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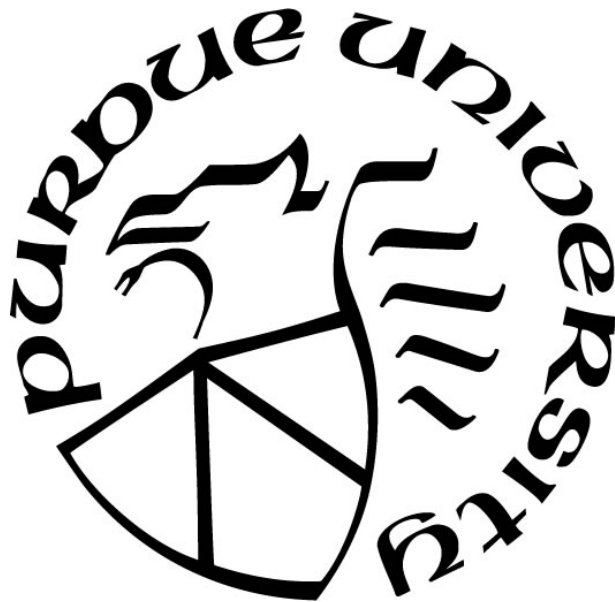Follow this and additional works at: https://docs.lib.purdue.edu/open_access_theses

MANCHURIAN ROOTSTOCKS INFLUENCE EAB RESISTANCE WHEN GRAFTED TO NORTH AMERICAN ASH

by

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A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science

Department of Entomology

West Lafayette, Indiana

May 2018

THE PURDUE UNIVERSITY GRADUATE SCHOOL
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ACKNOWLEDGMENTS

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ABSTRACT

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Degree Received: May 2018
Title: Influence of Manchurian Rootstocks in Grafting for Emerald Ash Borer Resistance in North American Ash.
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Emerald ash borer (EAB; *Agrilus planipennis* Fairmaire Coleoptera: Buprestidae) is an invasive phloem-boring pest from Asia that has killed tens of millions of North American ash trees (*Fraxinus* spp.). In its native range, EAB only attacks highly stressed ash trees, functioning as a secondary pest. Because Asian ash shares a co-evolutionary history with EAB, they possess a suite of secondary defensive compounds that prevent EAB from successfully colonizing healthy trees. However, all North American ash trees, regardless of health, are susceptible to EAB, and typically die within four years of infestation. Propagating ash resistant to EAB is crucial to maintaining the North American ash resource. Because many secondary metabolites utilized in plant defense are synthesized in the roots, interactions between the roots and shoots of the tree are critical in host plant resistance. Grafting utilizes this root-shoot connectivity to confer desirable traits, such as resistance to a pest or pathogen, from the rootstock of a plant to its scion. In chapter 1, I review the literature on EAB and its life history, mechanisms of host plant resistance, the practice of horticultural grafting, and root-shoot interactions in grafted plants. In chapter 2, I determine the extent to which resistant Manchurian ash rootstocks confer EAB resistance to susceptible green ash scions. The resistance capability of the parent stock for grafted trees was validated by assessing woodpecker damage and canopy dieback. Adult EAB survivorship, total leaf area consumption, and fecundity, as well as morphological tree characteristics, were measured in the field on grafted and buffer green and Manchurian ash in Indiana in 2017. Grafting trees with resistant rootstocks holds promise for propagating EAB-resistant ash trees. Although total leaf area consumed was not affected by graft combination, beetles caged on conspecific green ash lived longer and laid more eggs than beetles caged on trees with a Manchurian ash scion or rootstock. Beetle survival did not differ between conspecific Manchurian ash and heterospecific green and Manchurian trees. Beetles caged on trees with Manchurian scions and green rootstocks laid more eggs than beetles on caged on trees

with green scions and Manchurian rootstocks, and no beetles caged on conspecific Manchurian ash laid eggs. This result demonstrates that, although any grafted tree with a Manchurian rootstock or scion will be more resistant than a conspecific green tree, rootstock has a greater effect than the scion on traits such as fecundity. My findings demonstrate that EAB resistance can be conferred from rootstock to scion, and that grafting could be used to develop EAB-resistant ash.

CHAPTER 1- LITERATURE REVIEW ON EMERALD ASH BORER AND HOST PLANT RESISTANCE

1.1 Introduction

Emerald ash borer (EAB; *Agrilus planipennis* Fairmaire, Coleoptera: Buprestidae) is an invasive phloem-boring pest from Asia. Since its initial discovery in North America in 2002, EAB has killed hundreds of millions of ash trees (*Fraxinus* spp.) and billions more are at risk (Herms and McCullough 2014). To date, attempts to eradicate EAB or halt its spread in North America have been unsuccessful, and it has become established in thirty-one states and three Canadian provinces (emeraldashborer.info, 2018). In its native range, EAB is a secondary pest that primarily infests stressed or dying ash, but in North America, EAB attacks healthy as well as stressed ash (Poland and McCullough 2006, Baranchikov et al. 2008). Emerald ash borer is capable of colonizing all North American ash species, but its host preference for adult feeding, oviposition, and larval performance varies within the genus (Anulewicz et al. 2008, Rebek et al. 2008, Pureswaran et al. 2009, Tanis and McCullough 2015). Larvae feed on the vascular tissue of the tree which cuts off the movement of nutrients and leads to thinning of the canopy and the ultimate death of the tree within two to four years of infestation (Herms and McCullough 2014).

Like many plants, ash trees possess mechanisms to defend themselves against insect attack, though these defenses vary both quantitatively and qualitatively among different ash species (Anulewicz et al. 2008, Rebek et al. 2008, Tanis and McCullough 2015). Because plant defense compounds against leaf-feeding insects are synthesized in the root system and transported to the above-ground portion of the tree, grafting a susceptible North American scion to a resistant Asian rootstock could yield a North American ash tree resistant to EAB (Erb et al. 2009). To better understand the potential for developing ash varieties resistant to EAB, here I review the

natural history of EAB and its invasion of North America. Mechanisms of host plant resistance, the practice of horticultural grafting to propagate insect resistance in plants, and root-shoot interactions in grafted plants will also be discussed.

1.2 Emerald Ash Borer (*Agrilus planipennis* Fairmaire)

Exotic invasive species are organisms that are established outside of their native range and cause economic damage. They are highly prevalent in the United States, with an estimated 50,000 invasive species causing $137 billion in damage annually (Pimentel et al. 2000). However, no exotic invasive species has caused destruction in North American forests on the scale of EAB, which invaded North America in the 1990s (Siegert et al. 2014). In the United States, many cities planted ash trees after destruction of the popular street tree American elm (*Ulmus Americana* L.) by Dutch elm disease, making urban forests exceedingly vulnerable to attack by EAB (Herms and McCullough 2014, Griffin et al. 2017). The cost of treating, removing, and replacing the 17 million urban ash trees in the US from 2009-2019 is $10.7 billion (Kovacs et al. 2011). Ecological impacts of wild ash disappearing from forest landscapes would also be catastrophic, as at least 282 arthropod species feed on ash (Gandhi and Herms 2010b).

The native range of EAB includes the Russian Far East, Korea, Japan, and China (Wei et al. 2004). Emerald ash borer spends its entire life cycle on ash trees (Poland and McCullough 2006). EAB females lay between 40 and 70 eggs, which are deposited individually on the bark of ash trees (Wang et al. 2010). Upon hatching, larvae bore into the tree to feed in the cambium and phloem, creating serpentine galleries in the phloem and outer sapwood. These serpentine galleries slow the movement of nutrients and water throughout the tree (Herms and McCullough 2014). Larvae complete four instars before pupating, then emerge from ash trees as adults. After one to two weeks of maturation feeding, adult EAB spend the remainder of their six-week lifespan mating and laying eggs (Wang et al. 2010). The eggs hatch ten to fourteen days later,

and larvae chew through the bark and begin to feed (Cappaert et al. 2005). Depending on the vigor of the host plant, it can take one or two years for EAB to complete development (Wang et al. 2010). If its host tree is highly stressed, a larva will spend summer feeding, then enter a prepupal stage in autumn to overwinter in the outer sapwood or bark (Villari et al. 2016). In a healthy host tree, larvae spend their first winter in an early instar and feed for another summer before overwintering as pupae (Cappaert et al. 2005). Climate can also influence larval development, with the development of beetles in northern habitats typically requiring two years (Crook and Mastro 2010). Regardless of the length of their life cycle, EAB must overwinter as prepupae before they can pupate in mid- to late spring and emerge as adult beetles, ready to feed and find a mate (Herms and McCullough 2014).

No long-range pheromones are known to be involved in EAB mating behavior (Otis et al. 2005). Instead, males utilize visual cues to locate females already on a host plant, then drop onto the female in what is known as paratrooper copulation (Lelito et al. 2007). Males differentiate between other male beetles and female beetles using the female-specific contact pheromone 3-methyltricosane, and often fly away after making contact with another male (Lelito et al. 2009, Wang et al. 2010, Crook and Mastro 2010). Because mating occurs on the larval host tree, a mutual attraction to host volatiles is essential to unite the sexes (Crook and Mastro 2010).

Ash trees produce volatile organic compounds (VOCs), which influence host location and colonization in EAB (Rodriguez-Saona et al. 2006) and vary both quantitatively and qualitatively among ash species (Markovic et al. 1996, Rodriguez-Saona et al. 2006, de Groot et al. 2008, Crook and Mastro 2010, Chen et al. 2011). In electroantennogram experiments, a suite of these were antennally-active and likely play a role in host location behavior (Rodriguez-Saona et al. 2006). For example, hexanal is an antenally active compound emitted by Manchurian (*Fraxinus*

mandschurica Rupr.), black (*Fraxinus nigra* Marshall), green (*Fraxinus pennsylvanica*

Marshall), and white (*Fraxinus americana* L) ash (Kolich 2014). Manchurian ash produces at

least sixteen antennally active VOCs, some of which are only found in Manchurian ash (e.g., 2-

butoxy ethanol and (*Z*)-ocimene) (Rodriguez-Saona et al. 2006). Examples of VOCs not

produced by Manchurian ash include (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate, which are found

in white and green ash (de Groot et al. 2008). Antennally active VOCs can be valuable

components of EAB management strategies. For example, (*Z*)-3-hexenol is highly attractive to

male EAB, and it has been incorporated into a successful lure used for trapping EAB in the field

(Grant et al. 2010). These variations in VOC profiles among ash reflect varying EAB host

suitability among different species of ash (Crook and Mastro 2010).

Susceptibility to EAB is highly variable within the genus *Fraxinus*. North American ash

species such as black, white, and green ash are far more susceptible to attack and colonization by

EAB than Manchurian and Chinese ash (*Fraxinus chinensis* Roxb.), which are native to Asia

(Cappaert et al. 2005, Anulewicz et al. 2008, Rebek et al. 2008, Tanis and McCullough 2015).

Popular ornamental cultivars of ash, including 'Autumn Purple' and 'Summit', are varieties of

vulnerable white and green ash species, respectively (Santamour and McArdle, 1983). In a study

conducted in an ash plantation infested with EAB, blue ash (*Fraxinus quadrangulata* Michx.),

which is native to North America, had higher survivorship rates than white, green, and black ash

(Tanis and McCullough 2012, Tanis and McCullough 2015). The Manchurian and blue ash used

in the study also had significantly fewer larval galleries than green or black ash, and 71% of the

blue ash had no larval galleries, suggesting that blue ash is a less preferred and less suitable host

for EAB (Tanis and McCullough 2015). Beetles caged on blue ash had significantly lower

survivorship than those caged on white ash (Tanis and McCullough 2015). Asian ash species

likely possess secondary defensive compounds, developed through coevolution, which defend against EAB infestation and are responsible for the variations in host plant suitability (Chen et al. 2011). Because these defenses can be induced in North American ash, it is thought that, while they possess the same defensive mechanisms as Asian ash, they do not respond quickly enough when attacked by EAB to prevent infestation (Villari et al. 2016). These variations in host plant resistance among different species of ash could be utilized to develop resistant varieties of ash (Rebek et al. 2008, Koch et al. 2011).

1.3 Host Plant Resistance

Host plant resistance is the term given to the suite of defenses present in plants to repel insect attack or kill colonizing insects. Due to intense pressure from insects, there is a stunning diversity of plant defensive mechanisms against insects (Gong and Zhang 2013). These defenses can be constitutive and always present, or inducible and produced in response to insect attack. Some defenses create physical barriers to protect the plant, such as thorns, resin production to entrap invading insects, or sap flow (Morewood et al. 2004). Biochemical defensive compounds are secondary metabolites involved in host plant resistance are not directly related to primary growth and reproduction, but are still essential for the plant to thrive (Berenbaum 1995). They can range from antifeedants to discourage feeding to toxic compounds that kill herbivores (Gatehouse 2002). Plants develop these resistance mechanisms through generations of coevolution with insect pests (Feeny 1976, Jermy 1984). However, North American ash trees lack a coevolutionary history with EAB (Gandhi and Herms 2010a). As such, most North American ash are largely unable to defend themselves and experience widespread mortality from EAB (Wei et al. 2003, Jacobs 2007, Rebek et al. 2008, Villari et al. 2016).

When valuable native plants are threatened by an exotic invasive pest, variations in host plant resistance in the affected species can be used to develop resistant plants. In the twentieth century,

both American chestnut (*Castanea dentata* Borkh.) and American elm were devastated by

chestnut blight (*Cryphonectria parasitica* Barr*)* and Dutch elm disease (DED), respectively

(Strobel and Lanier 1981, Paillet 2002). However, the development of resistant phenotypes

through selective breeding could facilitate the restoration of these species to American forests

(Merkle et al. 2006, Jacobs 2007, Griffin et al. 2017). For example, crossbreeding DED-

susceptible American elm trees to resistant Asian elm, as well as selective breeding of individual

DED-resistant American elm, has produced several DED-resistant elm cultivars (Merkle et al.

2006). Some of these cultivars, such as 'Princeton' and 'New Harmony', survived at rates greater

than 80% in sixteen different locations around the United States over a study period of ten years,

which is promising for the future of American elm (Griffin et al. 2017). Similarly, a program of

backcross breeding between susceptible American chestnut and resistant Chinese chestnut has

produced hybrid trees that are resistant to chestnut blight yet are otherwise indistinguishable

from American chestnut (Jacobs 2007).

Although selective breeding holds promise for the regeneration of species like American

chestnut and American elm, there are other techniques that could be used to create resistant

plants. One of these techniques is grafting, in which the root system of one plant (called the

rootstock) and the shoot of another (called the scion) are fused together and grow as a unified

composite plant (Goldschmidt 2014). Although a successfully grafted plant functions as a single

individual, there are interactions between the rootstock and scion that influence the physiological

and chemical properties of the scion, such as promoting resistance to a pest or using a dwarfing

rootstock to increase fruit yield (Tworkoski and Miller 2007, Muñoz et al. 2008, Goldschmidt

2014).

