullough 2006, Anulewicz et al. 2008). For example, *Fraxinus* species of Asian origin that coevolved with the beetle are most resistant to attack, and only stressed trees are generally affected (Gould et al. 2005). EAB is capable of killing otherwise healthy North American ash and colonizes trees as small as saplings – most affected trees die within four years of colonization (Cappaert et al. 2005, Poland and McCullough 2006).

EAB locate suitable hosts via volatile organic compounds (VOCs) released by the leaves and shoots of their host (Rodriguez-Saona et al. 2006, Crook et al. 2008, Crook and Mastro 2010). EAB is capable of infesting all species of *Fraxinus*, but susceptibility is variable between species. The cause of this variability may be due to quantitative and qualitative differences in volatile emission. These subtle differences in volatile chemistry are recognized by EAB and also the parasitoids being used for their biological control. Another proposed explanation for the variation in susceptibility is differences in phloem chemistry between *Fraxinus* species. Phenolic compounds may serve a variety of purposes, such as digestibility reduction and as toxins. Manchurian ash, which is the most resistant species, has higher concentrations of lignins and hydroxycoumarins than those of its North American relatives (Eyles et al. 2007, Whitehill et al. 2012). Due to their structure, lignins are difficult for insects to digest and may also cause mandibular wear (Feeny 1975, Rhoades and Cates 1976, Peeters 2002). Hydroxycoumarins have been found to deter herbivorous insects as well, and may function in trehalase inhibition and have anti-microbial activity (Leszczynski et al. 1995, Silva et al. 2006, Rodriguez et al. 2000, Cipollini et al. 2011). If these properties of resistance could be conferred to North American species, EAB resistance could be achieved.

Grafting has historically been used to confer certain properties of a rootstock to a scion. Roots synthesize secondary metabolites that are involved in defending plants against herbivory (Erb et al. 2009), and are integral in the production of constitutive and induced defenses. Prior to the arrival of EAB, green ash was considered the universal donor of rootstocks and was used as a rootstock for a wide variety of ash cultivars (Ball 2004). As one of the ash species most susceptible to EAB attack, this could have unfortunate consequences for relatively resistant scions grafted on to this rootstock. For example, autumn purple ash, a white ash scion grafted on to green ash, is more susceptible to EAB than white ash (Rebek et al. 2008). If a species with

more resistance such as Manchurian or blue ash were used as donor rootstock instead, resistant properties might be translocated from the root to the shoot.

The *long-term* goal of this research is to pave the way toward developing systems for propagating *Fraxinus* scions that are resistant to EAB and exploit potential phytochemical connectivity between roots and shoots. There is currently a *critical need* to identify the mechanisms of EAB resistance in ash and methods for conferring this resistance and propagating trees. My *objective in this proposal* is to identify changes in VOC emission induced by grafting, determine the extent to which EAB parasitoids respond to these VOC's, and to determine the extent to which rootstock dictates the phloem chemistry of the scion. My *central hypothesis* is that grafting susceptible ash species to resistant rootstock will confer resistant properties to the scion via root/shoot connectivity, and that resistant and susceptible species differ in their VOC and phloem composition. The *rationale* for this study is that 1) VOC and phloem analysis may elucidate EAB resistance mechanisms in ash species; and 2) these differences in VOC emission may affect the attraction of natural enemies to the host. I propose the following specific aims:

Specific Aim #1: Determine the extent to which rootstock dictates phloem chemistry of the grafted chimera. My working hypothesis is that due to the root-shoot connectivity, the rootstock will influence the phloem chemistry of grafted scions.

Specific Aim #2: Determine the extent to which rootstocks influence the composition of volatile organic compounds (VOCs) released by the leaves and branches on the scion when EAB larvae are feeding on the tree. My working hypotheses are that: a) the VOCs released by the scion will be similar to those released by the donor rootstock ash species and b) EAB larval feeding will alter the composition of VOCs released by the chimera.

Specific Aim #3: Determine the extent to which EAB parasitoids respond to EAB-induced volatile compounds of reciprocal grafts. My working hypotheses are that: a) EAB parasitoids will preferentially respond to volatiles induced by EAB larval feeding b) grafts containing native (i.e., Asian) rootstock or chimera will be preferred.

Through the completion of these objectives, I expect to determine the utility of grafting for propagating resistant ash trees and the response of biological control agents already released against EAB to grafted trees. VOC's are important to the host location behavior of EAB and their parasitoids and I expect to discover more about the contribution of rootstock to both the volatile and phloem chemistry of *Fraxinus*. The changes in phloem chemistry of the scion may also contribute to the mortality of eggs and performance of larvae.

B. Rationale and Significance

Ash is not only an important forest tree and street tree in urban forests, but also historically important in the production of baseball bats, tool handles, and other wood products. Unfortunately, the spread of EAB will continue to devastate North American ash. It is estimated that from 2009 to 2019, $10.7 billion could be spent on ash treatment, removal, and replacement of trees if current EAB range expansion continues (Kovacs et al. 2010). Grafting of ash to

produce resistant trees and the elucidation of the resistance mechanisms would ensure that *Fraxinus* is not lost from North America. This work will also uncover the ways in which grafted trees will attract EAB parasitoids and further protect them from EAB related mortality. This project is *novel* and *significant* because it is the first to use grafting as a method for increasing the resistance of a hardwood tree to an invasive woodboring pest.

