HOST PLANT INTERACTIONS BETWEEN EMERALD ASH BORER AND FIVE *FRAXINUS* SPECIES.

By

Sara R. Tanis

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ABSTRACT

HOST PLANT INTERACTIONS BETWEEN EMERALD ASH BORER AND FIVE *FRAXINUS* SPECIES.

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Agrilus planipennis Fairmaire (Coleoptera: Buprestidae), a secondary pest of stressed or declining ash (*Fraxinus*) trees in its native Asia, is the most destructive forest insect to ever invade North America. To date, tens of millions of ash trees have been killed in 19 states and two Canadian provinces. North American ash species are generally less resistant to *A. planipennis* than Asian species. *Fraxinus quadrangulata* Michx. and *Fraxinus americana* L. were originally abundant in two woodlots in southeast Michigan, but survival rates of the two species following *A. planipennis* invasion differed profoundly. Overall, 63% and 71% of *F. quadrangulata* trees were alive at the sites. In contrast, only 16% (all <11 cm diameter) of the *F. americana* trees survived at one site and all were dead at the second site.

Urban trees are often planted in confined spaces and may experience varying degrees of environmental stress. Fertilizer and paclobutrazol (PB), a gibberellin inhibitor, may be applied to enhance tree vigor. Effects of fertilizer and PB were assessed on the physiology and growth of North American (*F. americana* and *F. quadrangulata*) and Asian (*Fraxinus mandshurica* Rupr.) ash trees in a plantation established in 2006. *Fraxinus quadrangulata* had 33% less radial growth, 13% more root biomass and 25% lower foliar nitrogen than *F. americana* or *F. mandshurica*. Ratios of pre: post treatment radial growth in control and fertilized trees were 33 and 43% higher, respectively, than PB trees. Aboveground growth of *F. quadrangulata* and *F. mandshurica* were reduced by PB treatment while *F. americana* was unaffected. Fertilized *F.*

quadrangulata trees had higher relative chlorophyll content and nitrogen concentrations than control or PB trees, but other species did not respond to fertilizer.

Suitability of the ash species for adult *A. planipennis* feeding and survival and larval density was also determined. Survival of adult *A. planipennis* caged on *F. quadrangulata* was lower and beetles consumed less leaf area than beetles caged on other species. North American *Fraxinus nigra* Marsh. and *Fraxinus pennsylvanica* Marsh. trees were heavily colonized by *A. planipennis* larvae. In contrast, *F. quadrangulata* and *F. mandshurica* were rarely colonized, while *F. americana* were moderately colonized. Results confirm North American *F. quadrangulata* has resistance to *A. planipennis* similar to *F. mandshurica*.

Systemic trunk injected insecticides are often used to protect ash trees from *A. planipennis*, but wounds and injury are a concern. We examined basal trunk sections from 22 *F. pennsylvanica* and 24 *F. americana* trees macro-injected with a low or medium rate of emamectin benzoate in 2008 only or 2008+2009. Only 12 of the 233 injection sites had evidence of injury and there was no sign of pathogen infection. Confocal laser scanning microscopy (CLSM) examination showed xylem discoloration was not indicative of tissue damage. Cross-sectional area of earlywood lumen was 55% larger in *F. quadrangulata* than in *F. pennsylvanica* trees. Interspecific differences in xylem anatomy likely influence efficiency of trunk injections.

Phenolic compounds and reactive oxygen species (ROS) are produced by plants in response to injury, stress or insect damage. Autofluorescence indicative of phenolics was detected in *F. quadrangulata* and *F. mandshurica* phloem, but not in *F. pennsylvanica* phloem. Fluorescence of ROS was detected in all the phloem of all three ash species, but *F. mandshurica* produced three times more than the other species. A continuous layer of sclerenchymatous cells was observed in *F. quadrangulata* and *F. mandshurica* and may affect *A. planipennis* larvae.

This one is for you, Muff.

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INTRODUCTION

Agrilus planipennis Fairmaire (Coleoptera: Buprestidae), a secondary pest of stressed or declining ash (*Fraxinus*) trees in its native Asia, has become the most destructive forest insect to ever invade North America (Aukema et al. 2011; Kovacs et al. 2011). To date, tens of millions of *Fraxinus* trees have been killed in 19 states and two Canadian provinces (Pugh et al. 2011; EAB.info 2013).

North American ash species are generally less resistant to *A. planipennis* than Asian ash species, which may be attributable to the lack of a co-evolutionary history (Bryant et al. 1994; Gandhi and Herms 2010a; Nielsen et al. 2011) and the scarcity of native phloem-feeding congeners (Drooz 1985). In large, forested tracts of parkland in southeastern Michigan, the origin of A. planipennis in North America (Siegert et al. 2009), mortality rates of North American *Fraxinus pennsylvanica* Marsh., *Fraxinus americana* L., and *Fraxinus nigra* Marsh. trees exceeded 99% (Herms et al. 2009; Gandhi and Herms 2010b; Knight et al. 2010). In 2009, We revisited two woodlots where *Fraxinus quadrangulata* Michx. and *F. americana* trees were originally sampled in 2004–2005 by Anulewicz et al. (2007). We observed large, apparently healthy specimens of *F. quadrangulata* in both areas, which was surprising given the nearly complete mortality of native ash trees in these counties and across southeastern Michigan. In Chapter One, we assess tree survival by diameter class of *F. quadrangulata* and *F. americana* in the two naturally regenerated woodlots.