As a vegetative and asexual propagation technique, grafting provides complete control over the genetic outcome of the plant, without the uncertainty that arises from crossbreeding (Pallardy 2008). Ash trees are typically monoecious, with the exception of dioecious blue ash (Wallander et al. 2008). Northern Treasure is an F_1 hybrid cross between susceptible female black ash and resistant male Manchurian ash (Davidson and Ronald 2001). Due to its resistant Manchurian parent, there was hope that Northern Treasure would be resistant to EAB.

However, Northern Treasure proved to be highly susceptible to EAB, with 100% EAB-induced tree mortality within a three-year study (Rebek et al. 2008). The many advantages of grafting make it a promising candidate for developing EAB-resistant North American ash.

1.4 Grafting: Concepts and Applications

Grafting is the fusion of the vascular tissue of two plants into a single composite individual. It is used in agriculture, horticulture, and forestry as a method of asexual plant propagation (Goldschmidt 2015). Grafting as an agricultural technique has been employed for millennia, with references dating back to the fifth century BCE, although some evidence suggests that the practice could have begun earlier (Mudge et al. 2009). Ancient societies grafted many types of perennial plants, ranging from fruit trees to grape vines, but the grafting of annual plants, like many vegetables, has become increasingly common in the past century (Rivero et al. 2003).

Successful graft formation depends on the capacity of vascular tissues of the scion to unite with those of the roots. This is often determined by phylogenetic relatedness between the plants. Plants belonging to different families are generally incompatible and can rarely be grafted (Goldschmidt 2015). Because North American and Asian ash are congeners, grafts among individuals belonging to the genus are likely to be successful.

One of the most common goals in grafting is to develop a plant with desirable characteristics found in multiple cultivars or species. Grafting can produce plants resistant to abiotic or biotic

stress factors, such as disease or insect herbivores, as well as promote desirable traits like higher fruit yield or faster growth rates (Baldry et al. 1982, Ortiz et al. 1986, Garcia-Sanchez et al. 2006, Duke et al. 2006, Tworkoski and Miller 2007, Muñoz et al. 2008). Popular ash cultivars, such as Autumn Purple, are grafted, and are often grown on fast-growing green ash rootstock, showing that ash is a good candidate for grafting to boost host plant resistance (Ball 2004). Grafting could be used to grow a North American ash scion on an Asian ash rootstock, resulting in a North American ash tree with the resistance capabilities of Asian ash.

1.5 Root-Shoot Interactions in Grafted Plants

In grafted plants, interactions between the rootstock and scion produce a plant with physiological and chemical characteristics influenced by both the rootstock and scion (Goldschmidt et al. 2014). However, because many defensive compounds are synthesized in plant roots and transported to the above ground parts of a plant, the resistance capabilities of the rootstock could strongly affect the overall host plant resistance of the grafted plant (Erb et al. 2009). This root-shoot connectivity seen in grafted plants, and its effects on host plant defense, could be used to develop an EAB-resistant North American ash tree. If a resistant Manchurian rootstock could transmit its chemical defenses to a susceptible North American scion when grafted together, the resulting tree could have the EAB resistance capabilities of Asian ash with the appearance of a North American ash.

The ability of rootstocks to influence various characteristics of scions is well-documented (Baldry et al. 1982, Ortiz et al. 1986, Garcia-Sanchez et al. 2006, Tworkoski and Miller 2007). For example, rootstocks affect scion growth and can be used to promote or reduce plant growth. In apple (*Malus* spp.) trees, dwarfing rootstocks are used to control tree size in high-density plantings, which increases fruit size and overall yield (Tworkoski and Miller 2007). In the Mediterranean, certain rootstocks improve fruit yield in salt-sensitive mandarin orange trees

(*Citrus reticulata* Blanco) and (*C. sinensis* [L.] Osbeck × *Poncirus trifoliata* [L.] Raf.), which are typically grown in low-quality, saline environments (Garcia-Sanchez et al. 2006). Rootstocks not only influence tree growth and performance, they also affect characteristics of fruit produced by the scion. Different rootstocks affect trait such as size and acidity in citrus fruits like lemons and oranges (Baldry et al. 1982, Ortiz et al. 1986). However, scions also affect traits of the grafted plant. In young apple trees, scion type influences the duration of tree growth, while rootstock type affects growth rate and tree weight (Vyvyan 1955).

Grafting can promote resistance against insect pests in cultivars or species that normally are susceptible to infestation. In clementine trees (*Citrus clementina* Hort. *ex* Tan.), certain rootstock varieties confer greater resistance against leafminers (*Phyllocnistis citrella* Stainton) to scions (Muñoz et al. 2008). When fed on leaves from grafted mahogany (*Swietenia macrophylla* King and *Khaya senegalensis* A. Juss**)** and cedar (*Cedrela odorata* L. and *Toona Ciliata* M. Roem) trees, larval survivorship of mahogany shoot borer (*Hypsipyla grandella* Zeller) was lower on trees with a susceptible scion grafted onto a resistant rootstock than a conspecific susceptible grafted tree, with survivorship comparable to that of a conspecific resistant grafted tree (Perez et al. 2010). Resistant rootstocks are also used to grow lodgepole pine (*Pinus contorta* var. *latifolia* Cerezke) resistant to the Warren root collar weevil (*Hylobius warreni* Wood) (Duke et al. 2006). As scion-rootstock connectivity is better understood, particularly the influence of the rootstock on various characteristics expressed by the scion, it seems likely that EAB resistance could be conferred from rootstock to scion in ash trees, making grafting a valuable potential tool for preserving the North American ash resource (Kolich 2014).

1.6 Summary

Emerald ash borer is a devastating exotic insect pest that could extirpate the North American ash resource (Rebek et al. 2010, McCullough and Herms 2014). Variation in host plant resistance among different ash species holds potential for developing resistant ash cultivars via grafting. Although host plant resistance in ash has been extensively studied, the effects of grafting a susceptible scion to a resistant rootstock have not been investigated (Rebek et al. 2008, Chen et al. 2010; Tanis and McCullough 2012, Tanis and McCullough 2015, Peterson et al. 2015). However, because the cultivars used in other resistance studies, such as Autumn Purple, are grafted, and green ash is a commonly used rootstock in ash grafting, it is possible that the effects of susceptible rootstocks have already been measured, albeit inadvertently (Ball 2004). Because of the potential grafting holds for propagating resistant North American ash, there is a critical need to understand the effects of grafting on EAB fitness.

Previous work (Kolich 2014) offers some insight into how different graft combinations affect EAB fitness, but does not test the question directly. This study focused on the effects of grafting both within and between species on VOC profiles and adult EAB leaf consumption, using green, white, black, and Manchurian ash. Of the nine VOCs found in all graft combinations, there were both qualitative and quantitative differences in volatile profiles among graft combinations. Green ash scions on Manchurian ash rootstocks and conspecific green ash produced significantly more (*E*)-2-hexenal and significantly less hexanal and (*Z*)-3-hexenol than conspecific Manchurian ash. These compounds are all antenally-active and likely influence host location, making these variations potentially meaningful in terms of EAB behavior. Additionally, the influence exerted on the scion by the rootstock indicates that other chemical attributes of the tree, such as defensive compounds, could also be affected by grafting. In adult EAB feeding assays conducted on excised leaves, beetles caged on conspecific green ash trees and Manchurian ash scions on green ash

rootstock consumed more leaf area than beetles caged on green ash scions on Manchurian ash rootstock and conspecific Manchurian trees (Kolich 2014). However, no survivorship or fecundity assays were performed on these grafted trees, meaning that there remains a critical knowledge gap regarding EAB performance on grafted trees. The effects of grafting on host location and suitability for EAB could have implications for the development of resistant North American ash.

Although the results of Kolich (2014) are promising, more work is needed to determine the potential efficacy of grafting as a tool to develop resistant North American ash. No survivorship or fecundity bioassays have been conducted on grafted ash trees, which are critical to determining how grafting affects host plant resistance in ash trees and subsequent effects on EAB fitness. Bioassays of different EAB fitness measures, like adult survivorship, feeding, and fecundity conducted on grafted ash trees would help determine the extent to which resistance can be conferred from rootstock to scion. Additionally, measuring various characteristics of grafted trees, such as height or canopy index, would facilitate a deeper understanding of how grafting influences trees. Studying the effects of grafting North American and Asian ash on EAB fitness could elucidate whether grafting is a viable option for maintaining the North American ash resource.

CHAPTER 2. EAB PERFORMANCE AND TREE CHARACTERISTICS ON GRAFTED *FRAXINUS* spp.

2.1 Abstract

The invasive emerald ash borer (EAB; *Agrilus planipennis* Fairmaire Coleoptera: Buprestidae) is a phloem-boring pest from Asia that has killed tens of millions of North American ash trees (*Fraxinus* spp.). Because of its co-evolutionary history with Asian ash EAB only attacks highly stressed ash trees in its native range and functions as a secondary pest. However, all North American ash trees are susceptible to EAB because they lack the secondary defensive compounds possessed by Asian ash and typically die within four years of infestation. To maintain the North American ash resource, we must seek ways of propagating ash resistant to EAB. Interactions between the root and shoot of a tree are critical to host plant resistance, as many secondary metabolites utilized in plant defense are synthesized in the roots. This root-shoot connectivity can be exploited using grafting to confer desirable traits, such as resistance to a pest or pathogen, from the rootstock of a plant to its scion. In this study, I sought to determine the extent to which resistant Manchurian ash rootstocks confer EAB resistance to susceptible green ash scions. By assessing woodpecker damage and canopy dieback, we validated the resistance capability of the parent stock for grafted trees. I measured adult EAB survivorship, total leaf area consumption, and fecundity, as well as morphological tree characteristics on grafted and buffer green and Manchurian ash in Indiana in 2017. Beetles caged on conspecific green ash lived longer and laid more eggs than beetles caged on trees with a Manchurian ash scion or rootstock, although total leaf area consumed per beetle was not affected by graft combination. Beetle survival did not differ between conspecific Manchurian ash and heterospecific green and Manchurian trees. Beetles caged on trees with Manchurian scions and green rootstocks laid more

eggs than beetles on caged on trees with green scions and Manchurian rootstocks, and no beetles caged on conspecific Manchurian ash laid eggs. Our findings show that a grafted tree with a Manchurian rootstock or scion will be more resistant than a conspecific green tree, rootstock has a greater effect than the scion on traits such as fecundity. EAB resistance is conferrable from rootstock to scion, and grafting could be used to develop EAB-resistant ash.

2.2 Introduction

Emerald ash borer (EAB; *Agrilus planipennis* Fairmaire, Coleoptera: Buprestidae) is an invasive wood-boring insect that threatens the North American ash resource (*Fraxinus* spp.; Herms and McCullough 2014). Since its arrival in North America in the early 1990s, EAB has spread to thirty-one states and three Canadian provinces (emeraldashborer.info, 2018). EAB has killed hundreds of millions of ash trees, and billions more are at risk (Herms and McCullough 2014). Adult beetles lay their eggs in the crevices of ash bark, and upon hatching, larvae bore into the phloem to feed, creating serpentine galleries in the phloem and outer sapwood. These galleries slow the movement of nutrients and water throughout a tree, girdling the branches and trunk. Girdled branches are nutrient-starved and do not produce leaves. The tree canopy thins as larval density increases, and ultimately, the tree dies (Tluczek et al. 2011). Trees typically die within two to four years of infestation (Herms and McCullough 2014).

In its native range of eastern Asia, EAB functions as a secondary pest, attacking and killing stressed ash (Wei et al. 2004, Baranchikov et al. 2008). Yet in North America, EAB readily attacks and kills healthy or stressed ash (Poland and McCullough 2006). No single species of ash is preferred by EAB for feeding and oviposition, and larval performance also varies within the genus *Fraxinus* (Anulewicz et al. 2008, Rebek et al. 2008, Pureswaran and Poland 2009). In general, North American ash species such as green (*F. pennsylvanica*

Marshall), white (*F. americana* L), and black ash (*Fraxinus nigra* Marshall), are highly

susceptible to EAB, and seem to lack the secondary defensive compounds that protect healthy

Asian ash from EAB (Bonello et al. 2007, Eyles et al. 2007, Rebek et al. 2008, Chen and Poland

2010). The exception is blue ash (*F. quadrangulata* Michx.), a North American ash species with

resistance levels comparable to that of Asian ash (Tanis and McCullough 2012, Tanis and

McCullough 2015). This variation in resistance may be attributed to differences in the volatile

organic compound (VOC) profiles and defensive compounds among ash species (Villari et al.

2016).

In order to locate and colonize suitable *Fraxinus* hosts, EAB detect VOCs emitted by the

leaves and bark, both constitutively and in response to herbivore feeding (Crook and Mastro

2010). As with defensive compounds found in the phloem, VOCs also vary qualitatively and

quantitatively among ash species (Rodriguez-Saona et al. 2006, de Groot et al. 2008, Chen and

Poland 2011). Manchurian ash emits at least sixteen VOCs that are antennally-active to both

male and female EAB in electroantennogram experiments, which is twice the number of known

compounds produced by green and white ash. Manchurian ash is resistant to EAB and high VOC

emission may signal such to colonizing adult beetles (Crook and Mastro 2010). Additionally,

Manchurian ash possesses nine phenolic compounds not found in susceptible green or white ash,

including esculin, a trehalase inhibitor that interferes with insect metabolic function (Silva et al.

2006, Cipollini et al. 2011). Adult EAB oviposit less on Manchurian ash than on North American

species, and their leaves are less preferred by foraging adults (Rebek et al. 2008, Pureswaran and

Poland 2009, Rigsby et al. 2014, Tanis and McCullough 2015, Rigsby et al. 2017). Effectively

exploiting these variations in phloem and foliar chemistry, and their desirable effects on EAB

attraction and fitness, could be essential for developing North American ash varieties that are resistant to EAB.

Grafting, the fusion of the vascular tissue of two plants into a single composite individual, is a common technique used to promote desirable traits in plants (Goldschmidt 2014). In grafted plants, the root system, known as the rootstock, affects chemical and physiological traits expressed by the above-ground portion of the plant, called the scion. Rootstocks can promote dwarfing phenotypes, increase fruit yield, and protect the plant against pathogens and insect herbivores (Duke and Lindgren 2006, Garcia-Sanchez et al. 2006, Tworkoski and Miller 2007, Muñoz et al. 2010, Perez et al. 2010). Roots play an essential role in plant defense. The root-shoot-root loop transports many foliar defensive compounds that are synthesized in the roots to the leaves (Erb et al. 2009). Previous work by Kolich (2014) demonstrated that the volatile profiles of North American scions grafted onto Asian rootstocks were quantitatively different from those of conspecific grafted (grafts of the same species) North American and Asian trees. However, the effects of heterospecific grafting (grafts of two different species) on host plant resistance among different species of ash are unknown. Additionally, grafting does affect other characteristics of plants, such as height, which can be particularly important when propagating ornamental trees.