II. Review of Previous Work

A. Review of Literature that is Relevant to this Project

(1) Host Plant Resistance to EAB. With no evidence of long-range pheromones (Rodriguez-Saona et al. 2006, Lelito et al. 2007), it appears that EAB locate suitable hosts via the volatiles released by ash (Rodriguez-Saona et al. 2006, Crook et al. 2008, Crook and Mastro 2010). Even very small amounts of green leaf volatiles (GLVs) produce an antennal response in EAB using electroantennogram detection, suggesting that volatiles are an integral part of host location (Rodriguez-Saona et al. 2006). All North American ash species are vulnerable to EAB but susceptibility is variable, with blue ash exhibiting more resistance than the more susceptible green and white ash (Cappaert et al. 2005, Poland and McCullough 2006). EAB utilizes Manchurian and Chinese Ash in its native range, but only infests stressed trees and prefers the North American species such as green and velvet ash that have been introduced to the region (Yu 1992, Duan et al. 2012). The ability to perceive and respond to volatile organic compounds (VOCs) released by host plants plays an important role in the host selection and reproduction of many insects, and different genotypes of the same plant emit different volatiles (Dicke and Baldwin 2010). These differences in VOCs may play a role in the differential attraction of EAB to the various *Fraxinus* species.

Host plant resistance is the term given to the suite of adaptations that plants have evolved to protect themselves from herbivory. For example, Manchurian and Korean ashes are able to cause EAB mortality through the formation of callous which encapsulates and kills the larvae (Duan et al. 2010). This type of herbivore-induced defense is common in plants that have coevolved with their pests and VOCs are often included in this category. VOCs can act as repellants, or herbivore-induced defense, but may also act as attractants (Unsicker et al. 2009). For example, the volatiles released by *Nicotiana attenuata* attract predators to the plant which feed on the herbivores that attack it. However, GLVs and terpenoids also attract flea beetles, which damage the plant as well (Halitschke et al. 2007). In Y-tube olfactometer tests, parasitoids of EAB have been found to be attracted to the volatiles of ash, and not attracted to host odors (Wang et al. 2010). Research continues to analyze the VOCs in North American ash species to better understand why they lack the resistance of their Asian relatives.

(2) Role of Roots in the Defense Against Herbivores. Roots have been found to play an important role in defending plants from herbivores. For example, the roots of tobacco produce much more nicotine than the shoot does (Solt 1957), and nicotine is transported via the xylem for storage in leaf vacuoles to defend the plant from herbivory (Morita et al. 2009, Shoji et al. 2000). Trees are also capable of this root to shoot defense network. For example, simulated herbivory of hybrid poplar leaves (*Populus trichocarpa* Torr. & A. Gray x *Populus deltoides* Bartr. ex Marsh) induced levels of a trypsin inhibitor and increased transcription of herbivory defense genes in the roots (Major and Constable 2007). Green ash is a common donor of rootstock for the propagation of a wide variety of grafted ash cultivars (Ball 2004). However, as one of the most susceptible

ash species to EAB attack, this could have unfortunate consequences on the defenses and resistance of scions grafted on to this rootstock. Grafting susceptible North American species onto resistant Asian species instead of green ash rootstock could be a solution to this problem, and could influence the way that ash is cultivated in the future.

Examples of successful grafting systems can be found in many plant groups. When the tree *Citrus clementina* Hort. ex. Tan. is grafted onto differing citrus rootstocks, it appears that the rootstock induces physiochemical changes to the scion that influence resistance of the chimera to herbivory by the citrus leafminer, *Phyllocnistis citrella* Stainton (Muñoz et al. 2008). In several solanaceous plants, including nightshade, tropane alkaloids are produced in the roots and transported to the leaves (Ziegler and Facchini 2008). In reciprocal grafts with other species, the alkaloid patterns of chimeras mirror those of the rootstock, suggesting the rootstock confers secondary metabolites to the grafted shoots (Bais et al. 2001).

(3) Volatile Organic Compounds and Phytochemicals. Induced volatiles and phytochemicals also play an important role in the resistance of ash to EAB. North American ash species including black, green and white ash differ in their foliar chemistry compared to the more resistant Manchurian ash (Rebek et al. 2008, Pureswaran and Poland 2009). These differences include the amounts of phenolics (Eyles et al. 2007) and protease inhibitors (Chen and Poland 2010) that are found constitutively throughout the tree. Nutritive content may play a role in the preference of EAB to some ash species over others. In feeding experiments, EAB that fed on black ash had shorter lives than those that fed on green and white ash. In dual-choice tests, green and white ash were also preferred over black ash (Chen and Poland 2010). Moreover, in six-choice bioassays EAB concentrated and fed preferentially on green, black, and white ash compared with blue, European, and Manchurian ash (Pureswaran and Poland 2009). This preference implies that there may be a fitness advantage for EAB to feed on non-native hosts.