In the Midwestern United States, *Fraxinus* trees can comprise 20 to 50% of the urban forest canopy (MacFarlane and Meyer 2003; Poland and McCullough 2006). Like other landscape trees, urban ash trees are often planted in locations with limited space and may experience varying degrees and types of environmental stress (Chaney 2005a). Fertilizer and

paclobutrazol (PB), a gibberellin inhibitor, may be applied to enhance tree vigor. In Chapter Two, I assess effects of fertilizer and PB on the physiology and growth of four North American (*F. americana, F. nigra, F. pennsylvanica* and *F. quadrangulata*) and one Asian (*Fraxinus mandshurica* Rupr.) ash species growing in a plantation established in 2006.

Ash species are evolutionarily diverse (Wallender 2008) and have varying levels of inherent resistance to *A. planipennis* (Rebek et al. 2008; Anulewicz et al. 2007; Tanis and McCullough 2012). *Fraxinus nigra* and *F. pennsylvanica* are highly preferred by *A. planipennis* (Cappaert et al. 2005, Limback 2010) while *F. americana* is considered moderately preferred (Anulewicz et al. 2007). To date, *F. quadrangulata* appears to be the least preferred North American species (Anulewicz et al. 2007, 2008; Tanis and McCullough 2012). In Asia, *A. planipennis* functions as a secondary pest, typically attacking stressed ash trees (Liu et al. 2003). In North America, *A. planipennis* colonizes both healthy and stressed *Fraxinus* trees (Poland and McCullough 2006), but preferentially attacks stressed trees (McCullough et al. 2009a, 2009b). Many tree care companies promote plant growth regulator treatments to reduce tree growth and ameliorate stress (Chaney 2005a, 2005b). In Chapter Three, I assess differences in *A. planipennis* host preference and suitability of the four North and the Asian ash species and among trees treated with PB, fertilizer or left as untreated controls in the controlled plantation.

Systemic insecticides applied as trunk injections are often used to protect landscape ash trees from *A. planipennis*, but wounds and injury are a concern particularly for macro-injection methods. TREE-äge, a product with emamectin benzoate as the active ingredient, is increasing used because it provides ash trees with multiple years of protection against *A. planipennis* (McCullough et al. 2011). Trunk injected insecticides can also cause internal tissue discoloration (Mota-Sanchez et al. 2009; Tanis et al. 2012) which may be indicative of tissue wounding (Smith

and Lewis 2005). In Chapter Four, I evaluate possible injury and tissue discoloration associated with emamectin benzoate injection sites on *F. pennsylvanica* and *F. americana* trees injected in 2008 only or in 2008 and again in 2009.

Interspecific variability in xylem architecture may affect the efficiency of trunk injections (Mota-Sanchez et al. 2009; Tanis et al. 2012). Uptake of trunk injected insecticides can be slowed by friction as products pass through the xylem lumen or through the bordered pits. In a related study in Chapter Four, I examined cross sectional area of earlywood vessels and bordered pit densities in *F. quadrangulata* and *F. pennsylvanica* with confocal laser scanning microscopy (CLSM) to determine if xylem anatomy could influence trunk injection success.

Interspecific variability in ash resistance to *A. planipennis* has sparked interest in ash physiology and defensive chemistry. *Fraxinus quadrangulata* does not share an evolutionary history with *A. planipennis*, yet it has resistance similar to *F. mandshurica*, a species that co-evolved with EAB in China. In contrast, *F. pennsylvanica* is highly vulnerable to *A. planipennis* infestation. Phenolic compounds and reactive oxygen species (ROS) are often produced by plants in response to injury, stress or insect damage. *Agrilus planipennis* larvae feed on the phloem and cambium tissues of *Fraxinus* trees (Cappaert et al. 2005) and could be affected by interspecific differences in phloem defensive chemical production or anatomy. In Chapter Five, I narrow the scope of my host preference research down to the tissue level. I use CLSM to assess interspecific differences in phenolic compound autofluorescence, ROS production and phloem architecture of *F. pennsylvanica*, *F. quadrangulata* and *F. mandshurica*.

CHAPTER ONE

Differential persistence of blue ash and white ash following emerald ash borer invasion

Tanis, S.R. and D.G. McCullough. 2012. Differential persistence of blue ash and white ash following emerald ash borer invasion. Canadian Journal of Forest Research. 42(8): 1542-1550.