In this study, I tested the hypothesis that resistant Asian rootstocks confer EAB resistance to susceptible North American scions in grafted trees by comparing adult EAB survivorship, herbivory, and fecundity on grafted and buffer Manchurian and green ash trees. I also determined the extent to which ash rootstocks affected height, canopy index, diameter, leaf area, and leaflet number- important morphological traits of the scion. Understanding the extent to which root-

shoot connectivity confers traits from rootstock to scion will ultimately lead to techniques for

propagating North American ash species that are resistant to EAB.

2.3 Materials and Methods
2.3.1 Evaluation of EAB Resistance in Parent Stock
In 2006, the parent stock trees of the grafted ash used in this study were planted at the

Richard R. Lugar Forestry Farm (Tippecanoe Co., IN). Parent stock trees were originally

purchased as saplings from Bailey Nurseries (Newport, MN), Lawyer Nursery (Plains, MT), and

Musser Forests, Inc. (Indiana, PA). In total, 113 trees (45 white ash, 45 green ash, and 23

Manchurian ash) were planted in a completely randomized design. Because these trees were

never treated with insecticides, they were infested by emerald ash borer sometime after its initial

detection in Tippecanoe County in 2011. This infestation allowed me to use them as proxies for

the resistance capabilities for the grafted trees used in this study. To evaluate the resistance

capabilities of these trees, canopy dieback and woodpecker damage were used as measures of

EAB larval density (Hughes et al. 2015). As EAB larval density increases in a tree, branches are

girdled and no longer produce leaves, causing canopy dieback. Woodpeckers will also peck

holes and peel back the bark of heavily infested trees to eat EAB larvae. Therefore, a tree that is

susceptible to EAB and has been heavily infested will have high canopy dieback and

woodpecker damage. I assessed woodpecker damage on parent stock on 9 May 2017 to establish

the baseline resistance to EAB of the grafted trees used in this study. Woodpecker damage was

categorized as 0 (no woodpecker damage present on the tree), 1 (woodpecker damage present but

not abundant, with holes on branches in the canopy), or 2 (woodpecker damage abundant, with holes on branches in the canopy and the trunk). DBH was measured at 1.3 m above the ground with diameter tape on 9 May 2017. On 6 July 2017, I measured the percentage of canopy dieback in all trees by visually assessing branch dieback (Rebek et al. 2008).

2.3.2 Field Site and Experimental Design

The study was conducted at the Purdue University Harrold Woodland (Whitley Co., IN). Trees used in the study were grafted from parent stock growing at the Richard R. Lugar Forestry Farm (Tippecanoe Co., IN) in April 2010. These grafts represented all combinations of black, Manchurian, Chinese, green, and white ash. Grafted trees were kept in a shade frame under drip fertigation with 0.115 g of Peters' Professional Fertilizer (12-12-12; N-P-K) per tree per day at the Purdue University John S. Wright Forestry Center (Tippecanoe Co., IN) until they were planted at the study site in May 2011 (Kolich 2014). There were four replicates of grafted trees planted in a randomized complete block design. The 125 saplings represented four replicates of all 20 heterospecific graft combinations and five replicates of conspecific grafts of each species (Table 2.1). Saplings of each species (n=25) were planted randomly along two perpendicular rows between blocks to serve as buffer rows. Trees were planted in rows three meters apart, with trees six meters on center.

From 2011-2015, trees were sprayed twice annually in the spring with a foliar application of Astro (36.8% permethrin, Astro® 3.2 EC, FMC Agricultural Solutions, CA) to prevent *A. planipennis* infestation. The product was applied according to label instructions (0.85 mL/L) and sprayed to runoff. On 23 May 2016, branches thicker than 1 cm, and therefore vulnerable to attack by EAB, were painted with Astro (36.8% permethrin, Astro® 3.2 EC, FMC Agricultural Solutions, CA) using a 6.35 cm wide paintbrush to protect them from EAB without contaminating leaves used in the adult performance bioassays. On 11 April 2017, all trees were

visually inspected to determine whether the rootstock and scion were alive. Dead trees that lacked leaves were not used in bioassays. On 11 April 2017, all trees were fertilized with approximately 113.4 grams of Greenskeeper's Secret All-Purpose Fertilizer (12-12-12; N-P-K), which was applied uniformly around each tree, from the bole to the dripline.

2.3.3 Source of Beetles

The three cohorts of beetles used in this study were reared from naturally infested ash logs harvested prior to EAB emergence. On 15-17 February 2017, EAB-infested green and white ash trees were harvested at Richard G. Lugar Forestry Farm (Tippecanoe Co., IN). Infested trees were felled with a chainsaw, immediately sectioned into ~70 cm bolts, and kept in cold storage at 4°C at Throckmorton-Purdue Agricultural Center (Tippecanoe Co., IN). On 11 May, 12 June, and 23 July, logs were placed in in rearing chambers made from cardboard tubes with plastic plugs (2.44 m by 3 m by 2.44 m; 26°C, 55% RH and 14:10 h L:D cycle) and checked twice daily. Emerged beetles were removed and fed white ash foliage in an environmental chamber at 29°C, with 75% relative humidity and a 14:10 h (L:D) cycle until used in experiments.

Beetles began to emerge from logs two weeks after removal from cold storage. In the May cohort, beetles were separated by sex based on morphological characteristics immediately after emergence (Rutledge and Keena 2012). One male and three female beetles were placed in plastic cups (Walmart, Bentonville, AZ) that were 12 cm tall, with a 6 cm diameter base and 9.8 cm rim, containing a piece of filter paper, trimmed to fit the cup (9 cm diameter, Whatman, Maidstone, United Kingdom) and a white ash leaf. Leaf petioles were cut diagonally and placed it in a 7.4 mL vial filled with water. Each cup was covered by a piece of screen mesh (10 cm x 10 cm, Walmart, Bentonville, AZ) beneath an unbleached coffee filter (Meijer, Grand Rapids, MI), held in place with a rubber band. Leaves and filter paper were replaced and dead beetles were removed every four days. In July, beetles were not separated by sex until 24 hours before each

bioassay. In July, all beetles that emerged each day were placed together in a larger plastic

container (29.3 cm in diameter, 76.3 cm high). Each container had a piece of filter paper and four

white ash leaves in a smaller plastic container (3.3 cm wide and 9.2 cm high) filled with water.

After use, all containers were cleaned with antibacterial hand soap (active ingredient triclosan

0.10%, Great Value™, Walmart, Bentonville, AZ) and warm water. In August, beetles were

placed in groups of eight, with two males and six females, in the same plastic containers used in

July.

2.3.4 EAB Bioassays

Field studies were conducted from 5-19 June, 8-22 July, and 9-23 August 2017. Cages used

in bioassays were constructed according to the design outlined in Tanis and McCullough 2015

(Figure 2.1). Four beetles (one male and three females) were placed in each cage, and then

transported to the field site in coolers. In July and August, the coolers contained ice packs

covered in a layer of cardboard and newspaper. Two cages were placed on separate intact leaves

on different branches per tree. The opening of each cage using binder clips, and each cage was

secured in place by rope cords tied around branches. In June, five- to seven-day-old beetles were

starved for 24 hours prior to being placed in coolers and transported to the field site in the bed of

the truck. Over half of the beetles used in the June bioassay died between being placed on the

tree and the first time I assessed survivorship, possibly due to stress from lack of available food

or from being transported. Consequently, five- to seven-day old beetles had a white ash leaf

placed in their cage to eat while being transported to the field in July and August, and the coolers

were kept in the air-conditioned cab of the truck.

Thirty-eight grafted trees and 12 buffer trees were used in June, 21 grafted trees and 7 buffer

trees were used in July, and 4 grafted trees were used in August (Table 2.2). At the conclusion of

each 14-day trial, all surviving beetles were transported back to the lab in coolers in the cab of

the truck. They were immediately separated by sex and placed in individual rearing containers, as previously described, with a leaf from the tree they had occupied during the field assay. Beetles were kept in an environmental chamber at 29°C, with 75% relative humidity and a 14:10 h (L:D) cycle. Survivorship was assessed every other day and leaves were changed every four days until all beetles had died.

When leaves were changed, the coffee filter was inspected to determine whether beetles had laid eggs. If eggs were present on the coffee filter, it was replaced with a fresh filter. Eggs were examined under a dissecting microscope, counted and assessed to determine whether they were fertile. Fertilized eggs are brown, whereas unfertilized eggs are yellow and desiccate over time (Rutledge and Keena 2012). Each filter paper was stored in an individual Petri dish in an environmental chamber with conditions described above for four weeks. They were checked every other day for three weeks, and the number of larvae that hatched were counted. All adult beetles that were alive at the conclusion of the field study were dissected and examined under a Leica M165C stereomicroscope (Danaher, Wetzlar, Germany) for spermatophores or eggs that had not been laid. If present, spermatophore length was measured and eggs were counted.

2.3.5 Adult Performance Bioassays

For the duration of each 14-day trial, I checked and recorded the number of live beetles per cage every other day in order to generate a survivorship curve for beetles on intact plants. Every four days during the 14-day trial, the cage was moved to a new leaf on the tree, and the old leaf was removed, placed in a plastic bag, and transferred to a cooler with ice packs to keep it cool until it could be refrigerated in the laboratory at -7°C. Leaves were scanned using a flatbed scanner and individual leaf area was assessed using Image J software (National Institutes of Health 1.51n, Bethesda, MD, 2017). Leaf area consumed by each group of beetles was determined by subtracting the area of remaining leaf tissue from an estimate of the leaf area prior

to feeding. The leaf area prior to beetle feeding was estimated in Image J by tracing and filling in the leaf area consumed by beetles during the bioassays. In order to convert pixels into square cm, area measurements were spatially calibrated against known values, according to standard scale (Table 2.3).

2.3.6 Grafting and Tree Performance

To determine the extent to which grafting affects overall performance of ash trees, I evaluated the diameter, height, and canopy index of each tree at Harrold Woodland on 7 June 2017. The diameter of each scion was measured at 15 cm above the soil with diameter tape, and a canopy size index was calculated using the formula (canopy height + canopy width at widest point + canopy width perpendicular to widest point)/3 (Oliver et al. 2010).

2.3.7 Effects of Grafting on Leaf Morphology

To determine the effects of grafting on leaf morphology, I measured leaf characteristics of grafted and buffer trees. On 15 September 2017, three leaves from each of the Manchurian and green ash (n=12) and three leaves from each buffer green and Manchurian ash (n=6) were collected and brought back to the lab. I counted the number of leaflets per leaf counted and measured leaf area measured using a flatbed scanner as described previously. I compared average leaf area and leaflet number between buffer trees and among grafted trees to determine the extent to which grafting within and among species affects leaf morphology.

2.3.8 Data Analysis

All data were analyzed using SAS (SAS® 9.4 Institute Inc., Cary, NC, 2013) and figures were created in Sigma Plot (Systat 13.0.0, Software Inc., San Jose, CA, 2014). Due to a limited number of beetles in 2017, Manchurian and green ash were prioritized for bioassays and morphology measurements, because Manchurian ash is the most resistant and green ash is the least resistant to EAB. Bioassays conducted on black and white ash were excluded from data

analysis. Three complete replicates of grafted Manchurian and green ash were alive and used in this study (Table 2.3). Assumptions of normality for all data were tested with residual plots and the Shapiro-Wilk test.

To determine whether species affected woodpecker damage, I performed a non-parametric one way analysis (PROC NPAR1way). I performed an ANOVA (PROC ANOVA) followed by a Tukey HSD test to determine whether species affected canopy thinning or DBH in the parent stock trees planted at Lugar. To determine if significant variation were present in survivorship among all four combinations of grafted Manchurian and green ash, as well as Manchurian and green buffer trees and between conspecific grafted trees, I performed a repeated measures ANOVA (ANOVA; PROC MIXED), using a controlled spherical model with the Kenward-Roger approximation to calculate degrees of freedom. Cumulative density of EAB was calculated as the number of beetle days using the following equation: beetle days:

Beetle days $= \Sigma \ ((N_{(x+1)} + N_{(x)})/2) * D_{(x+1)x})$

N is the population of beetles on sampling day x and $D_{(x+1)x})$ is the number of days between sampling dates, making beetle days the average number of beetles between each pair of observations multiplied by the number of days between observations (Prado et al. 2015). If a source of variation was significant, a Tukey HSD test was performed to separate means.

I used the same repeated measures analysis of variance procedure to determine if significant variation were present in total leaf area consumed among all four combinations of grafted Manchurian and green ash, as well as buffer Manchurian and green trees. If a source of variation was significant, a Tukey HSD test was performed to separate means. To determine whether there was a difference in the mean number of beetles laying eggs and number of eggs laid among all

four combinations of grafted Manchurian and green ash, as well as buffer green and Manchurian ash, a two-sample t-test was performed.

I used an ANOVA (ANOVA; PROC ANOVA) to determine whether there was significant variation in canopy index, tree height, and diameter among all four combinations of grafted Manchurian and green ash, or between grafted and buffer ash, followed by a Tukey HSD test when there was significant variation between species or among graft combinations. To determine the extent to which grafting affects leaflet number and leaf area among all graft combinations of green and Manchurian ash, as well as between grafted and buffer ash, I performed an ANOVA (ANOVA; PROC ANOVA) was performed, followed by a Tukey HSD test when there was significant variation in leaflet number and leaf area.

2.4 Results
2.4.1 Evaluation of EAB Resistance in Parent Stock

Average woodpecker damage was significantly affected by species of ash trees planted at Lugar Farm, with Manchurian ash having no woodpecker damage and green ash (*Fraxinus pennsylvanica* Marshall) having extensive damage (ANOVA, $F_{3,158}$=43.76, P<0.0001, Tukey's test, P<0.05, Figure 2.2). Diameter at breast height (DBH) was significantly smaller in Manchurian ash, although it did not vary among other species (ANOVA, $F_{3,158}$=3.41, P=0.0191, Tukey's test, P<0.05, Figure 2.3). Canopy dieback was significantly affected by species, with green ash having the highest percentage of canopy dieback and Manchurian the lowest (ANOVA, $F_{3,158}$=75.55, P<0.0001, Tukey's test, P<0.05, Figure 2.4).