(4) Biological Control of EAB and Host Volatiles. Volatiles induced by herbivory may also act as attractants for a variety of predators and parasitoids. In terms of EAB, parasitoid wasps in their native range are responsible for helping to maintain EAB population levels. *Spathius agrili* is a gregarious idiobiont ectoparasitoid, capable of reaching levels of parasitism as high as 60% in some areas of China (Wang 2005). *Oobius agrili*, a more recently discovered egg parasitoid and biological control agent, has proven to be more difficult in terms of assessment of its parasitism rates. Researchers have developed a method of placing egg-sentinel logs (bolts of ash with known numbers of EAB eggs on them) in the field to assess the success of parasitism and establishment of the species in an area (Duan et al. 2012). *Tetrastichus planipennisi*, a koinobiont endoparasitoid, has been found to succeed in parasitizing up to 40% of EAB larvae. Together with *O. agrili*, a 73.6% reduction in EAB populations has been achieved in green ash in parts of China (Lui et al. 2007).

These natural enemies have been released in the US with some success, but difficulties in establishment have occurred. The most effective strategy for releasing parasitoids is to release them when EAB populations are low to moderate in size and parasitoids have ample time to establish before EAB populations crash and all ash in the area are killed (L. Bauer, pers. comm.). For this reason, a reservoir of surviving ash is needed for the maintenance of parasitoid populations. These reservoirs are most effective when they include a mixture of susceptible and resistant trees. Green and Manchurian ash plantings in China are believed to aid in the parasitism rate of *T. plannipennisi* and *O. agrili* enough to allow both ash species to survive (Lui et al.

2003). Sustainable control of EAB may be possible if the proper forest composition is present. It is unclear however which volatiles they respond most strongly to and how grafted trees differ in VOC output.

B. Preliminary Studies

(1) Characterization of Leaf Volatiles (VOCs) of Scion Wood. In a preliminary experiment, we collected volatiles from four grafted trees (i.e.; 1. green scion x green rootstock; 2. Manchurian scion x Manchurian rootstock; 3. Manchurian scion x green rootstock; and 4. green scion x Manchurian rootstock). We found both quantitative and qualitative differences in the volatile profiles of these trees and the profiles of the reciprocal crosses differ from those of both the donor rootstock and scion (See Figs. 1 & 2). In fact, there is a total loss of four compounds when Manchurian ash scions are grafted onto green ash rootstock when compared to the pure Manchurian ash grafts (see Table 1, Figs.1 & 2), suggesting that the rootstock has some influence on the volatile profiles of the chimera. However, it appears that when green scions are grafted onto Manchurian ash rootstock, the differences are less dramatic. Nevertheless, these 9 differences in volatile profiles from chimeras may be even more pronounced when the defense system of a tree is induced by feeding from adult EAB. Results of these preliminary volatile collections are significant because they demonstrate changes that may occur in grafted trees.

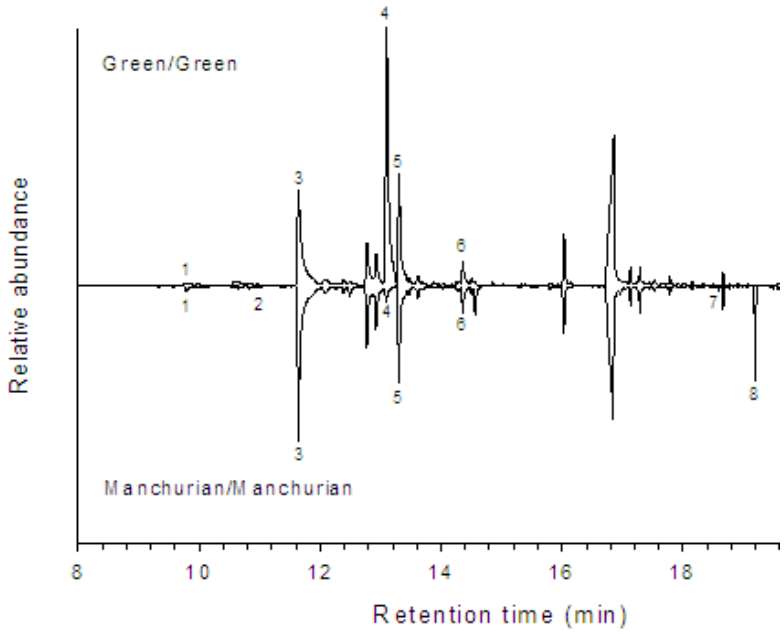

Fig. 1: Representative total ion chromatogram of volatile organic compounds collected from green ash scion grafted onto green ash rootstock (top) and Manchurian ash scions grafted onto Manchurian ash rootstock (bottom).