ABSTRACT

Catastrophic mortality of North American ash (*Fraxinus* spp.) caused by *Agrilus planipennis* Fairmaire has been attributed to the lack of coevolved resistance between native ash species and this Asian invader. Although *A. planipennis* host preference or tree resistance can vary, all North American ash species are presumably highly vulnerable to *A. planipennis*. WE inventoried live and dead blue ash (*Fraxinus quadrangulata* Michx.) and white ash (*Fraxinus americana* L.) in two southeastern Michigan woodlots several years after the *A. planipennis* invasion to assess their survival. *Agrilus planipennis* populations in this area peaked in approximately 2005, and the region is now characterized by nearly complete ash mortality. At the Plymouth site, 71% of the original 380 blue ash were alive, whereas only 29 saplings of the original 187 white ash were alive. At the Superior Township site, 63% of the original 210 blue ash were living, whereas all 125 white ash were dead. More than 80% of the blue ash had evidence of previous *A. planipennis* colonization, but 87% appeared healthy in 2011. Tree diameter did not consistently affect survival, and live and dead trees of both species were distributed across sites, indicating that differential survival was not attributable to localized conditions.

INTRODUCTION

Emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), a phloem-boring beetle native to Asia (Chinese Academy of Science, Institute of Zoology 1986; Yu 1992), was first identified in North America in southeastern Michigan, USA, and southern Ontario, Canada, in 2002 (Cappaert et al. 2005). Tens of millions of ash (*Fraxinus* spp.) trees in forested and urban settings have been killed by *A. planipennis* in Michigan alone, and infestations have been found in 14 additional states and in Quebec, Canada (Emerald Ash Borer Information Website, www.emeraldashborer.info, accessed 6 March 2012). Recent analyses showed that *A. planipennis* is the most costly forest insect to invade the US, with annual economic impacts in excess of US $900 billion (Aukema et al. 2011; Kovacs et al. 2011).

Ash trees are injured by *A. planipennis* larvae feeding on phloem and cambium in serpentine galleries (Cappaert et al. 2005). On most trees, *A. planipennis* initially colonizes the upper canopy (Cappaert et al. 2005; McCullough and Siegert 2007a). As *A. planipennis* densities build, larval feeding eventually leads to mortality of the entire tree. External evidence of *A. planipennis* infestation includes distinctive D shaped holes left by emerging *A. planipennis* beetles, larger holes left by woodpeckers preying on late instar larvae, cracks in the outer bark above larval galleries, epicormic shoots on large branches or the trunk, and progressive canopy dieback (Cappaert et al. 2005; Poland and McCullough 2006; McCullough et al. 2009a).

North American ash species are generally less resistant to *A. planipennis* than Asian ash species, which may be attributable to the lack of a co-evolutionary history (Bryant et al. 1994; Lieutier 2008; Gandhi and Herms 2010a; Nielsen et al. 2011) and the scarcity of native phloem-feeding congeners (Drooz 1985). In its native range, *A. planipennis* is considered a secondary pest and typically colonizes only declining or stressed ash trees (Liu et al. 2007; Wang et al.

2010; Cipollini et al. 2011). In North America, *A. planipennis* preferentially colonizes stressed ash (McCullough et al. 2009a, 2009b) but will readily colonize and kill healthy ash trees (Cappaert et al. 2005; Poland and McCullough 2006). In large, forested tracts of parkland in southeastern Michigan, mortality rates of North American green ash (*Fraxinus pennsylvanica* Marsh.), white ash (*Fraxinus americana* L.), and black ash (*Fraxinus nigra* Marsh.) trees exceeded 99% by 2009 (Herms et al. 2009; Gandhi and Herms 2010b; Knight et al. 2010).

To date, populations of *A. planipennis* have encountered five native ash species in eastern North America, and although all appear to be suitable hosts, interspecific variation in *A. planipennis* host preference or ash tree resistance has been observed in field settings (Cappaert et al. 2005; Anulewicz et al. 2007). Preferential colonization of some North American species was first noted by Anulewicz et al. (2007), who quantified *A. planipennis* exit holes and canopy condition annually in sites in southeastern Michigan from 2003 to 2005. They found that when green ash and white ash trees co-occurred, green ash trees were preferentially colonized, and as they succumbed, *A. planipennis* densities increased on white ash trees.

In 2004 and 2005, Anulewicz et al. (2007) compared *A. planipennis* densities on white ash and blue ash (*Fraxinus quadrangulata* Michx.) trees in two southeastern Michigan woodlots in which both species were relatively abundant. They reported that average densities of *A. planipennis* exit holes and woodpecker attacks were six fold higher on white ash trees than on blue ash trees. Some white ash trees in both woodlots had been killed by *A. planipennis* before sampling began in 2004 and others were declining in 2005 (Anulewicz et al. 2007). They noted that 57% of the blue ash trees sampled in 2005 had *A. planipennis* exit holes, bark cracks, or holes left by woodpeckers preying on larvae (A.C. Anulewicz and D.G. McCullough, unpublished data).

Other studies have similarly suggested that blue ash may be less preferred by or less suitable for *A. planipennis* than other North American ash species. In a laboratory trial with clipped leaves, adult *A. planipennis* beetles appeared to become more discriminatory over the course of 48 h and ultimately spent less time and consumed less blue ash and Manchurian ash foliage compared with four other ash species (Pureswaran and Poland 2009). Similarly, when adult *A. planipennis* were caged with intact leaves on live trees for 14 days, beetle survival was higher on white ash trees (67%) than on blue ash trees (42%) (Tanis and McCullough 2012).