2.4.2 EAB Bioassays

Adult EAB survivorship on grafted green ash over the course of a 38-day study was significantly greater than grafted Manchurian ash when the scion (ANOVA, $F_{1,5.7}$=6.75, P=0.0427, Figure 2.5A) or the rootstock (ANOVA, $F_{1,5.7}$=6.27, P=0.0483) was green ash. There was also a significant effect of day on survivorship (ANOVA, $F_{19,373}$=66.77, P<0.0001), but no

effect of scion*rootstock (ANOVA, $F_{1,5.69}=0$, $P=0.9656$), scion*day ($F_{19,372}=1.03$, $P=0.4230$), rootstock*day (ANOVA, $F_{19,372}=0.31$, $P=0.9981$), or scion*rootstock*day (ANOVA, $F_{1,373}=0.60$, $P=0.9099$). The last surviving beetles caged on conspecific green ash beetles lived six days longer than on conspecific Manchurian ash or heterospecific grafted trees. Cumulative beetle days on grafted trees were significantly affected by rootstock (ANOVA, $F_{1,4.07}=5.51$, $P=0.0129$, Figure 2.5B), with beetles living longer on conspecific green ash than any other graft combination. Although there was a significant effect of rootstock*month (ANOVA, $F_{1,6.81}=8.23$, $P=0.0247$) on beetle days, scion (ANOVA, $F_{1,4.07}=5.15$, $P=0.08484$), scion*rootstock interaction (ANOVA, $F_{1,4.07}=3.16$, $P=0.1487$), month (ANOVA, $F_{1,4.07}=8.17$, $P=0.0651$), scion*month (ANOVA, $F_{1,6.81}=0.67$, $P=4414$, $P=$), and scion*rootstock*month (ANOVA, $F_{1,6.81}=0.46$, $P=0.5195$) did not affect beetle days.

When survivorship on buffer green and Manchurian ash was compared to survivorship on conspecific grafted green and Manchurian ash, survivorship was highest on grafted green ash and lowest on Manchurian ash, with a significant effect of species (ANOVA, $F_{1,7.62}=8.29$, $P=0.0216$, Figure 2.6A), grafting (ANOVA, $F_{1,7.62}=5.49$, $P=0.0487$), and day (ANOVA, $F_{19,390}=72.07$, $P<0.0001$). There was no effect of species*grafting (ANOVA, $F_{1,7.62}=1.58$, $P=0.2453$) or species*day (ANOVA, $F_{19,390}=1.19$, $P=0.2589$). Adult EAB lived longer on conspecific green ash trees than on green or Manchurian ash and conspecific grafted Manchurian ash, with a significant effect of species (ANOVA, $F_{1,4.23}=116.85$, $P=0.0003$, Figure 2.6B), grafting (ANOVA, $F_{1,4.23}=69.47$, $P=0.0009$), and month (ANOVA, $F_{1,8.11}=9.87$, $P=0.0135$). Total beetle days were not affected by species*grafting (ANOVA, $F_{1,4.23}=6.14$, $P=0.0650$), species*month (ANOVA, $F_{1,8.11}=0.80$, $P=0.3973$), grafting*month (ANOVA, $F_{1,8.11}=0.84$, $P=0.3848$), or species*grafting*month (ANOVA, $F_{1,8.11}=1.08$, $P=0.3297$).

Total leaf area consumed by adult EAB was not affected by scion species (ANOVA, $F_{1,13.7}=0.03$, $P=0.8684$, Figure 2.7) or rootstock species (ANOVA, $F_{1,13.7}=0.11$, $P=0.3100$). Additionally, there was no significant effect of scion*rootstock (ANOVA, $F_{1,13.7}=1.75$, $P=0.2076$), month (ANOVA, $F_{1,13.7}=0.57$, $P=0.4644$), scion*month (ANOVA, $F_{1,13.7}=1.06$, $P=0.3203$), rootstock*month (ANOVA, $F_{1,13.7}=0.27$, $P=0.6094$), or scion*rootstock*month (ANOVA, $F_{1,13.7}=1.73$, $P=0.2095$) on herbivory. There was a significant effect of species*grafting (ANOVA, $F_{1,14}=5.15$, $P=0.0396$, Figure 2.8) on total leaf area consumed in conspecific grafted green and Manchurian ash. However, species (ANOVA, $F_{1,14}=2.58$, $P=0.1304$), grafting (ANOVA, $F_{1,14}=2.62$, $P=0.1281$), month (ANOVA, $F_{1,14}=0.97$, $P=0.3416$), species*month (ANOVA, $F_{1,14}=0.77$, $P=0.3960$), grafting*month (ANOVA, $F_{1,14}=0.32$, $P=0.5828$), and species*grafting*month (ANOVA, $F_{1,14}=0.25$, $P=0.6278$) did not affect herbivory. Beetles caged on buffer Manchurian ash consumed significantly less than beetles caged on grafted Manchurian and green ash, as well as buffer green ash (Figure 2.8).

Regardless of grafting, no female beetles caged on Manchurian ash laid eggs or contained eggs when dissected (Tables 2.4 and 2.5). Eight beetles caged on green ash laid a total of 322 eggs, accounting for roughly 80% of all the eggs laid in the fecundity bioassay (Tables 2.4 and 2.5). One female caged on a grafted tree with a green scion and Manchurian rootstock laid 17 eggs (Table 2.4). Although females on a conspecific green ash tree laid an average of 34.16 eggs, while females on trees with a Manchurian scion and green rootstock laid an average of 16 eggs, there was no significant difference in average number of eggs laid per female between the two graft combinations (t-test, $t=0.87$, $P=0.41$).

2.4.3 Grafting and Tree Performance

All green and Manchurian trees planted in 2011 were alive for the duration of the study (n=8). Of the sixteen grafted trees originally planted, only one tree with a green rootstock died and all trees with Manchurian rootstocks survived. In contrast, all eight green scions were still living, while only six of the eight trees with Manchurian scions survived.

Canopy index among grafted green and Manchurian ash was significantly affected by scion species (ANOVA, $F_{1,12}$=6.10, P=0.0295, Figure 2.9) and rootstock species (ANOVA, $F_{1,12}$=6.63, P=0.0243). Conspecific green ash had significantly larger canopy indices than any other graft combination, and canopy indices did not vary among the other three graft combinations. In comparisons of canopy index among green and Manchurian ash, there was a significant effect of species, with green ash having larger canopy indices regardless of grafting (ANOVA, $F_{1,13}$=18.24, P=0.0009, Figure 2.10).

Among grafted trees, tree height was not affected by rootstock or scion (ANOVA, $F_{3,12}$=0.89, P=0.4739, Figure 2.11). However, when the heights of green and Manchurian ash were compared, there was a significant effect of species (ANOVA, $F_{1,13}$=13.86, P=0.0026, Figure 2.12) but not of grafting (ANOVA, $F_{1,13}$=0.02, P=0.8902) on height. Regardless of grafting, green ash were significantly taller than Manchurian ash.

In grafted trees, scion diameter did not vary significantly among graft combinations (ANOVA, $F_{3,12}$=2.09, P=0.1546, Figure 2.13). In comparisons of diameter among green and Manchurian ash, there was no significant variation (ANOVA, $F_{3,13}$=2.01, P=0.1623, Figure 2.14).

2.4.4 Effects of Grafting on Leaf Morphology

Among grafted green and Manchurian ash, there was no significant variation in number of leaflets per leaf (ANOVA, $F_{3,8}$=0.80, P=0.5282, Figure 2.15). When leaflet number per leaf of

green and Manchurian ash was compared, there was a significant effect of species (ANOVA, $F_{1,12}$=5.32, P=0.0417, Figure 2.16) and of the interaction between species and grafting (ANOVA, $F_{1,12}$=5.32, P=0.0465). Green ash had significantly fewer leaflets per leaf than buffer Manchurian ash or grafted green and Manchurian ash.

Average leaf area did not vary significantly among grafted green and Manchurian ash (ANOVA, $F_{3,8}$=0.39, P=0.7632, Figure 2.17) or conspecific grafted and buffer green and Manchurian ash (ANOVA, $F_{3,9}$=1.46, P=0.2902, Figure 2.18).

2.5. Discussion

Evaluation of woodpecker damage and canopy dieback among the parent stock of trees used in this study indicated that Manchurian ash was indeed resistant to EAB, and that green ash was susceptible (Figs. 2.2 and 2.4). This is consistent with other studies that evaluated the resistance capacity of various ash species to EAB (Rebek et al. 2008, Rigbsy et al. 2014, Tanis and McCullough 2015). The woodpecker and canopy dieback assays also revealed intermediate levels of EAB resistance in white ash (Figs. 2.2 and 2.4), as seen in other EAB resistance studies (Anulewicz et al. 2008, Rebek et al. 2008, Tanis and McCullough 2015). This assessment of resistance to EAB established that the Manchurian ash used in the rest of the study should have greater resistance to EAB than the green ash.

The results of the adult EAB survivorship bioassay did not support my hypothesis that EAB resistance is conferrable from rootstock to scion. Instead, I found that, regardless of whether Manchurian ash was present in the rootstock, scion, or both, any grafted tree containing Manchurian ash would be more resistant than a conspecific green ash tree. Although beetle survivorship over time was affected significantly by both rootstock and scion species, beetles

caged on conspecific green ash had higher overall survivorship than beetles caged on conspecific Manchurian or heterospecific grafted trees (Figure 2.5A). When total beetle days were compared, beetles caged on conspecific green ash lived longer than beetles on any other graft combination, and there was a significant effect of rootstock but not scion on beetle days (Figure 2.5B). Although a Manchurian ash rootstock seemed to be able to confer resistance to a susceptible green scion, the green rootstock did not confer susceptibility to the Manchurian scion.

When comparing buffer green ash with conspecific grafted green ash, grafting affected both survivorship over time and overall beetle days, with beetles surviving longer on grafted green ash than buffer green ash (Figure 2.6A and 2.6B). However, survivorship and beetle days did not vary between buffer and conspecific grafted Manchurian ash, indicating that grafting itself does not affect the mechanisms of resistance present in Manchurian ash. Because green ash are not able to resist EAB effectively, the stress of grafting could further weaken their defensive capabilities, resulting in higher beetle survival.

The results from the adult EAB leaf consumption bioassay did not support my hypothesis that EAB resistance is conferrable from rootstock to scion. Total leaf area consumed did not differ between buffer green and Manchurian ash, or among the four combinations of grafted green and Manchurian ash (Figures 2.7 and 2.8). In six-choice feeding assays, adult EAB preferred to feed on North American ash species, such as green, black, and white ash, over Asian species like Manchurian ash, and consumed more leaf area on green ash than Manchurian ash (Pureswaran and Poland 2009). However, in my study, beetles fed on leaves of a single tree for the duration of the bioassay, making it a no-choice feeding assay. Beetles that were caged on Manchurian ash did not live as long as beetles on conspecific green ash. This suggests that the

chemical defenses present in Manchurian ash are toxic to EAB and negatively impact their survival. However, they do not appear to affect the total leaf area consumed by beetles forced to feed on less suitable Manchurian ash. The negative effects seen on beetle survivorship suggest that the mechanisms present in Manchurian ash can be classified as antibiosis, the term for defenses that decrease fecundity, survivorship, or herbivory (Villari et al. 2016). The reduced preference for Manchurian ash seen in the herbivory choice assays conducted by Pureswaran and Poland (2009) are antixenotic in nature, as they show a decrease in behavioral preferences (Villari et al. 2016). My findings, in conjunction with Pureswaran and Poland (2009) suggest that the mechanisms of resistance against EAB could be both antixenotic and antibiotic, with beetles having less preference for Manchurian ash and reduced fitness when forced to feed on Manchurian ash.

Finally, the EAB fecundity bioassay also supported my hypothesis that an EAB-resistant rootstock could confer resistance to a susceptible scion. No beetles caged on conspecific grafted Manchurian ash produced eggs, suggesting that the antibiotic defensive properties that caused higher mortality on grafted trees containing Manchurian ash also affected fecundity (Table 2.4, Villari et al. 2016). More eggs were laid, both per beetle and in total, by beetles caged on conspecific green ash than any other graft combination (Table 2.4). In buffer trees, no beetles laid eggs on Manchurian ash (Table 2.5). The lack of eggs laid by beetles caged on Manchurian ash was consistent with previous studies investigating EAB oviposition preference and larval development. These studies found that fewer eggs were laid on Manchurian ash than North American ash species, and that Manchurian ash had fewer larval galleries and adult exit holes than North American ash (Rigsby et al. 2014, Tanis and McCullough 2015). More eggs were laid by beetles caged on trees with Manchurian scions and green rootstocks than beetles caged on

trees with green scions on Manchurian rootstocks. The greater influence of rootstock species than scion species on fecundity indicates that resistance to EAB, in the form of defensive compounds that reduced beetle survivorship and fecundity, is conferred from the rootstock to the scion.

In my assessment of tree characteristics, conspecific green ash had significantly larger canopy indices than any other graft combination, and both buffer and conspecific grafted green ash had larger indices than buffer and conspecific Manchurian ash (Figs. 2.9 and 2.10). Among graft combinations, height did not vary, but conspecific and buffer green ash were taller than conspecific and buffer Manchurian ash (Figs. 2.11 and 2.12). In the evaluation of the DBH of the parent stock planted at Lugar, Manchurian ash was significantly smaller than any other ash species (Figure 2.3). Although leaflets per leaf did not vary among graft combinations, buffer green ash had fewer leaflets per leaf than buffer Manchurian ash, as well as conspecific grafted green and Manchurian ash (Figs. 2.15 and 2.16). Diameter and leaf area were not affected by species or grafting (Figs. 2.13, 2.14, 2.17, 2.18). These findings suggest that, when morphological traits are affected by different graft combinations, the presence of Manchurian ash results in a tree with characteristics more similar to Manchurian ash than green ash, as seen in the EAB bioassays. Although a green ash scion can be made resistant via grafting to a Manchurian ash rootstock, it will look more like a Manchurian ash tree than a green ash tree.

In conclusion, this study demonstrated that grafting trees with resistant rootstocks holds potential for the propagation of EAB-resistant North American ash trees. Overall, my EAB bioassays demonstrated that, although the amount of leaf area consumed was not affected by graft combination in a no-choice assay, beetles caged on grafted trees with a Manchurian ash scion or rootstock live a shorter life and lay fewer eggs than a beetle caged on conspecific

grafted green ash. These findings are consistent with previous studies demonstrating that Manchurian ash is a less preferred and less suitable host for EAB than green ash (Rebek et al. 2008, Pureswaran and Poland 2009, Rigsby et al. 2014, Tanis and McCullough 2015, Rigsby et al. 2017). The decreased beetle fitness observed on grafted trees with a Manchurian ash rootstock or scion, as compared to conspecific grafted green ash, suggests that grafting Manchurian ash to green ash results in a tree with a greater capacity to resist EAB than green ash. Although tree characteristics were not always affected by graft combination, conspecific green ash was generally different from any graft combination including Manchurian ash. Expanding this study to include other ash species would determine whether this transferal of resistance is unique to grafted green and Manchurian ash or is possible with other resistant species in the genus. If similar results can be obtained by grafting green ash to blue ash (*Fraxinus quadrangulata* Michx.), a North American ash species that is resistant to EAB, it could be possible to propagate resistant North American ash trees using only native ash, without the influence on tree characteristics seen when grafting Manchurian and green ash. Grafting can confer resistance to EAB from rootstock to scion, and therefore holds great potential as a tool for developing EAB-resistant ash and preserving the North American ash resource.