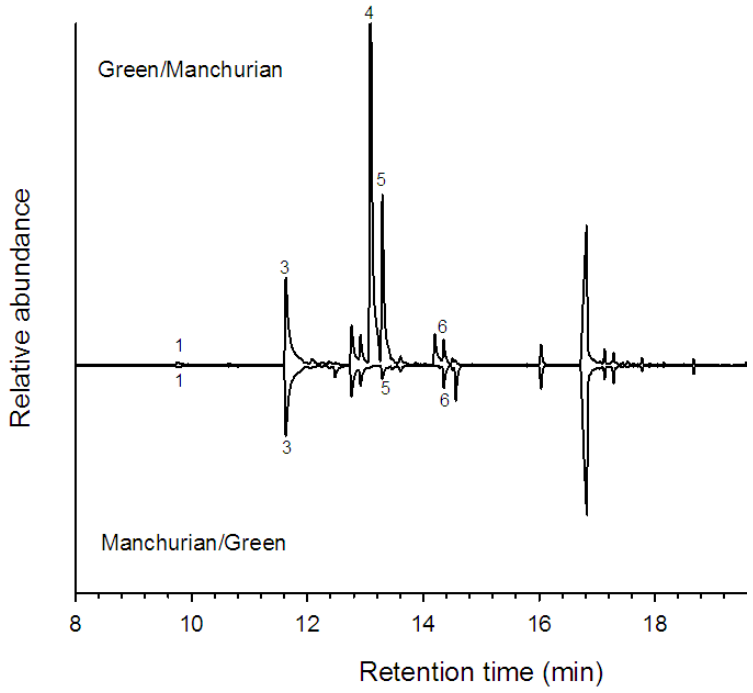

Fig. 2: Representative total ion chromatogram of volatile organic compounds collected from the following reciprocal grafts: green ash scion grafted onto Manchurian ash rootstock (top) and Manchurian ash scions grafted onto green ash rootstock (bottom).

Table 1: Volatile compounds extracted from headspace collections of grafted green and Manchurian (Man.) ash scion and rootstock (n= >2 per combination).

		(Scion/Rootstock)			
Peak no.[a]	**Compound**	**Green/Green**	**Green/Man.**	**Man./Man.**	**Man./Green**
1	**Hexanal**	+[b]	+	+	+
2	**(*E*)-2-Hexenal**	-	-	+	-
3	**(*Z*)-3-Hexenyl acetate**	+	+	+	+
4	**(*E*)-β-Ocimene**	+	+	+	-
5	**(*Z*)-β-Ocimene**	+	+	+	+
6	**Nonanal**	+	+	+	+
7	**Cubebene**	-	-	+	-
8	**β-Caryophyllene**	-	-	+	-

[a]Peaks are numbered in order of elution from a DB-5 capillary column and correspond to those in Figs. 1 and 2.
[b]Presence of a compound is indicated by a +; absence by -

III. Approach

Field Site and Experimental Design. Saplings of five different ash species (Table 2) were purchased from Lawyer Nursery (Plains, MT); Bailey Nurseries (Newport, MN); and Musser Forests, Inc. (Indiana, PA) or harvested from the Purdue University Department of Forestry and Natural Resources ash plantation. Reciprocal and conspecific scion and rootstock grafts were performed in April 2010 and maintained under drip fertigation in a shade frame at the Purdue University John S. Wright Forestry Center (Tippecanoe Co., Indiana) and then transferred to a cold dome until planted. In May 2011, the ash planting was arranged at the Purdue University Harrold Woodland (Whitley Co., IN). A completely randomized block design with four replicates (blocks) was used in a 2x2 arrangement and all combinations of chimeras (n=20) and conspecific grafts (n=5) were planted in a 5x5 arrangement in each block. Five ash saplings of each species (propagated from rootstock) were randomized between blocks and serve as buffer rows. The inter-row spacing is 6 m and distance between each row is 3 m.

Table 2: *Fraxinus spp*. that served as scion and rootstock donors in reciprocal grafts of ash.

Common Name	Species Latin Name	Species Group	Susceptible to EAB*?
White Ash	*Fraxinus americana*	North American	Yes
Black Ash	*F. nigra*	North American	Yes
Green Ash	*F. pennsylvanica*	North American	Yes
Chinese Ash	*F. chinensis*	Asian	No
Manchurian Ash	*F. mandshurica*	Asian	No

Aim 1: Determine the extent to which rootstock dictates phloem chemistry of the grafted chimera.

Experimental Design (Aim no. 1). To determine the degree to which rootstock influences the phloem chemistry of grafted trees, we will take phloem tissue samples from above and below the graft junction of trees for constitutive phenolic analysis in the lab. 100 mg tissue samples will be collected from three replicates of each the 25 unique graft combinations. We will harvest tissue once in early June and again in August – corresponding with the hatching of EAB eggs and the presence of 3rd and 4th instar larvae respectively. Tissue will be collected from the trees, placed on ice and transported to the lab where phloem tissue was excised immediately, frozen in liquid nitrogen, and stored at −80 °C until sample extraction (Whitehill et al. 2012).

Phenolic Extraction. Soluble phenolics will be extracted according to Eyles et al. (2007) and Cipollini et al. (2011). Briefly, stem phloem will be ground in liquid nitrogen, weighed to 100 mg and extracted twice with 500 µl of 100% methanol over 24-48 hr in the dark at 4°C. Extracts

were centrifuged for 5 min at 13,400×g and the two supernatants were pooled into~1 ml of extract. OR The pooled extract was transferred to a 1.5-ml microcentrifuge tube and centrifuged (12,000×g for 5 min) to remove solids. Samples were stored at −20°C and analyzed within 1 wk of extraction.