In 2009, we revisited the two woodlots where blue ash and white ash trees were originally sampled in 2004–2005 by Anulewicz et al. (2007). We observed large, apparently healthy specimens of blue ash in both areas, which was surprising given the nearly complete mortality of native ash in these counties and across southeastern Michigan (Herms et al. 2009; Gandhi and Herms 2010b; Knight et al. 2010). In 2010–2011, we returned to the two woodlots to systematically quantify and compare survival and canopy condition of blue ash and white ash trees. We assessed size, distribution, and basal area of live and dead ash trees of both species in each woodlot to evaluate the potential influence of site-related variables on tree survival or condition.

METHODS

Study sites

Ash trees were inventoried in a 6.8 ha woodlot in Plymouth Township, Wayne Co., Michigan, and a 4.8 ha woodlot in Superior Township, Washtenaw Co., Michigan. Soils at both sites are predominantly silty clay loam with 2%–6% slopes (U.S. Department of Agriculture 2012b). Understory vegetation in the two sites included herbaceous ephemerals (*Trillium grandiflorum* (Michx.) Salisb., *Erythronium americanum* Ker, and *Podophyllum peltatum* L.) and occasional areas of shrubs (*Asimina triloba* (L.) Dunal, *Crataegus* spp., and *Rosa multiflora* Thunb.).

Ash inventory

In September 2009, we identified and flagged all *Fraxinus* spp. trees within each woodlot with colored tape to indicate whether the trees were alive or dead. Live trees had at least one living shoot or epicormic sprout on the trunk or branches. In October 2009, following leaf drop, 12 contiguous 50 × 50 m cells were established in each woodlot to facilitate overstory inventories. Plots were excluded from low lying, swampy areas where no trees occurred. We recorded species and GPS coordinates (eTrex Legend, Garmin Ltd., Olathe, Kansas, USA) and measured diameter at breast height (DBH) (1.3 m aboveground) for all living and dead (standing and down) ash trees ≥2.5 cm DBH. A total of 17 dead, fallen ash trees (three at Plymouth; 14 at Superior Township) were too decayed for species determination and were excluded from maps and analyses. An estimated 25 dead white ash trees had been removed and used for firewood by the owners of the Superior Township property in 2007–2009 and could not be inventoried. The property owner at the Plymouth site indicated that no trees (regardless of species) had been removed for firewood. Dead trees were examined and bark was removed when necessary to

assess larval galleries and confirm that death was caused by *A. planipennis*. Distribution of live and dead blue ash and white ash trees was mapped using GPS coordinates (R statistical software; R Foundation for Statistical Computing, Vienna, Austria).

In May 2010, following leaf expansion, we returned to evaluate canopy condition of live ash trees. Canopy dieback was visually estimated (10% increments) for live ash in both sites following protocols of Zarnoch et al. (2004), where dieback represents the percentage of the tree crown comprised of dead branches, excluding natural branch pruning. Dieback ratings were completed by one person to ensure consistency. Trees were grouped by canopy dieback classes for presentation. Trees with <30% dieback were considered to be relatively healthy, those with 31%–60% dieback exhibited "moderate" canopy dieback, and trees with >60% canopy dieback were considered to be severely declining and unlikely to recover (Herms et al. 2009).

Variable radius plots and relative importance values

In July 2011, we established a variable radius plot (10 BAF wedge prism) in the center of each 50 × 50 m cell at each site. We recorded species and measured DBH of live and dead (standing and down) trees ≥2.5 cm DBH and visually estimated canopy dieback for live trees within each plot. Data from these plots were consolidated to estimate basal area by genus (including ash and non-ash trees) and total basal area for each site. Relative importance values were calculated for the five most abundant genera at each site. Relative importance values represent the contribution of a given genus to the overstory and were calculated as the sum of the relative frequency (number of plots containing the genus as a percentage of the total number of occurrences for all genera within all plots) + relative density (number of individuals of the genus as a percentage of the total number of individuals of all genera) + relative dominance (total basal area of the genus as a percentage of the total basal area for all genera) (Kent and Coker 1992).

Statistical analysis

To simplify presentation, ash trees were grouped by DBH into four classes corresponding to young recruits (≤10 cm), small trees (10.1–20 cm), pole-sized trees (20.1–30 cm), and merchantable trees (>30.1 cm). We used a binomial t test to determine if DBH differed between ash species and between living and dead blue ash trees in each site. Linear relationships between DBH and canopy dieback were evaluated for live blue ash in both sites. Data were analyzed using SAS statistical software (SAS Institute Inc., Cary, North Carolina) at α of $P < 0.05$.

RESULTS

Blue ash and white ash trees were originally abundant in the two woodlots, but survival rates of the two species following invasion by *A. planipennis* differed profoundly. We inventoried a total of 567 and 335 *Fraxinus* spp. trees in the Plymouth and Superior Township sites, respectively, in the 50 × 50 m cells. At the Plymouth site, 270 of the 380 blue ash trees (63%) survived, including at least 50% of the blue ash trees in all diameter classes (Fig. 1.1a). In contrast, only 29 of 187 white ash trees (16%) at the Plymouth site survived, and all were <11 cm DBH (Fig. 1.1a). At the Superior Township site, 132 of the 210 blue ash trees (71%) were alive, including at least 50% of trees in each diameter class, while all 125 white ash trees were dead (Fig. 1.1b). Evidence of previous *A. planipennis* infestation was observed on 83% and 87% of live blue ash trees at the Plymouth and Superior Township sites, respectively, and 10 of the 29 live white ash at the Plymouth site.