Table 2.1 Ash (*Fraxinus*) species used in 2017 performance EAB adult performance bioassays at the Purdue University Harrold Woodland (Whitley Co., IN).

Common Name	Scientific Name	Susceptible to EAB?*	Species Group
Green ash	*F. pennsylvanica* Marshall	Yes	North American
Black ash	*F. nigra* Marshall	Yes	North American
White ash	*F. americana* L.	Yes	North American
Chinese ash[+]	*F. chinensis* Roxburgh	No	Asian
Manchurian ash	*F. mandschurica* Ruprecht	No	Asian

*See Rebek et al. 2008

[+]The Chinese ash in this study was later identified as green ash, and was excluded from data analysis.

Table 2.2 Number of ash trees of each graft combination and buffer species used in 2017 adult EAB performance bioassays at the Purdue University Harrold Woodland (Whitley Co., IN). Trees were grafted in 2010 and planted in 2011.

Graft combination (scion: rootstock)/Species	June	July	August
Green: Green	3	2	1
Green: Manchurian	3	2	1
Manchurian: Green	3	2	1
Manchurian: Manchurian	3	2	1
Green: Black	1	1	0
Black: Green	2	1	0
Black: Black	2	1	0
Green: White	3	1	0
White: Green	2	2	0
White	3	0	0
White: Black	1	1	0
Black: White	3	1	0
White: Manchurian	1	1	0
Manchurian: White	4	2	0
Manchurian: Black	2	1	0
Black: Manchurian	2	1	0
Green	3	2	0
Manchurian	3	2	0
Black	2	1	0
White	4	1	0

Table 2.3 Settings used in measuring total leaf area and calculating leaf area consumed for adult herbivory bioassays and determining grafting effects on ash leaf morphology in ImageJ (National Institutes of Health 1.51n, Bethesda, MD, 2017) (After Kolich 2014).

Measure	Setting
Distance in pixels	112.02
Known distance	1.00
Pixel aspect ratio	1.0
Unit of length	cm
Image Type	8-bit
Threshold	Black and white
Process	Binary

Table 2.4 Percentage of gravid females (females who laid eggs or contained eggs when dissected), total number of eggs laid, and eggs laid per female during a 24-day fecundity assay conducted on leaves from grafted green and Manchurian ash. There was no significant difference between the average number of eggs laid by a female on a green/green tree and a female on a Manchurian/green tree (t=0.87, *P*=0.41).

Graft Combination (scion/rootstock)	Percentage of Gravid Females	Total Eggs	Eggs Per Female
Green/Green	37.5% (n=6)	205	34.16
Manchurian/Green	40% (n=4)	64	16
Green/Manchurian	9% (n=1)	17	17
Manchurian/Manchurian	0 (n=0)	0	0

Table 2.5 Percentage of gravid females (females who laid eggs or contained eggs when dissected), total number of eggs laid, and eggs laid per female during a 24-day fecundity assay conducted on leaves from buffer green and Manchurian ash.

Species	Percentage of gravid females	Total Eggs	Mean Eggs Per Female
Green	33% (n=2)	117	58.5
Manchurian	0 (n=0)	0	0

Figure 2.1 Cage used in adult EAB bioassays in 2017. A 10.9 cm diameter section was removed from the center of two 18.6 cm foam plates (Walmart, Bentonville, AZ), which were then stapled together, leaving an 8 cm opening between plates. Two pieces of screen mesh (14.5 x 14 cm) were hot-glued over the opening, and two pieces of 1 m clothesline cotton cord or jute twine (Walmart, Bentonville, AZ) were stapled on.
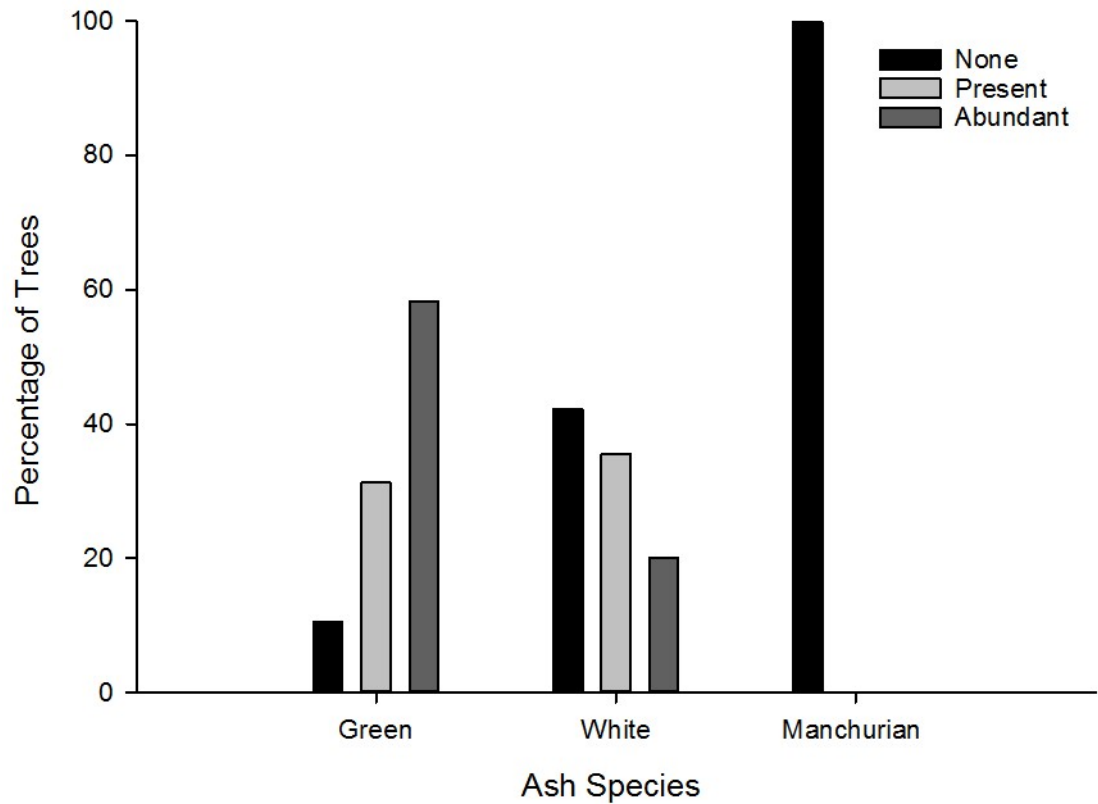
Figure 2.2 Mean (±SE) ranking of woodpecker damage on ash trees planted at Lugar Farm (Tippecanoe Co., IN) on a scale of 0 to 2 (0=absent, 1=present, 2=abundant). Density of woodpecker damage varied significantly among species (Kruskall-Wallis, χ^2=49.821, df=2, *P*<0.0001).
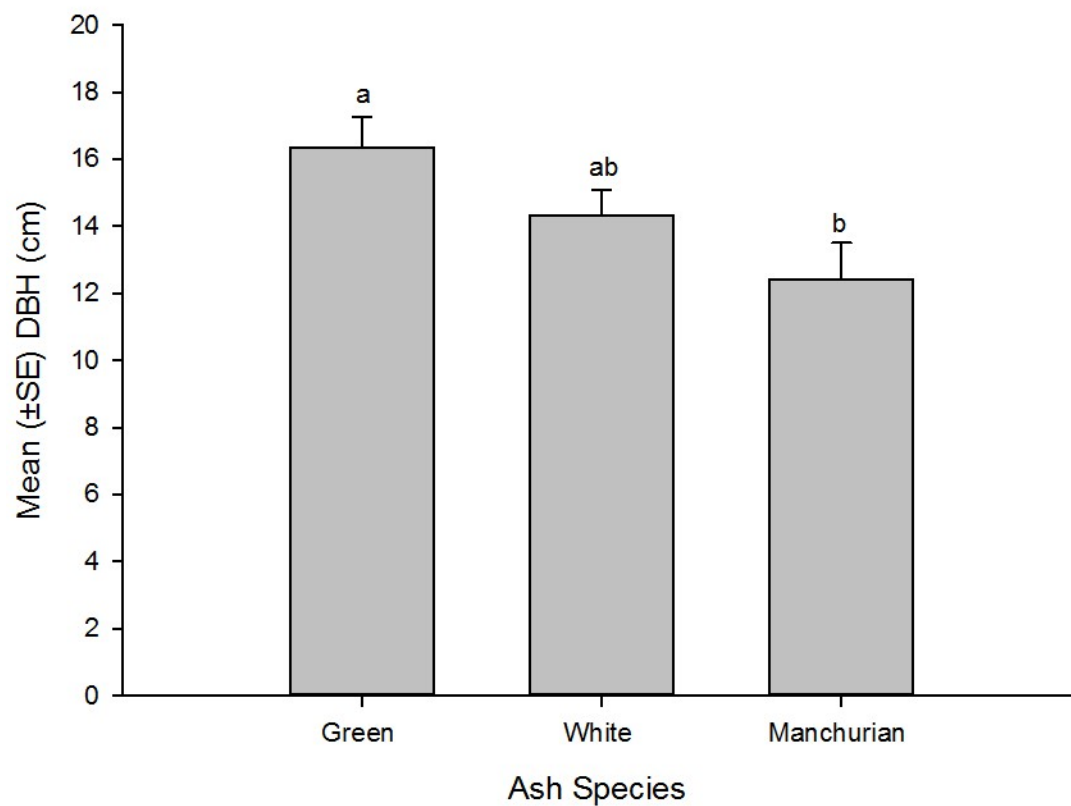
Figure 2.3 Mean (±SE) diameter at breast height (DBH) in centimeters (cm) of ash trees planted at Lugar Farm (Tippecanoe Co., IN). DBH varied significantly among species (ANOVA, $F_{2,113}=3.81$, $P=0.0250$). Letters indicate differences among ash species (Tukey's test, $\alpha<0.05$).
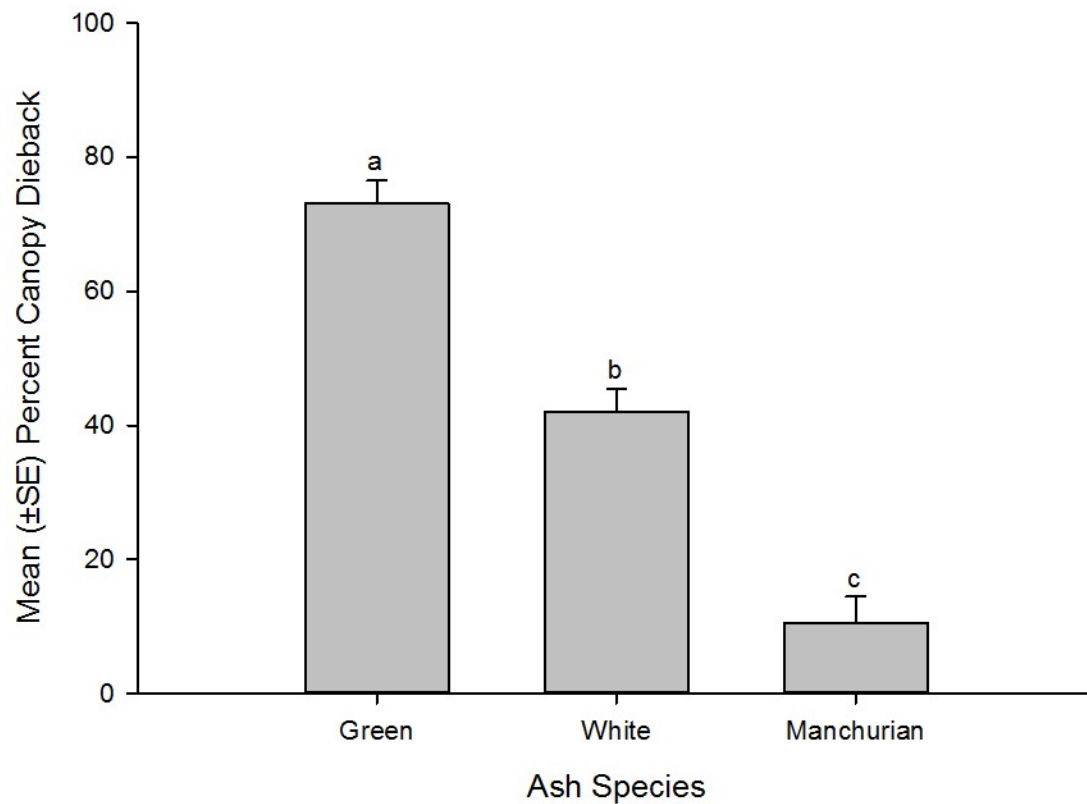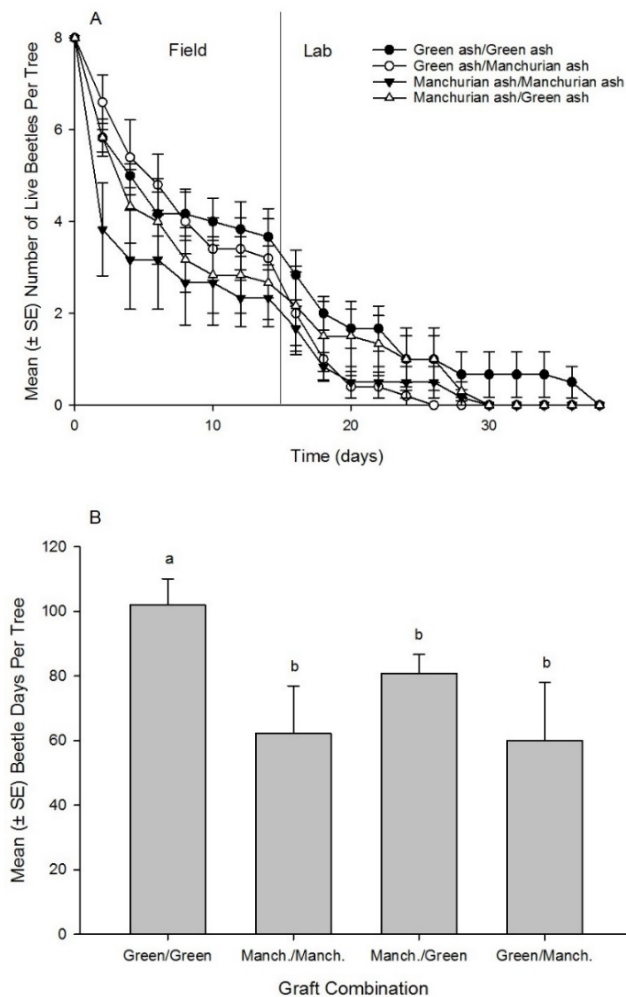
Figure 2.4 Mean (±SE) percent canopy dieback of ash trees planted at Lugar Farm (Tippecanoe Co., IN). Canopy dieback varied significantly among species (ANOVA, $F_{2,113}=62.19$, $P<0.0001$). Letters indicate differences among ash species (Tukey's test, $\alpha<0.05$).