Analysis of Phenolic Compounds. Extracts will be analyzed according to Whitehill *et al*. (2012). Briefly, phenolic extracts will be analyzed using HPLC-UV. The injection volume for all samples will be 10 µl. Samples will be passed through a Photodiode Array Detector (PDA; scanning range, 200–400 nm) with individual peaks quantified at 280 nm. Phenolic extracts from each ash species will also be analyzed using an HPLC-ESI-MS (Varian 500 MS; Palo Alto, CA, USA) in parallel with a PDA detector (Whitehill et al 2012). Individual compounds will be quantified following the methods of Eyles et al. (2007).

Statistical Analyses. Principal component analysis (PCA) will be used to observe the relationship between phenolic compound and ash species (Johnson and Wichern 2002). Univariate analysis of variance (ANOVA) will be used to analyze individual peak areas and mg g−1 FW concentrations of lignin (Whitehill et al. 2012).

Possible Pitfalls and Limitations. The trees in this study are not very mature and the excision of phloem tissue may prove detrimental to their health. These trees are subject to the vagaries of weather and also unprotected from other insect pests.

Aim 2: Determine the extent to which rootstocks influence the composition of volatile organic compounds (VOCs) released by the leaves and branches on the scion when EAB larvae are feeding on the tree.

Experimental Design (Aim no. 2). Head-space volatiles from twigs and leaves will be collected from trees in our study to identify; 1) the extent to which volatiles from the leaves of chimeras differ from those scions grafted onto their own rootstock; and 2) whether there are differences in the induced systemic response in volatile production by these grafts when they are fed upon by EAB larvae.

This will be accomplished in the three step experiment listed below:

1) Head-space leaf volatiles will be collected for 4 hours from 5-8 compound leaves of three trees from each of the 25 unique grafts. These volatile collections will be performed prior to the phloem tissue harvest to control for any changes in volatile emission induced by the excision of tissue. This collection will take place in early June.
2) Following the excision of phloem, 6 EAB eggs will be placed on two of the tree representatives of each graft to evaluate egg survival and larval performance and also to induce possible systemic changes in VOC emission.
3) In August, head-space leaf volatiles will be collected again for 4 hours from 5-8 compound leaves of the trees described above. This collection will allow us to determine if a change in VOCs was induced by larval feeding as a plant defense.

Methods for Collecting Headspace Leaf Volatiles and Analysis. 5-8 compound leaves and intact stems will be enclosed on the tree within a Tedlar® bag and volatiles will be collected on a filter containing 150 mg of 80/100 mesh HayeSep-Q® . A portable pump will be used to supply a

continuous stream of air over the material. A push-pull method will be used to ensure that a vacuum is not created during the 4 hours. Air will be pulled through one tube using a pump and pulled through another tube using a vacuum to create continuous flow over the leaves. Filters will be eluted with 150 μL of methylene chloride. The extract will then be analyzed by coupled gas chromatography-mass spectrometry (GC-MS) with electron impact ionization (EI, 70 eV) using a Hewlett-Packard 6890 GC (Hewlett-Packard, Sunnyvale, CA) equipped with a DB-5MS capillary column (30 m x 0.25 mm x 0.25 μm film; J&W Scientific, Folsom, CA) in splitless mode, interfaced to an HP 5973 mass selective detector, with helium carrier gas. The oven temperature will be programmed from 40°C/1 min, ramped at 10°C/min to 250°C, and held for 5 min at 250° C. Injector temperature will be 100°C and transfer line temperature held at 280º C. We will identify compounds by mass spectral fragments after comparing their retention times to those of authentic standards. The amount of each of these compounds will be quantified by comparing its peak area in the total ion chromatogram with that of internal standard and calibration curves generated with synthetic standards. Extracts will then be stored at 4°C until used in the bioassays described below.

Source of Eggs. The beetles used in this study were reared from naturally infested logs kept in cold storage and then placed in a wooden rearing box until emergence. Scouting for infested ash trees was conducted in September 2011 in various Indiana forests. Pole-size trees with DBH of 10-25 cm were harvested in February 2012 and cut into 60 cm length bolts. The ends of each bolt were waxed and all of the bolts were stored in a laboratory cold room at 4ºC. This process will be repeated in early 2013 to supply beetles for the second summer of the experiment. As beetles are needed for the study, bolts will be removed from storage and placed into a large wooden rearing box. The box is dark inside, except for a plastic tube along the side which lets light in and attracts the beetles to the collection cup. Adult EAB emerge between 4-6 weeks after removing bolts from cold storage. Male and female beetles will be separated and placed in 300 cm^3 screen cages and sustained on fresh foliage from blue ash (*Fraxinus quadrangulata* Michx.) and 10% sucrose solution (replaced every 2-3 days). Cages containing beetles will be stored at 25ºC in an environmental chamber (~70% relative humidity and photoperiod of 14:10 h L:D) until ready for mating. Pairs will be placed within cages together and allowed to mate. Coffee filters will be fixed to the lid of the cages for egg laying substrate and the eggs will be collected and placed in growth chambers at 25ºC until transfer to trees (~70% relative humidity and photoperiod of 14:10 h L:D).