Both blue ash and white ash trees were originally distributed throughout each woodlot. In Plymouth, blue ash trees originally occurred in all 12 grid cells and live blue ash were present in 11 of the cells (Fig. 1.2). Dead white ash occurred in every cell, and the 29 live white ash trees were scattered across 10 of the 12 grid cells (Fig. 1.2). At the Superior Township site, live and dead blue ash and dead white ash trees were present in all 12 grid cells (Fig. 1.3).

We calculated total *Fraxinus* spp. basal area using the ash inventory data from the grid cells. At the Plymouth site, 62% (2.5 $m^2 \cdot ha^{-1}$) of the total blue ash basal area (4.1 $m^2 \cdot ha^{-1}$) was alive. White ash trees in the Plymouth site originally accounted for 70% (9.7 $m^2 \cdot ha^{-1}$) of the total (living and dead) *Fraxinus* spp. basal area (13.7 $m^2 \cdot ha^{-1}$), but the 29 live white ash cumulatively accounted for <1% of the *Fraxinus* spp. basal area. At the Superior Township site,

81% (3.5 $m^2 \cdot ha^{-1}$) of the total blue ash basal area (4.3 $m^2 \cdot ha^{-1}$) was alive. Before the *A. planipennis* invasion, white ash comprised at least 44% (3.4 $m^2 \cdot ha^{-1}$) of the total (living and dead) *Fraxinus* spp. basal area (7.63 $m^2 \cdot ha^{-1}$) in this site, but all of these trees were killed.

Survival of the ash species at the two sites was not consistently related to tree DBH. At the Plymouth site, the overall mean (± standard error (SE)) DBH of all blue ash trees (live and dead), which averaged 8.9 ± 0.38 cm, was smaller than the overall mean of all white ash trees, which averaged 20.7 ± 1.13 cm ($t_{[1,564]} = 12.32$; $P < 0.001$). Dead blue ash trees, which averaged 11.0 ± 0.70 cm DBH, were larger than live blue ash, which averaged 8.1 ± 0.45 cm DBH ($t_{[1,379]} = 3.58$; $P < 0.001$). Most of the live blue ash (94%) and dead blue ash (92%) were ≤20 cm DBH (Fig. 1.1a). Only 2% and 4% of the live and dead blue ash trees, respectively, were >30 cm DBH (Fig. 1.1a). The largest living blue ash tree was 45.0 cm DBH, while the largest dead blue ash tree was 50.0 cm DBH. Average DBH of dead white ash at the Plymouth site was 23.7 ± 1.17 cm, and dead white ash trees were represented in all DBH classes (Fig. 1.1a). All 29 live white ash trees at the Plymouth site were <11 cm (Fig. 1.1a), with an average DBH of 4.7 ± 0.35 cm. Approximately 50% of the dead white ash were relatively small (<20 cm DBH), but 24% were at least 30 cm DBH and the largest dead white ash tree was 96.0 cm DBH.

At the Superior Township site, DBH of the dead white ash trees averaged (±SE) 16.0 ± 0.89 cm, which was larger than the overall mean of all blue ash trees (live and dead), which averaged 13.4 ± 0.06 cm ($t_{[1,333]} = 2.36$; $P = 0.019$). Average DBH of live blue ash was 15.2 ± 0.88 cm, which was larger than the DBH of dead blue ash, which averaged 10.2 ± 0.57 cm ($t_{[1,208]} = 4.10$; $P < 0.001$). Most of the live blue ash (67%) and nearly all of the dead blue ash

(98%) trees were <20 cm DBH (Fig. 1.1b). Only 7% of the live blue ash trees and a single dead blue ash tree were >30 cm DBH. The largest living blue ash tree was 46.5 cm DBH, while the largest dead blue ash tree was 31.0 cm DBH. Overall, 69% of the dead white ash trees were <20 cm DBH. Only 12% of the dead white ash trees were >30.1 cm DBH (Fig. 1.1b), and the largest dead white ash was 43.5 cm DBH.

The two woodlots included a mix of northern hardwood species, and until the *A. planipennis* invasion, both blue ash and white ash comprised a substantial portion of the overstory. We measured 34 *Fraxinus* spp. and 114 non-*Fraxinus* spp. trees at the Plymouth site and 22 *Fraxinus* spp. and 129 non-*Fraxinus* spp. trees at the Superior Township site (Table 1.1). Total basal area estimated from variable radius plots was 24.2 and 16.4 $m^2 \cdot ha^{-1}$ in the Plymouth and Superior Township sites, respectively. Overall, *Fraxinus* spp. ranked as the second and fourth highest contributing genus, accounting for 9% and 7% of the total basal area at the Plymouth and Superior Township sites, respectively (Table 1.1). Overstory composition at the sites was similar and dominated by *Acer* spp., *Quercus* spp., *Tilia americana* L., and *Carya* species. We also encountered a few stems of *Ulmus americana* L., *Juglans nigra* L., *Ostrya virginiana* (Mill.) K. Koch, and *Prunus serotina* Ehrh. at both sites. Two standing non-ash trees were dead (one *U. americana* and one *O. virginiana*) at the Superior Township site. No standing non-ash trees were dead in the plots at the Plymouth site.