Figure 2.5 A. Mean (±SE) adult EAB survivorship over 38 days on grafted green and Manchurian ash. Survivorship was significantly affected by scion (ANOVA, $F_{1,5.7}$=6.75, P=0.0427), rootstock (ANOVA, $F_{1,5.7}$=6.27, P=0.0483), and day (ANOVA, $F_{19,373}$=66.77, P<0.0001). There was no effect of scion*root (ANOVA, $F_{1,5.69}$=0, P=0.9656), scion*day ($F_{19,372}$=1.03, P=0.4230), root*day (ANOVA, $F_{19,372}$=0.31, P=0.9981), or scion*root*day (ANOVA, $F_{1,373}$=0.60, P=0.9099). B. Mean (±SE) total beetle days over 38 days on grafted green and Manchurian ash. Beetle days varied significantly by rootstock (ANOVA, $F_{1,4.07}$=5.15, P=0.0129) and rootstock*month (ANOVA, $F_{1,6.81}$=8.23, P=0.0247). Scion (ANOVA, $F_{1,4.07}$=5.15, P=0.0848), scion*root (ANOVA, $F_{1,4.07}$=3.16, P=0.1487), month (ANOVA, $F_{1,4.07}$=8.17, P=0.0651), scion*month (ANOVA, $F_{1,6.81}$=0.4414), and scion*root*month (ANOVA, $F_{1,6.81}$=0.46, P=0.5195) did not affect beetle days. Letters indicate differences among ash species (Tukey's test, α<0.05).
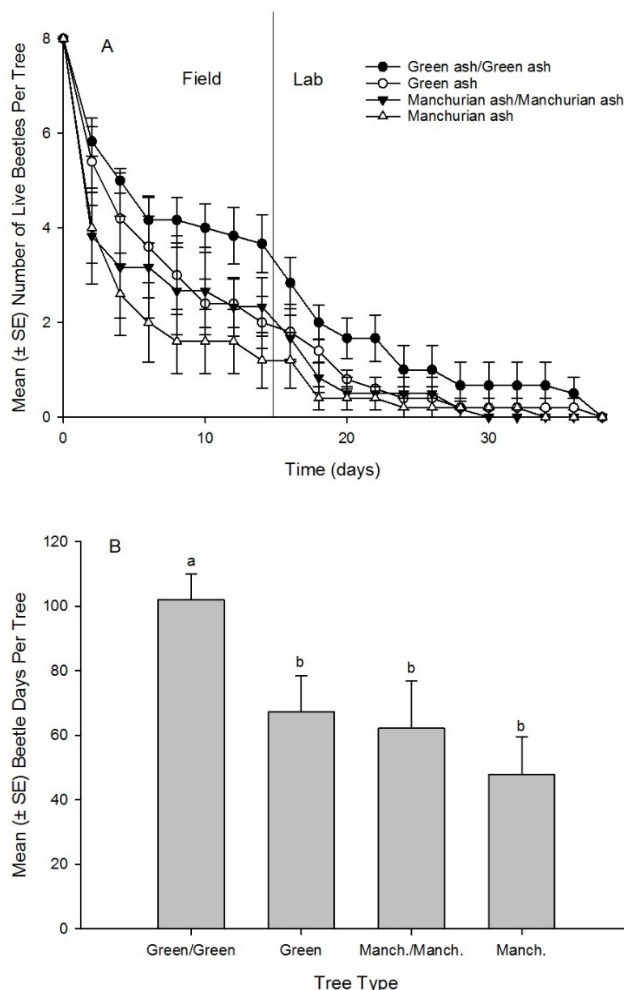
Figure 2.6 A. Mean (±SE) adult EAB survivorship over 38 days on buffer and conspecific grafted green and Manchurian ash. Survivorship was significantly affected by species (ANOVA, $F_{1,7.62}=8.29$, $P=0.0216$), grafting (ANOVA, $F_{1,7.62}=5.49$, $P=0.0487$), and day (ANOVA, $F_{19,390}=72.07$, $P<0.0001$), although there was no significant effect of species*grafting (ANOVA, $F_{1,7.62}$, $P=0.2453$) or species*day (ANOVA, $F_{19,390}=1.19$, $P=0.2589$). B. Mean (±SE) total beetle days over 38 days on buffer and conspecific grafted green and Manchurian ash. There was a significant effect of species (ANOVA, $F_{1,4.23}=116.85$, $P=0.0003$), grafting (ANOVA, $F_{1,4.23}=69.47$, $P=0.0009$), and month (ANOVA, $F_{1,8.11}=9.87$, $P=0.0135$) on beetle days. However, there was no effect of species*grafting (ANOVA, $F_{1,4.23}=6.14$, $P=0.065$), species*month (ANOVA, $F_{1,8.11}=0.80$, $P=0.3973$), grafting*month (ANOVA, $F_{1,8.11}=0.84$, $P=0.3848$), or species*grafting*month (ANOVA, $F_{1,8.11}=1.08$, $P=0.3297$). Letters indicate differences among ash species (Tukey's test, $\alpha<0.05$).
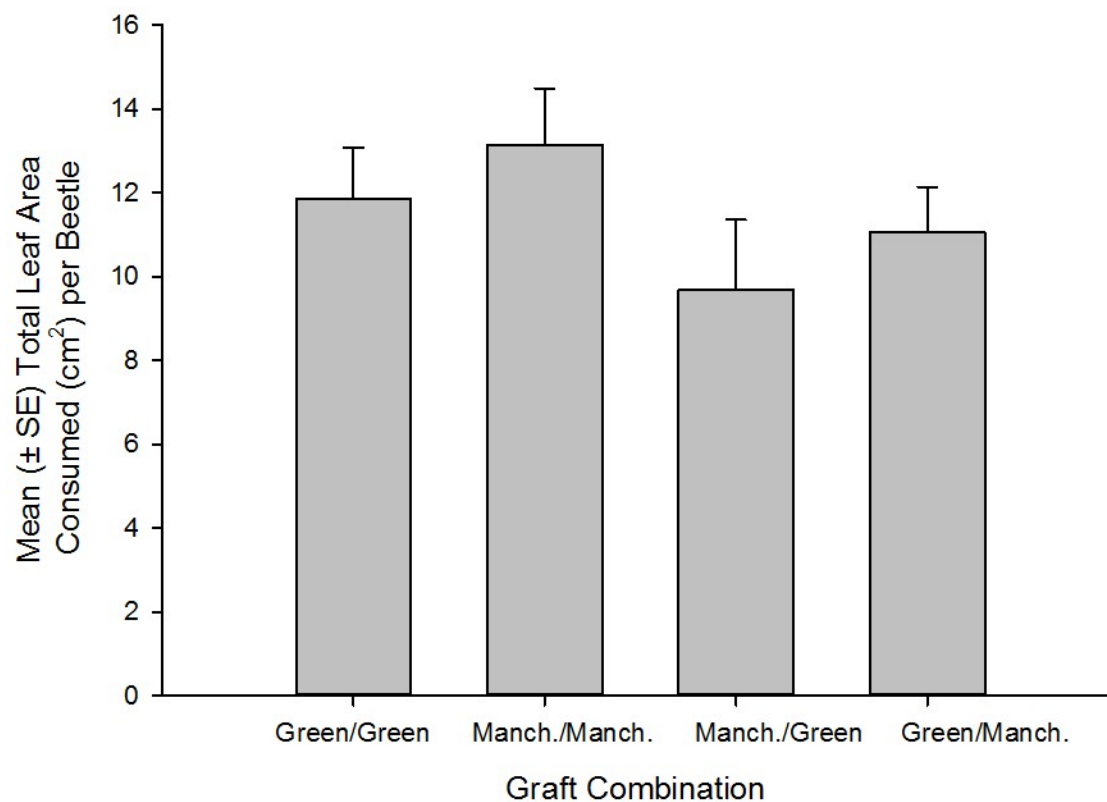
Figure 2.7 Mean ($\pm$ SE) total leaf area consumed (cm^2) per adult emerald ash borer on grafted green and Manchurian ash over a twelve-day field bioassay. Total herbivory was not significantly affected by scion species (ANOVA, $F_{1,13.7}=0.03$, $P=0.8684$), rootstock species (ANOVA, $F_{1,13.7}=0.11$, $P=0.3100$), scion*rootstock (ANOVA, $F_{1,13.7}=1.75$, $P=0.2076$), month (ANOVA, $F_{1,13.7}=0.57$, $P=0.4644$), scion*month (ANOVA, $F_{1,13.7}=1.06$, $P=0.3203$), rootstock*month (ANOVA, $F_{1,13.7}=0.27$, $P=0.6094$), or scion*rootstock*month (ANOVA, $F_{1,13.7}=1.73$, $P=0.2095$).