Quantifying Egg and Larvae Survival. According to Abell et al. (2012), the filter paper with EAB eggs laid on it will be secured to the trees using tree wrap and cotton balls to ensure close contact with the bark. 5 weeks later, the tree wrap and cotton will be removed and the coffee filters will be observed for egg hatching. In late August and following the final volatile collection, the area around the egg placement will be debarked to recover EAB larvae.

Statistical analysis. Concentrations of volatile compounds will be compared individually between grafts and herbivory treatments using multivariate analysis of variance (MANOVA; 8 StatSoft 2005). If equal variance assumptions of MANOVA are violated, we will either square root transform the data or use a nonparametric test. A t-test will be used to determine if there was a significant difference in the survival of eggs and larvae between the graft types.

Possible Pitfalls and Limitations. Collection of volatiles from the reciprocal ash grafts should be straight forward because we have optimized the equipment and methods for such work. Nevertheless, the trees in this study will be subject to the vagaries of weather, which may influence the emission of VOCs. We will hedge against this by simultaneously collecting volatiles from individual representatives from as many grafts as possible at the same time. We will also control for changes in VOC profiles with leaf phenology by collecting volatiles from mature leaves in early summer soon after leaf flush.

Aim 3: Determine the extent to which EAB parasitoids respond to EAB-induced volatile compounds of reciprocal grafts.

Experimental Design (Aim no. 3). A large glass petri dish (Pyrex; 14 cm x 2 cm) will be used to test the response of *S. agrili* and *T. planipennisi* to the crude solvent extracts of the volatiles collected according to Tooker et al. (2002). Filter paper containing crude solvent will be placed in the dish and wasps will be given 5 minutes to choose. Assays will be conducted between 8:30 and 17:00 h in the laboratory (~25°C) and the petri dish will be illuminated from above by cool white fluorescent tubes.

Supply of S. agrili and T. planipennisi. Parasitoids will be obtained from the USDA-APHIS EAB Biocontrol Laboratory in Brighton, Michigan as needed for assays. Wasps will be kept in a cage and stored at 25ºC in an environmental chamber (~70% relative humidity and photoperiod of 14:10 h L:D). A 1:4 honey solution will be supplied (replaced every 2-3 days) to sustain the wasps.

Bioassays. We will first test the attraction of female wasps to the extracts of those grafts that have the same rootstock and scion. To test the response of wasps to the volatiles of chimeras, they will be given the choice between the crude extracts of reciprocal crosses (e.g., green scion grafted onto Manchurian rootstock and the reciprocal). In this way, we can evaluate the bioactivity of all combinations of reciprocal grafts with ten trials. Odor sources will consist of a filter paper onto which we will pipette 50 µl of pooled crude solvent extracts and place in the petri dish. Assays containing extracts of conspecific graft (e.g. green scion grafted on green rootstock) will be no-choice tests, and a filter paper coated with solvent alone will serve as the control. Odor sources will be randomized between the sides of the petri dish after each bioassay to control for location effects, and the petri dish will be rinsed with acetone and air dried between trials. A newly eclosed, mated and naïve female wasp will be released in the canter of the petri dish and given 5 min to respond to the odor sources. We will record the following: 1) response; determined by the first time a wasp walks onto an odor source and remains for 30 sec; 2) retention time; i.e., the time a wasp spends on each side of the petri dish during the 5 min bioassay. For each trial, we will individually test the response of 30 wasps to each odor source.

Statistical Analysis: We will determine whether female wasps significantly respond to odor sources using a $\chi 2$ goodness-of-fit test (StatSoft 2005), and compare the time a wasp spends on each odor source using a parametric paired t-test (StatSoft 2005).

References

Abell, K. J., Duan, J.J., Bauer, L., Lelito, J.P., and Van Driesche, R.G. The effect of bark thickness on host partitioning between *Tetrastichus planipennisi* (Hymen: Eulophidae) and *Atanycolus spp.* (Hymen: Braconidae), two parasitoids of emerald ash borer (Coleop: Buprestidae). *Biological Control*, 63: 320-325.

Anulewicz, A.C., McCullough, D.G., Cappaert, D.L., and Poland, T.M. 2008. Host range of the emerald ash borer (*Agrilus planipennis* Farmaire) (Coleoptera: Buprestidae) in North America: results of multiple-choice field experiments. *Environmental Entomology*, 37: 230-241.

Bais, H. P., Loyola-Vargas, V. M., Flores, H. E., and Vivanco, J. M. 2001. Invited review: rootspecific metabolism: the biology and biochemistry of underground organs. *In Vitro Cellular & Developmental Biology–Plant*, 37: 730–741.

Ball, J. 2004. Arboreal schizophrenia. Arid Zone Times 11:1–4. http://www.aridzonetrees.com/images/AZTimes/Arboreal%20Schizophrenia.pdf (last accessed 4/4/2013).

Cappaert, D., McCullough, D.G., Poland, T. M., and Siegert, N.W. 2005. Emerald Ash Borer in North America: a research and regulatory challenge. *American Entomologist*, 51: 152-165.

Chen, Y. & Poland, T. M. (2010). Nutritional and defensive chemistry of three North American ash species: possible roles in the host performance and preference by emerald ash borer adults. *Great Lakes Entomologist*, 4: 20-33.