Previous *A. planipennis* larval feeding resulted in some level of canopy dieback on most of the live blue ash trees at the two sites, particularly those trees that were large enough (e.g., >10.1 cm DBH) to have been colonized when *A. planipennis* densities were peaking. At the Plymouth site, canopy dieback was minimal (<10%) on 131 of the 204 (64%) small blue ash trees (<10 cm DBH), while only 8% of these small trees had severe dieback (>60%) (Table 1.2).

Dieback on these small trees averaged (± SE) 23% ± 1.7%. Of the 50 blue ash trees that were 10.1–20 cm DBH, 16% had negligible dieback and 20% had severe dieback. For the 6% of the blue ash trees that were >20.1 cm DBH, canopy dieback averaged 43.8% ± 3.3%. Only two of these 16 trees had severe dieback, whereas dieback was low to moderate on the other 14 trees (Table 1.2). For live blue ash trees at Plymouth, DBH was linearly related to canopy dieback ($Y = 38.108 + 0.169x$) ($F_{[1,268]} = 39.15$; $P < 0.001$), although the regression explained relatively little variation ($R^2 = 0.127$). Ten of the 29 small live white ash trees in the Plymouth site had evidence of previous *A. planipennis* colonization. Canopy dieback was minimal (<10%), however, on 24 of the trees, and only a single tree had severe dieback.

At the Superior Township site, canopy dieback was minimal (<10%) on 18 of the 89 (20%) blue ash trees that were ≤20 cm DBH and severe (>60%) on 21 of these trees (24%) (Table 1.2). Canopy dieback for blue ash trees ≤20 cm DBH averaged 44% ± 3.1%. Of the 34 blue ash trees in the 20.1– 30 cm DBH class, most (83%) had low to moderate dieback (Table 1.2). Only eight blue ash trees at Superior Township were >30 cm DBH. Three of these trees had minimal dieback, five trees had low to moderate dieback, and one tree (41.2 cm DBH) had severe dieback (Table 1.2). Canopy dieback of blue ash trees at the Superior Township site was not related to DBH ($P = 0.46$).

Canopy dieback on the non-*Fraxinus* trees assessed in the variable radius plots was negligible. None of the non-ash trees had more than 10% canopy dieback at either site.

DISCUSSION

The long-term persistence of blue ash in southeastern Michigan, where high densities of *A. planipennis* have resulted in nearly complete mortality of other native ash species, is one of very few bright spots in the saga of the *A. planipennis* invasion in North America. Results from the original 2004–2005 survey of these sites led Anulewicz et al. (2007) to conclude that although white ash trees would succumb sooner, *A. planipennis* densities would continue to build on blue ash trees until the trees succumbed, eventually leading to the catastrophic mortality observed in forested areas across southeastern Michigan (Herms et al. 2009; Gandhi and Herms 2010b; Knight et al. 2010). We were surprised, therefore, to find that at least 60% of the blue ash trees at both sites were not only alive in 2011, but most appeared to be healthy. In contrast, every white ash tree in the Superior Township site was killed and the few surviving white ash trees in the Plymouth site were likely too small to have been colonized during the peak of the *A. planipennis* invasion. All of the dead ash trees in the two sites had been fully colonized by *A. planipennis*; larval galleries literally covered the trunks and branches.

In our experience, populations of *A. planipennis* are typically established in a site for at least three to four years before severe decline or mortality becomes apparent (Siegert et al. 2009; Pugh et al. 2011). Moreover, in newly infested sites with low *A. planipennis* densities, a high proportion of larvae requires two years for development (Siegert et al. 2010; Tluczek et al. 2011), which slows the population growth rate (Mercader et al. 2011a, 2011b). Evidence strongly suggests, therefore, that *A. planipennis* populations were established in our sites at least two years before this invasive pest was first identified in North America in 2002. The unexpectedly high survival of blue ash trees 10 or more years after invasion, therefore, indicates

that this species is more resistant than any other North American ash species that *A. planipennis* populations have encountered to date.