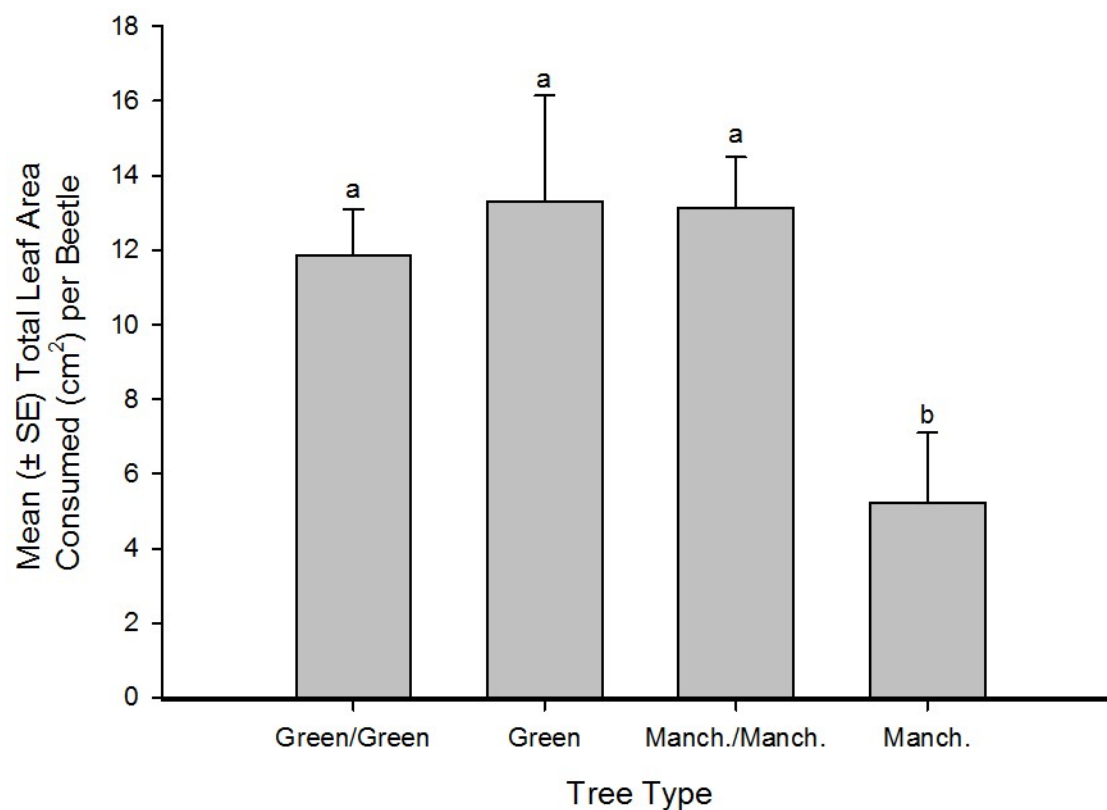
Figure 2.8 Mean ($\pm$ SE) total leaf area consumed (cm^2) per adult emerald ash borer on buffer and conspecific grafted green and Manchurian ash over a twelve-day field bioassay. Total herbivory was significantly affected by species*grafting (ANOVA, $F_{1,14}$=5.15, P=0.0396). Species (ANOVA, $F_{1,14}$=2.58, P=0.1304), grafting (ANOVA, $F_{1,14}$=2.62, P=0.0.1281), month (ANOVA, $F_{1,14}$=0.97, P=0.0.3416), species*month (ANOVA, $F_{1,14}$=0.77, P=0.3960), grafted*month (ANOVA, $F_{1,14}$=0.32, P=0.0.5828), and species*grafted*month (ANOVA, $F_{1,14}$=0.25, P=0.6278) did not affect herbivory. Letters indicate differences among ash species (Tukey's test, α<0.05).
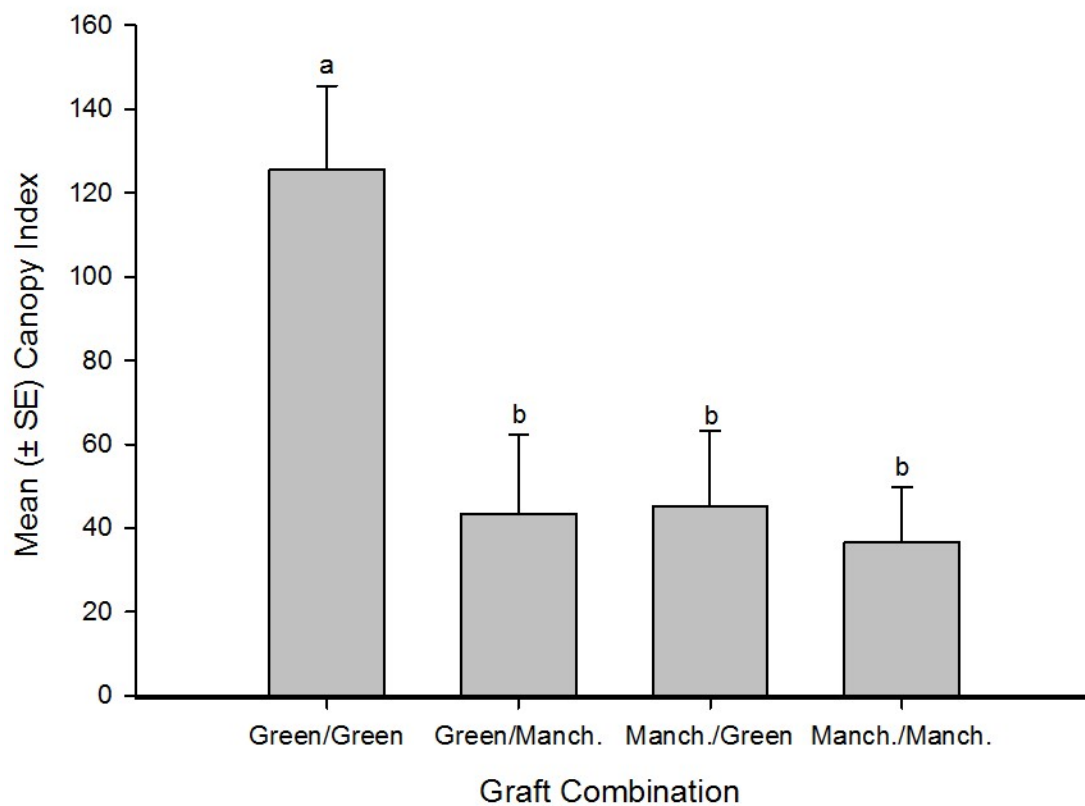
Figure 2.9 Mean (± SE) canopy index of grafted green and Manchurian ash trees. Scion species is listed first in the legend. Conspecific green ash had a larger canopy index than the other three graft combinations (ANOVA, $F_{3,12}=5.70$, $P=0.016$). Letters indicate differences among ash species (Tukey's test, $\alpha<0.05$).
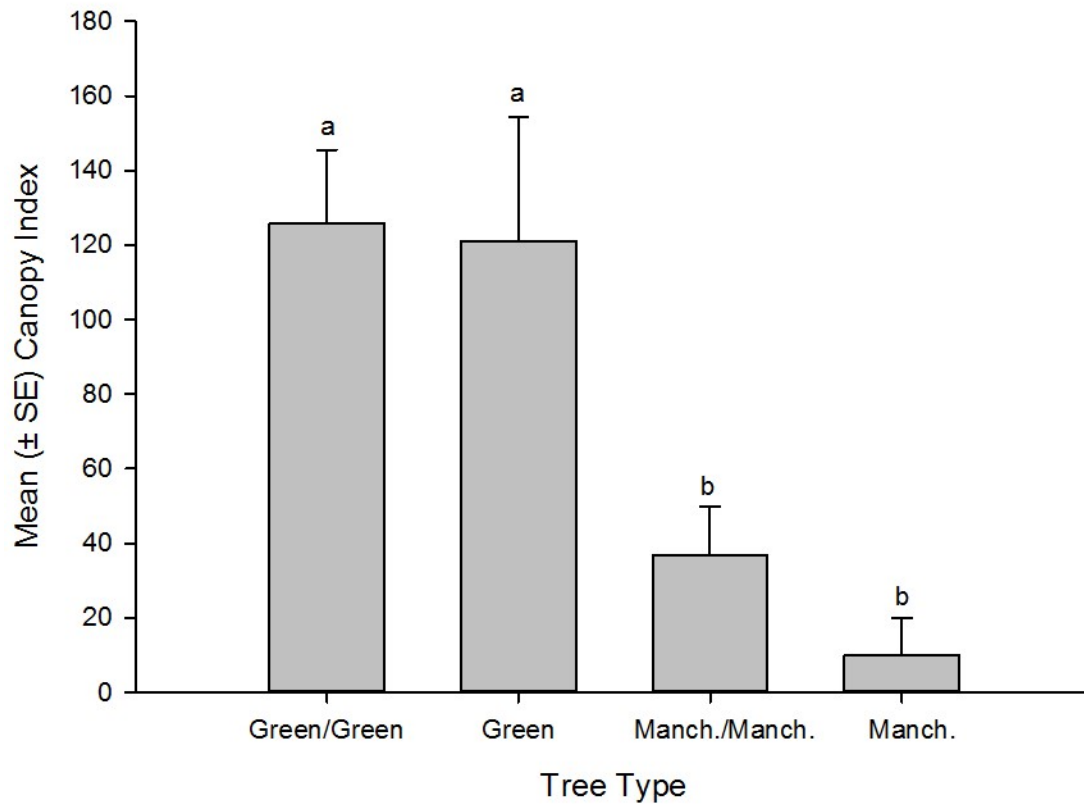
Figure 2.10 Mean (± SE) canopy index of buffer and conspecific green and Manchurian ash. Scion species is listed first in the legend. Green ash and conspecific grafted green ash had significantly larger canopy indices than Manchurian ash and conspecific grafted Manchurian ash (ANOVA, $F_{3,13}=6.29$, $P=0.0072$). Letters indicate differences among ash species (Tukey's test, $\alpha<0.05$).
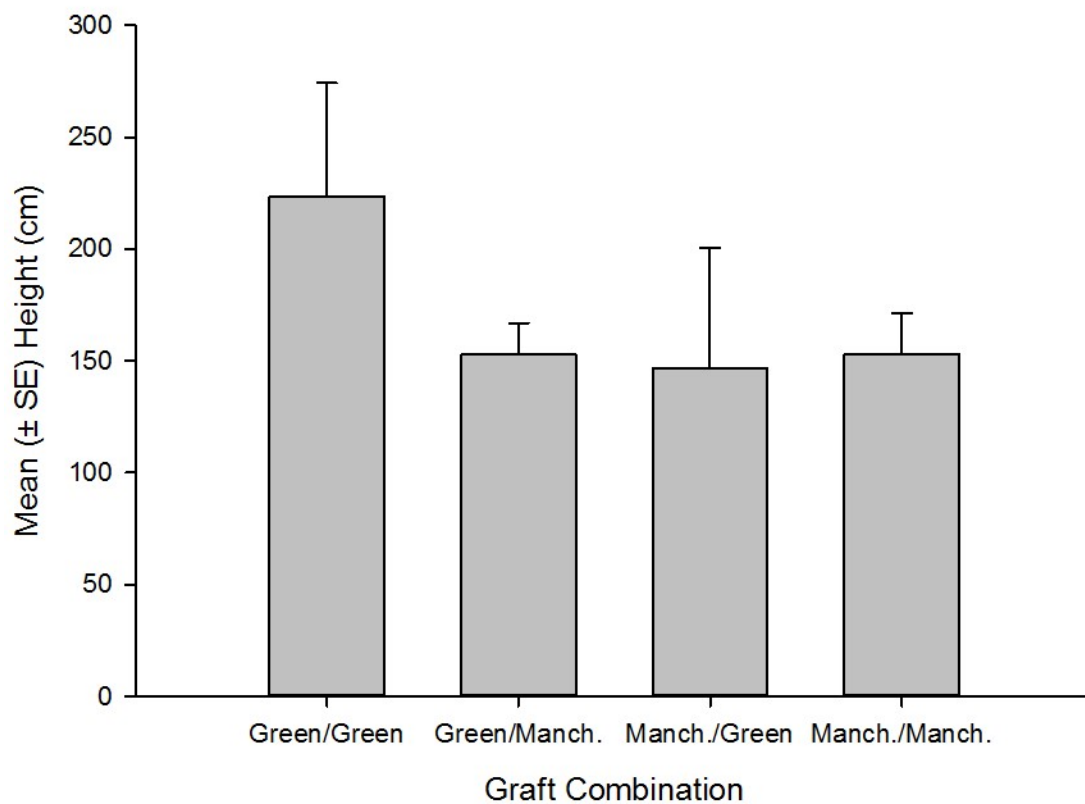
Figure 2.11 Mean (± SE) height of grafted green and Manchurian ash trees. Tree height did not vary significantly among grafted trees (ANOVA, $F_{3,12}=0.89$, $P=0.4739$). Scion species is listed first in the legend.
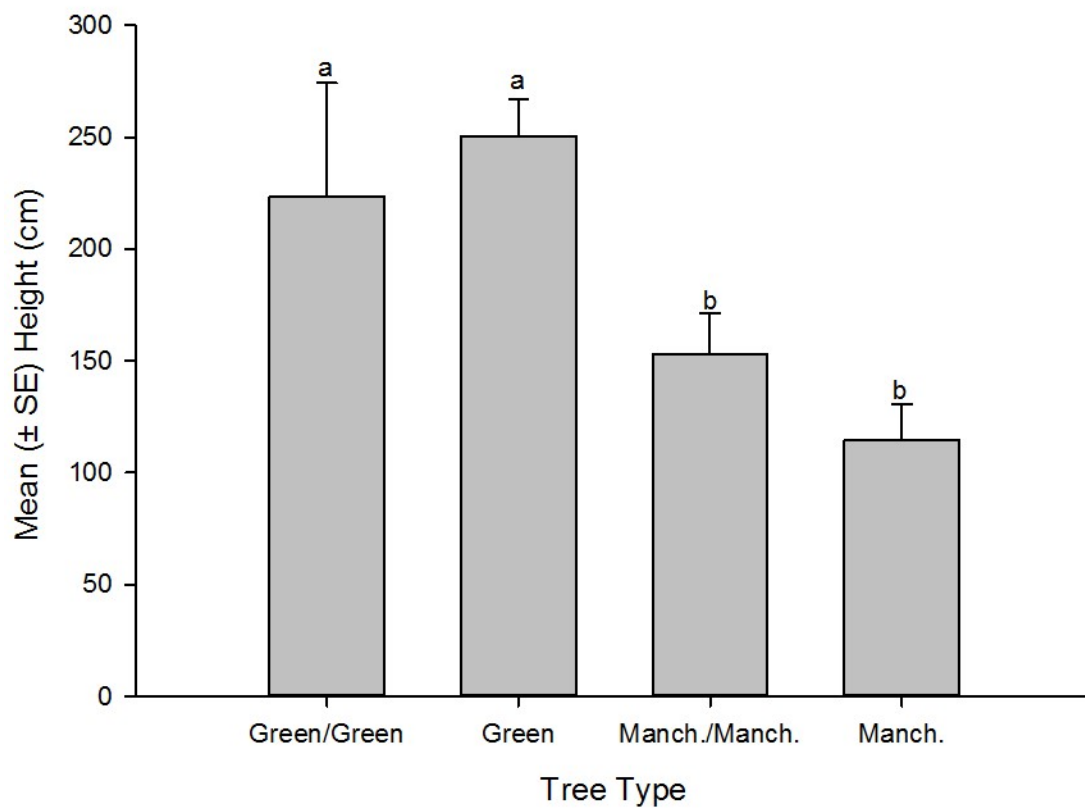
Figure 2.12 Mean (± SE) height of buffer and conspecific green and Manchurian ash. Green ash was significantly taller than Manchurian ash (ANOVA, $F_{3,13}=5.07$, $P=0.0153$). Letters indicate differences among ash species (Tukey's test, $\alpha<0.05$).
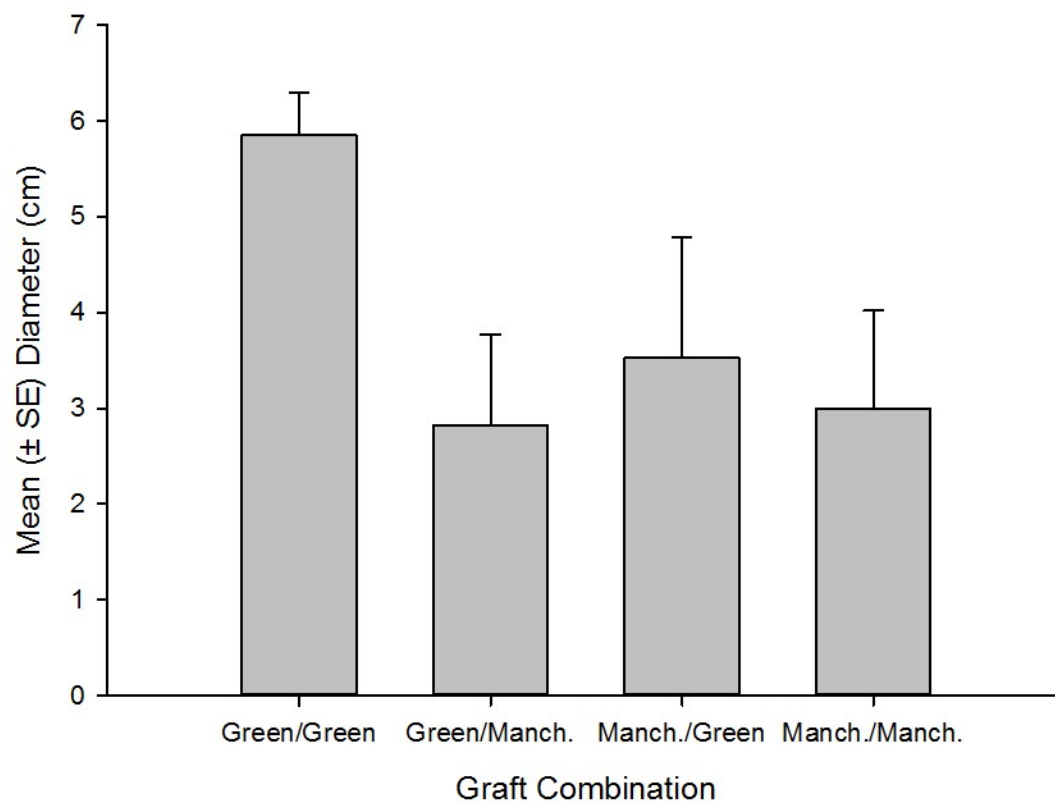
Figure 2.13 Mean (± SE) scion diameter (as measured with a caliper 10 cm above the graft union) of grafted green and Manchurian ash trees. Scion species is listed first in the legend. Scion diameter did not vary significantly among graft combinations (ANOVA, $F_{3,12}=2.09$, $P=0.1546$).