Cipollini, D., Wang, Q., Whitehill, J., Powell, J., Bonello, P., and Herms, D. 2011. Distinguishing defensive characteristics in the phloem of ash species resistant and susceptible to emerald ash borer. *Journal of Chemical Ecology*, 37: 450-459.

Crook, D. J., Khrimian, A., Francese, J. A., Fraser, I., Poland, T. M., Sawyer, A. J., & Mastro, V. C. (2008). Development of a host-based semiochemical lure for trapping emerald ash borer Agrilus planipennis (Coleoptera: Buprestidae). *Environmental Entomology*, 37: 356-36.

Crook, D. J. & Mastro V. C. (2010). Chemical ecology of the emerald ash borer *Agrilus planipennis. Journal of Chemical Ecology*, 36: 101-112.

Dicke, M. and Baldwin, I. T. 2010. The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. *Trends in Plant Science,* 15: 167-175.

Duan, J.J., Ulyshen, M.D., Bauer, L.S., Gould, J., & Van Driesche, R. (2010). Measuring the impact of biotic factors on populations of immature emerald ash borers (Coleoptera: Buprestidae). *Environmental Entomology*, 39: 1513-1522.

Duan, J.J., Yurchenko, G., & Fuester, R. (2012). Occurrence of emerald ash borer (Coleoptera: Buprestidae) and biotic factors affecting its immature stages in the Russian Far East. *Environmental Entomology*, 41, 245-254.

Erb, M., Lenk, C., Dengenhardt, J., and Turlings, T.C.J. 2009. The underestimated role of roots in defense against leaf attackers. *Trends in Plant Science,* 14: 653-659.

Eyles, A., Jones, W., Riedl, K., Cipollini, D., Schwartz, S., Chan, K., Herms, D.A., and Bonello, P. 2007. Comparative phloem chemistry of Manchurian (*Fraxinus mandshurica*) and two North American ash species (*Fraxinus americana* and *Fraxinus pennsylvanica*). *Journal of Chemical Ecology*, 33: 1430-1448.

Feeny, P.P. 1975. Biochemical coevolution between plants and their insect herbivores. Pages 3-19 in L.E. Gilbert and P.H. Raven (eds.). *Coevolution of Animals and Plants.* Austin, TX: University of Texas Press.

Gould, J., Tanner, J., Winograd, D., and Lane, S. 2005. Initial studies on the laboratory rearing of emerald ash borer and foreign exploration for natural enemies. Pages 73-74 in V. Mastro and R. Reardon (eds.), *Emerald ash borer research and technology development meeting*. U.S. Department of Agriculture, Forest Service, Morgantown, WV.

Halitschke, R., Stenberg, J.A., Kessler, D., Kessler, A., & Baldwin, I.T. (2008). Shared signals – 'alarm calls' from plants increase apparency to herbivores and their enemies in nature. *Ecology Letters,* 11: 24-34.

Johnson, R. and Wichern, D. A. 2002. Applied Multivariate Statistical Analysis, 5th ed. Prentice-Hall, Upper Saddle River.

Kovacs, K.F., Haight, R.G., McCullough, D.G., Mercader, R. J., Siegert, N.W., and Liebhold, A.M. 2010. Cost of potential emerald ash borer damage to US communities, 2009-2019. *Ecological Economics*, 69: 569-578.

Lelito, J. P., Fraser, I., Mastro, V. C., Tumlinson, J. H., Böröczky, K., & Baker, T. C. (2007). Visually mediated paratrooper copulations in the mating behavior of *Agrilus planipennis* (Coleoptera: Buprestidae), a highly destructive invasive pest of North American ash trees. *Journal of Insect Behavior,* 20: 537-552.

Leszczynski, B., Tjallingii, W.F., Dixon, A.F.G. and Swiderski, R. 1995. Effect of methoxyphenols on grain aphid feeding behavior. *Entomologia Experimentalis et Applicata,* 76: 157-162.

Lui, H. P, Bauer, L. S., Gao, R. T., Zhao, T., Petrice, T. H., & Haack, T. R. (2003). Exploratory survey for the emerald ash borer. *Agrilus planipennis* (Coloptera: Buprestidae) and its natural enemies in China. *Great Lakes Entomologist*, 36: 191-204.

Lui, H., Bauer, L.S., Miller, D.L., Zhaoc, T., Gaoc, R., Song, L., Luane, Q., Jine, R., & Gao, C. (2007). Seasonal abundance of *Agrilus planipennis* (Coleoptera: Buprestidae) and its natural enemies *Oobius agrili* (Hymenoptera: Encyrtidae) and *Tetrastichus planipennisi* (Hymenoptera: Eulophidae) in China. *Biological Control,* 42: 61-71.

Major, I. T. and Constabel, C. P. 2007. Shoot-root defense signaling andactivation of root defense by leaf damage in poplar. *Canadian Journal of Botony,* 85: 1171-1181.

Morita, M., Shitan, N., Sawada, K., Van Montagu, M.C.E., Inze, D., Rischer, H., Goossens, A., Oksman-Caldentey, K-M., Moriyama, Y., & Yazaki., K. (2009). Vacuolar transport of nicotine is mediated by a multidrug and toxic compound extrusion (mate) transporter in Nicotiana tabacum. *Proceedings of the National Academy of Sciences USA*, 106: 2447-2452.