We evaluated several factors to determine whether exposure to sun, local growing conditions, or other site-related variables were responsible for the differential mortality rates of the two ash species. Soil at the two sites consisted primarily of silty clay loam, which is suitable for both *Fraxinus* species (Burns and Honkala 1990; Farrer 1995). Previous observations have shown that adult *A. planipennis* activity, including oviposition, is consistently higher on trees exposed to full or nearly full sun than on shaded trees (Yu 1992; McCullough et al. 2009a, 2009b; Poland et al. 2011). Stress can alter volatiles emitted by ash trees, increasing their attraction to *A. planipennis* (Rodriguez-Saona et al. 2006; McCullough et al. 2009a, 2009b; Chen and Poland 2009). If white ash trees experienced poorer growing conditions (e.g., flooded, compacted soil, etc.) than blue ash trees, for example, survival rates could reflect differential attraction of *A. planipennis* to stressed versus healthier trees. Both blue ash and white ash trees, however, were relatively abundant in the two woodlots, and exposure and environmental conditions were similar. Live and dead ash trees of both species were distributed throughout both sites, and there were no apparent spatial patterns in ash mortality for either species. Furthermore, non-ash trees in both woodlots were healthy; only two dead non-ash trees were encountered in the plots in either site, and canopy decline was minimal on the non-ash overstory trees.

Similarly, there was no evidence that tree size consistently influenced mortality rates. Average DBH of live blue ash was slightly (3 cm) smaller than that of dead blue ash trees at the Plymouth site, but live blue ash were somewhat larger (5 cm), on average, than dead blue ash at the Superior Township site. Dead white ash were represented in all diameter classes at both

sites. Although we cannot determine whether the rate of white ash mortality differed among size classes, ultimately, all white ash trees were killed, with the exception of the 29 saplings at the Plymouth site. The unexpected persistence of blue ash trees in these sites, therefore, does not appear to be merely a function of differences between ash species in growing conditions or size.

It is especially notable that at least 60%–70% of the blue ash trees in both woodlots survived despite *A. planipennis* densities that were likely very high during the peak of the invasion. Using methods of McCullough and Siegert (2007a), we estimated that 114,750 and 53,980 *A. planipennis* beetles could have been produced by the dead white ash trees at the Plymouth and Superior Township sites, respectively, before they succumbed. An additional 24,680 and 15,050 *A. planipennis* adults could have emerged from the dead blue ash trees at Plymouth and Superior Township sites, respectively. Given these numbers, it is not surprising that nearly all of the surviving blue ash trees had some evidence of *A. planipennis* colonization. For example, at least 80% of the live blue ash trees >10 cm DBH in both sites had more than 10% canopy dieback. Most live blue ash appeared to be healthy in 2011, however, and less than 13% of these trees had severe dieback.

Although naturally regenerated blue ash and white ash trees were present in both woodlots, the two species differ considerably in terms of distribution, silvicultural traits, and morphological features. For example, distribution of blue ash is centered in Kentucky, Tennessee, and Missouri (USDA Hardiness Zones 4–7) (U.S. Department of Agriculture 2012a), where it occurs most commonly in mixed stands on limestone outcroppings (Bryant et al. 1980; Pallardy et al.1988; Prasad et al. 2007). Blue ash trees in the sites that we sampled, therefore, were growing near the northern edge of their native range. In contrast, the distribution of white ash extends across much of eastern North America, from Nova Scotia, Canada, south to northern

Florida (USDA hardiness Zones 3–9) (Burns and Honkala 1990). Whether blue ash will demonstrate similarly strong resistance to *A. planipennis* in other areas of its native range, particularly where it is relatively abundant, remains to be seen. Populations of *A. planipennis* were identified in Missouri in 2008, Kentucky in 2009, and Tennessee in 2010 (Emerald Ash Borer Information Website, www.emeraldashborer.info, accessed 6 March 2012), but to date, infestation and mortality rates for specific ash species have not been reported from these areas. Morphological differences between blue ash and white ash trees are also apparent. Blue ash and white ash were widely separated in a phylogenetic tree constructed for *Fraxinus* species based on ribosomal internal transcribed spacers and floral morphological characteristics (Wallander 2008). White ash, a dioecious species, was placed in the Melloides section, along with eight other North American species. Blue ash, along with *F. dipetala* and *F. anomala*, species native to the southwestern US, were placed in the Dipetalae section and are characterized by monoecious flowers and four distinctive cork ridges on woody shoots. Whether the other North American Dipetalae ash species are also relatively resistant to *A. planipennis* remains unknown.

Identification of specific traits or mechanisms underlying blue ash resistance could play a key role in the population dynamics of *A. planipennis* and survival of the genus *Fraxinus* in North America. Adult *A. planipennis* females feed on ash leaves for at least 14 days before oviposition begins and continue to leaf feed throughout their life span (Cappaert et al. 2005), laying a few eggs between bouts of feeding. Therefore, selection of hosts for leaf feeding by adult *A. planipennis* females likely has a strong effect on selection of hosts for oviposition (Mercader et al. 2011a). To date, most studies of *Fraxinus* resistance to *A. planipennis* have compared Asian ash species with North American green ash, white ash, and black ash (Eyles et al. 2007; Chen et al. 2011; Rebek et al. 2008; Whitehill et al. 2011). Pureswaran and Poland

(2009) reported that adult *A. planipennis* leaf consumption was lower when beetles were caged with blue ash and Manchurian ash leaves compared with leaves from green, white, or black ash trees. However, this study used detached leaves, perhaps negating potential inducible defensive responses. In a 14-day field trial, survival of *A. planipennis* beetles allowed to feed on intact leaves of live trees was lower on blue ash than on white ash (Tanis and McCullough 2012). More than 40% of the beetles that fed on blue ash, however, survived the maturation feeding period, and females could presumably have begun laying eggs.