Figure 2.14 Mean (± SE) diameter (as measured with a caliper 15 cm above the ground) of buffer and conspecific green and Manchurian ash. Diameter did vary not vary significantly among trees (ANOVA, $F_{3,13}=2.01$, $P=0.1623$).

Figure 2.15 Mean (± SE) number of leaflets per leaf on grafted green and Manchurian ash trees. Scion species is listed first in the legend. Leaflet number did not vary significantly among graft combinations (ANOVA, $F_{3,8}=0.80$, $P=0.5282$).
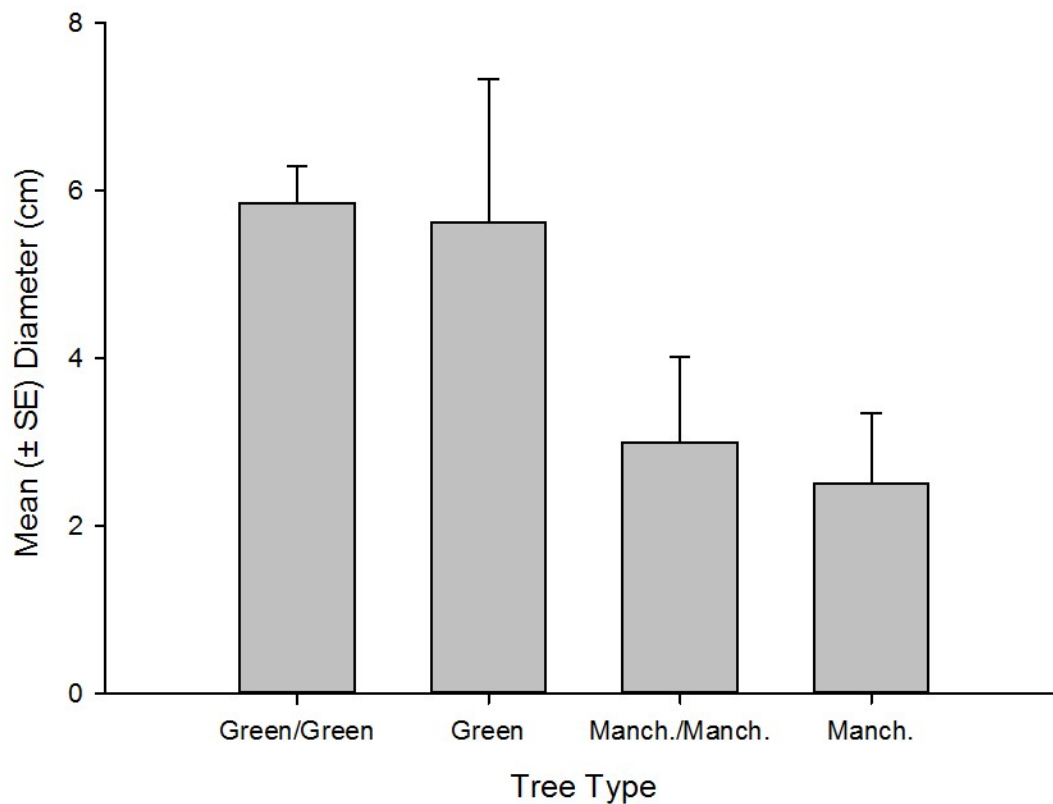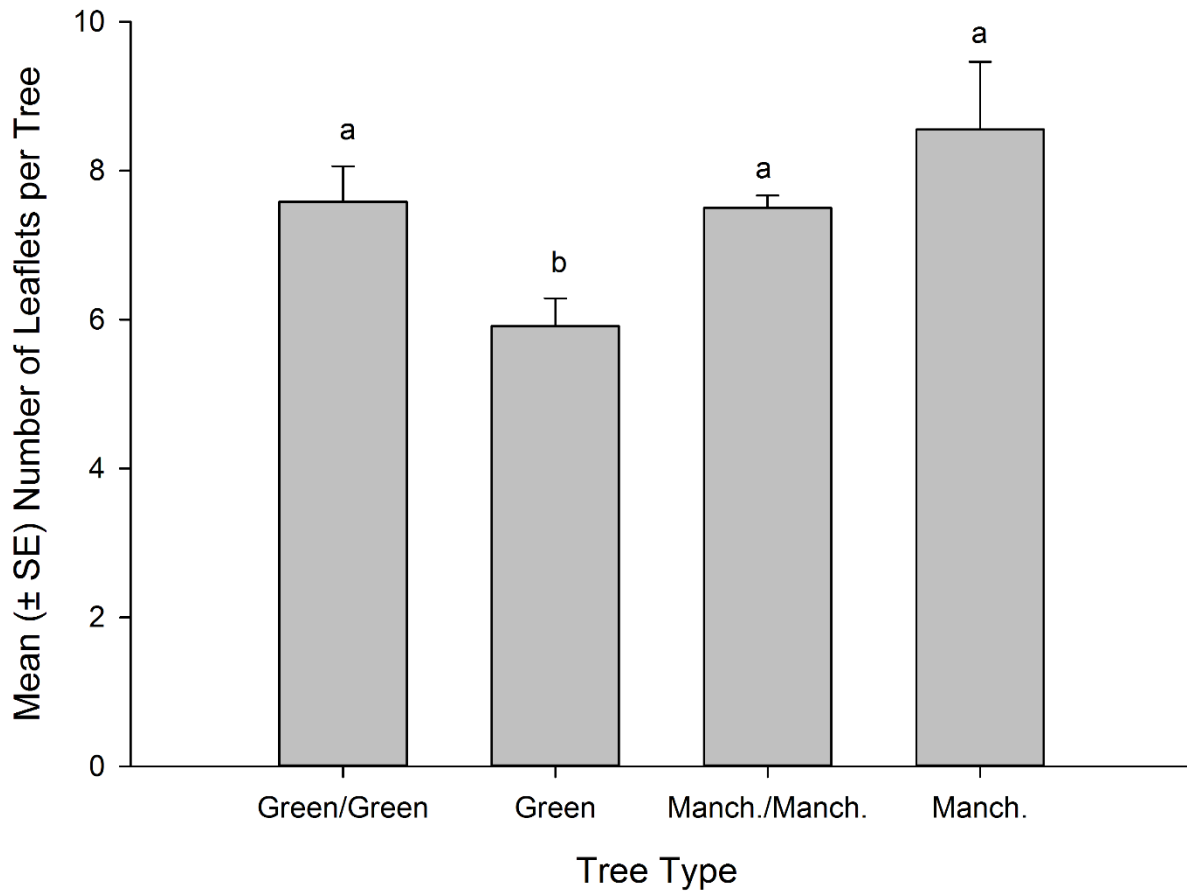
Figure 2.16 Mean (± SE) number of leaflets of buffer and conspecific green and Manchurian ash. Green trees had significantly fewer leaflets than all other trees (ANOVA, $F_{3,9}=4.08$, $P=0.0439$). Letters indicate differences among ash species (Tukey's test, $\alpha<0.05$).
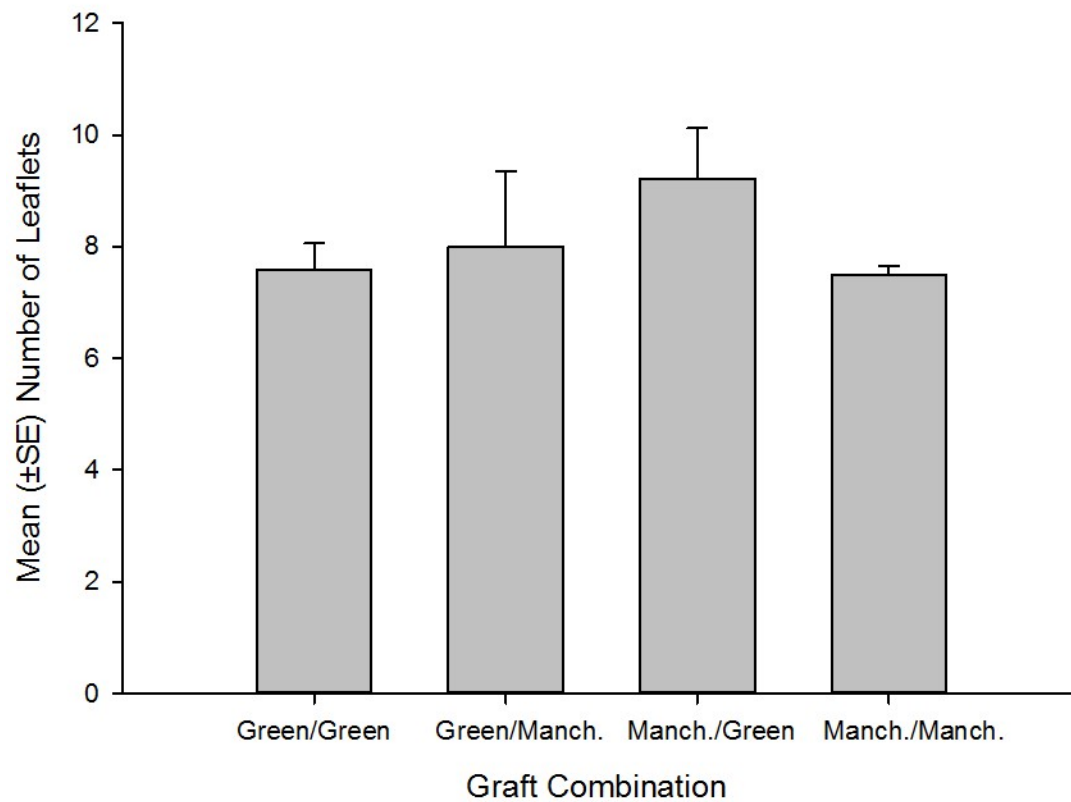
Figure 2.17 Mean (± SE) leaf area on grafted green and Manchurian ash trees. Scion species is listed first in the legend. There was no significant variation in leaf area among grafted green and Manchurian ash (ANOVA, $F_{3,8}=0.39$, $P=0.7632$).

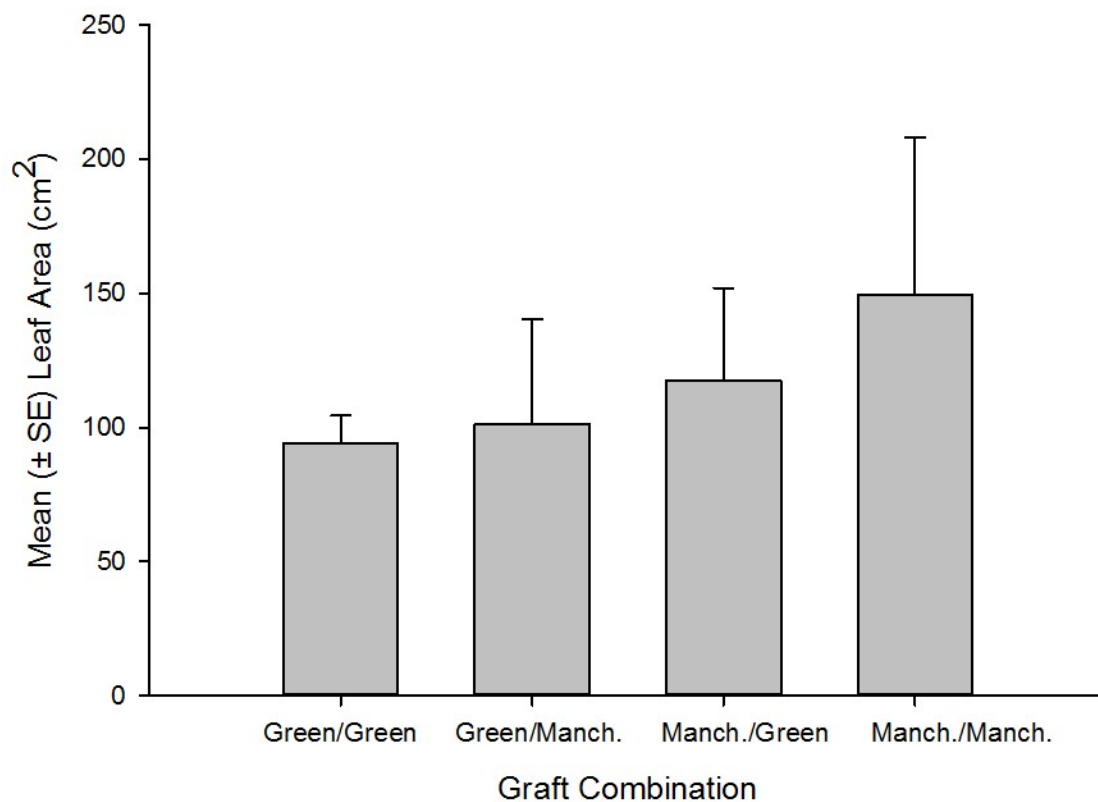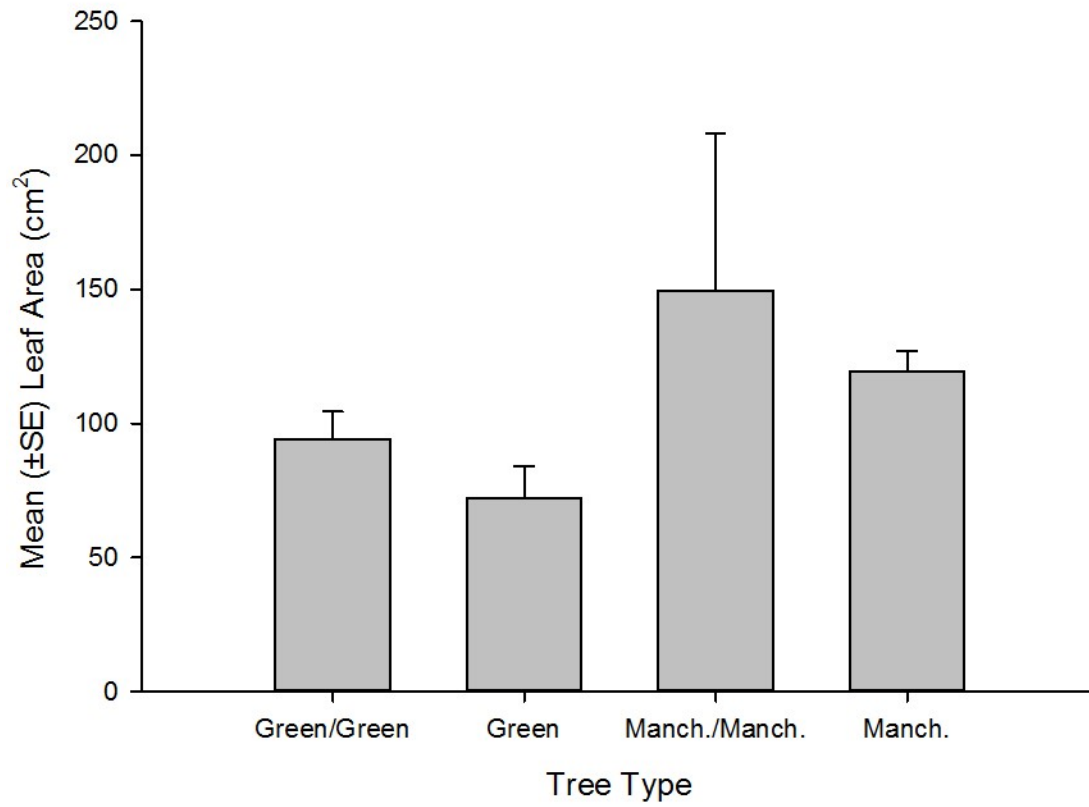Figure 2.18 Mean (± SE) leaf area of buffer and conspecific green and Manchurian ash. Leaf area did not vary among trees (ANOVA, $F_{3,9}=1.46$, $P=0.2902$).

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