MSU. 2013. Emerald Ash borer, http://www.emeraldashborer.info/ (last accessed 4/2/2013).

Muñoz, S. Trapero, García, Á. Hervalejo, Pérez, M. Jiménez, Boyero, J. R.,Vela, J. M., and Martínez-Ferri, E. 2008. Effects of rootstock and Flushing on the incidente of three insects on 'Clementine de Nules' citrus trees. *Environmental Entomology* 37: 1531-1537.

Peeters, P. 2002. Correlations between leaf structural traits and the densities of herbivorous insect guilds. *Biological Journal of the Linnean Society,* 77: 43-65.

Poland, T. M. and McCullough, D. G. 2006. Emerald ash borer; invasion of the urban forest and the threat to North America's ash resource. *Journal of Forestry*, 104: 118-124.

Pureswaran, D. S. and Poland, T. M. (2009). Host selection and feeding preference of *Agrilus planipennis* (Coleoptera: Buprestidae) on ash (*Fraxinus* spp.). *Environmental Entomology,* 38: 757-765.

Rebek, E. J., Herms, D. A., and Smitley, D. R. 2008. Interspecific variation in resistance to emerald ash borer (Coleoptera: Buprestidae) among North American and Asian Ash (*Fraxinus* spp.). *Environmental Entomology,* 37: 242-246.

Rodriguez, M.X., Buschmann, H., Iglesias, C., and Beeching, J.R. 2000. Production of antimicrobial compounds in cassava (*Manihot esculenta* Crantz) roots during post-harvest physiological deterioration. Pages 320-328 in L.J.C.B. Carvalho, A.M. Thro, A.D. Vilarinhos, (eds.). *Cassava Biotechnology. IVth International Scientific Meeting Cassava Biotechnology Network.* Embrapa, Brasilia.

Rodriguez-Saona, C., Poland, T. M., Miller, J. R., Stelinski, L. L., Grant, G. G., de Groot, P., Buchan, L., & Macdonald, L. (2006). Behavioral and electrophysiological responses of the emerald ash borer, *Agrilus planipennis*, to induced plant volatiles of Manchurian ash, Fraxinus mandshurica. *Chemoecology* 16: 75-86.

Rhoades D.F. and Cates, R.G. 1976. Toward a general theory of plant anti-herbivore chemistry. Pages 168-213 in J.W. Wallace and R.L. Mansell, (eds.). *Biochemical Interactions Between Plants and Insects.* New York: Plenum Press.

Shoji, T., Yamada, Y., and Hashimoto, T. 2000. Jasmonate induction of putrescine Nmethyltransferase genes in the root of *Nicotiana sylvestris*. *Plant and Cell Physiology* 41: 831–839.

Silva, M.C.P., Terra, W.R. and Ferreia, C. 2006. Absorption of toxic beta-glucosides produced by plants and their effect on tissue trehalases from insects. *Comparative Biochemistry and Physiology*, 143: 367-373.

Solt, M.L. (1957). Nicotine production and growth of excised tobacco root cultures. *Plant Physiology*, 32: 480-484.

StatSoft, Inc. 2005. STATISTICA (data analysis software system), version 7.1. www.statsoft.com.

Tooker, J. F., Koenig, W.A. and Hanks, L.M. 2002. Altered host plant volatiles are proxies for sex pheromones in the gall wasp *Antistrophus rufus*. *Proceedings of the National Academy of Science USA*, 99: 15486-15491.

Unsicker, S.B., Kunert, G., & Gershenzon, J. (2009). Protective perfumes: The role of vegetative volatiles in plant defense against herbivores. *Current Opinion in Plant Biology*, 12: 479-485.

USDA. 2010. Emerald Ash borer, http://www.nrs.fs.fed.us/disturbance/invasive_species/eab/biology_ecology/planipennis/ (last accessed 4/2/2013).

Wang, X.Y. (2005). Biology of the emerald ash borer and its biological control. *Postdoctoral Research Report: Chinese Academy of Forestry*, Beijing, China.

Wang, X.Y., Yang, Z.Q., Gould, J.R, Wu, H., & Ma, J.H. (2010). Host-seeking behavior and parasitism by *Spathius agrili* Yang (Hymenoptera: Braconidae), a parasitoid of the emerald ash borer. *Biological Control,* 52: 24-29.

Whitehill, J.G.A, Opiyo, S.O., Koch, J., Herm, D.A., Cipollini, D.F., Bonello, P. 2012. Interspecific comparison of constitutive ash phloem phenolic chemistry reveals compounds unique to Manchurian ash, a species resistant to Emerald ash borer. *Journal of Chemical Ecology*, 38: 499-511.

Yu, C.M. *Agrilus marcopoli* Obenberger. In: Xiao GR, editor. Forest Insects of China. 2nd edition. China Forestry Publishing House; 1992. pp. 400–401.

Ziegler, J. and Facchini, P. J. 2008. Alkaloid biosynthesis: metabolism and trafficking. *Annual Review of Plant Biology* 59: 735-769.