Although foliar traits or defenses may influence host selection by adult *A. planipennis*, most (87%) living blue ash trees in the two woodlots exhibited some evidence of previous *A. planipennis* colonization. It seems likely, therefore, that phloem-based compounds or other factors must affect the suitability of ash species for *A. planipennis* larval development or survival. When Anulewicz et al. (2007) surveyed these woodlots, they noted the presence of callus tissue, i.e., wound periderm, associated with some *A. planipennis* larval galleries on blue ash trees. Rapid production of wound periderm functions as a defense mechanism against *Agrilus anxius* Gory larvae, a North American species that colonizes paper birch (*Betula papyrifera* Marsh.) (Nielsen et al. 2011; Miller et al. 1991). We similarly observed wound periderm on trees in these sites and on ash trees in other areas. In our experience, however, this tissue seldom affects developing larvae and is rare on trees colonized by high densities of *A. planipennis* larvae. Whether fewer eggs were laid on blue ash trees in the sites that we sampled or whether most larvae failed to develop and emerge successfully from blue ash trees remains unknown.

The persistence of blue ash through the *A. planipennis* invasion has implications for both *A. planipennis* management and future research. Green ash and black ash trees, for example, are

more preferred and suitable hosts for *A. planipennis* than white ash (Cappaert et al. 2005; Chen and Poland 2010; Limback 2010), while our results show that blue ash is less preferred or a less suitable host for *A. planipennis* than white ash. Selecting green ash or black ash trees for traps or use as girdled trap trees, therefore, is presumably more likely to facilitate detection of *A. planipennis* than using white ash or blue ash (McCullough and Siegert 2007b). Resistance of blue ash may also have implications for biological control efforts underway across much of the *A. planipennis* infestation in the United States. Federal agencies have invested substantially in the importation, rearing, and release of Asian parasitoids for biological control of *A. planipennis* (Bauer et al. 2008; Duan et al. 2011, 2012). Native parasitoids, particularly *Atanycolus* spp., have also begun to parasitize *A. planipennis* larvae, sometimes accounting for high mortality in localized areas (Cappaert and McCullough 2009; Duan et al. 2009, 2011; Taylor et al. 2012). In areas with a substantial component of blue ash, the combination of relatively resistant hosts and specialized parasitoids could perhaps maintain *A. planipennis* populations at substantially lower densities than those typical of sites dominated by green, white, or black ash and may warrant evaluation (Barclay 1987; Barclay and Li 1991; Suckling et al. 2012). Additional studies to assess morphological features and constitutive or inducible defensesthat confer resistance in blue ash by acting on *A. planipennis* adults or larvae could potentially be propagated or enhanced in other ash species. Evaluating potential resistance of *F. dipetala* and *F. anomala*, the two North American ash species most closely related to blue ash (Wallander 2008), would also be useful.

Table 1.1: Number of trees, mean (±SE) diameter at breast height (DBH), total basal area and Relative Importance Values[1] for the five most common tree genera recorded in variable radius plots in woodlots in Plymouth and Superior Township, Michigan. Values for *Fraxinus* include dead and live trees.

Genus	**Number of Trees**	**DBH (cm)**	**Basal Area(m^2/ha)**	**Relative Importance Value[1]**
		Plymouth Site		
Quercus	40	51.2 ± 5.2	11.1	94.3
Fraxinus	**34**	**17.4 ± 5.3**	**2.2**	**53.5**
Acer	26	34.6 ± 3.0	2.9	40.3
Carya	17	38.4 ± 3.8	2.3	35.1
Tilia	11	37.2 ± 6.5	1.6	26.4
		Superior Township Site		
Acer	54	37.0 ± 2.1	6.8	59.5
Tilia	31	37.1 ± 2.3	3.7	40.7
Quercus	19	44.2 ±3.9	3.3	33.3
Fraxinus	**22**	**24.0 ± 1.9**	**1.1**	**24.5**
Carya	9	30.1 ± 4.6	0.8	16.0

[1]Relative Importance Values reflect the contribution of a given genus to the overstory and were calculated as the sum of the relative frequency + relative density + relative dominance following Kent and Coker (1992).

Table 1.2: Number of living blue ash and white ash trees by canopy dieback and diameter classes in woodlots surveyed in Plymouth and Superior Township, Michigan.

	Ash Diameter Class (cm DBH[1])			
Canopy Dieback Class	≤10.0	10.1 - 20.0	20.1 - 30.0	> 30.1
	Plymouth - Living Blue Ash			
≤10%	131	8	0	0
11-30	32	16	1	2
31-60	24	16	8	3
>60	17	10	2	0
	Plymouth - Living White Ash			
≤10%	23	1	0	0
11-30	4	0	0	0
31-60	0	0	0	0
>60	1	0	0	0
	Superior Township - Living Blue Ash			
≤10%	10	8	4	3
11-30	11	8	11	3
31-60	19	12	19	2
>60	13	8	0	1

[1]DBH refers to diameter at breast height, measured 1.3 m aboveground

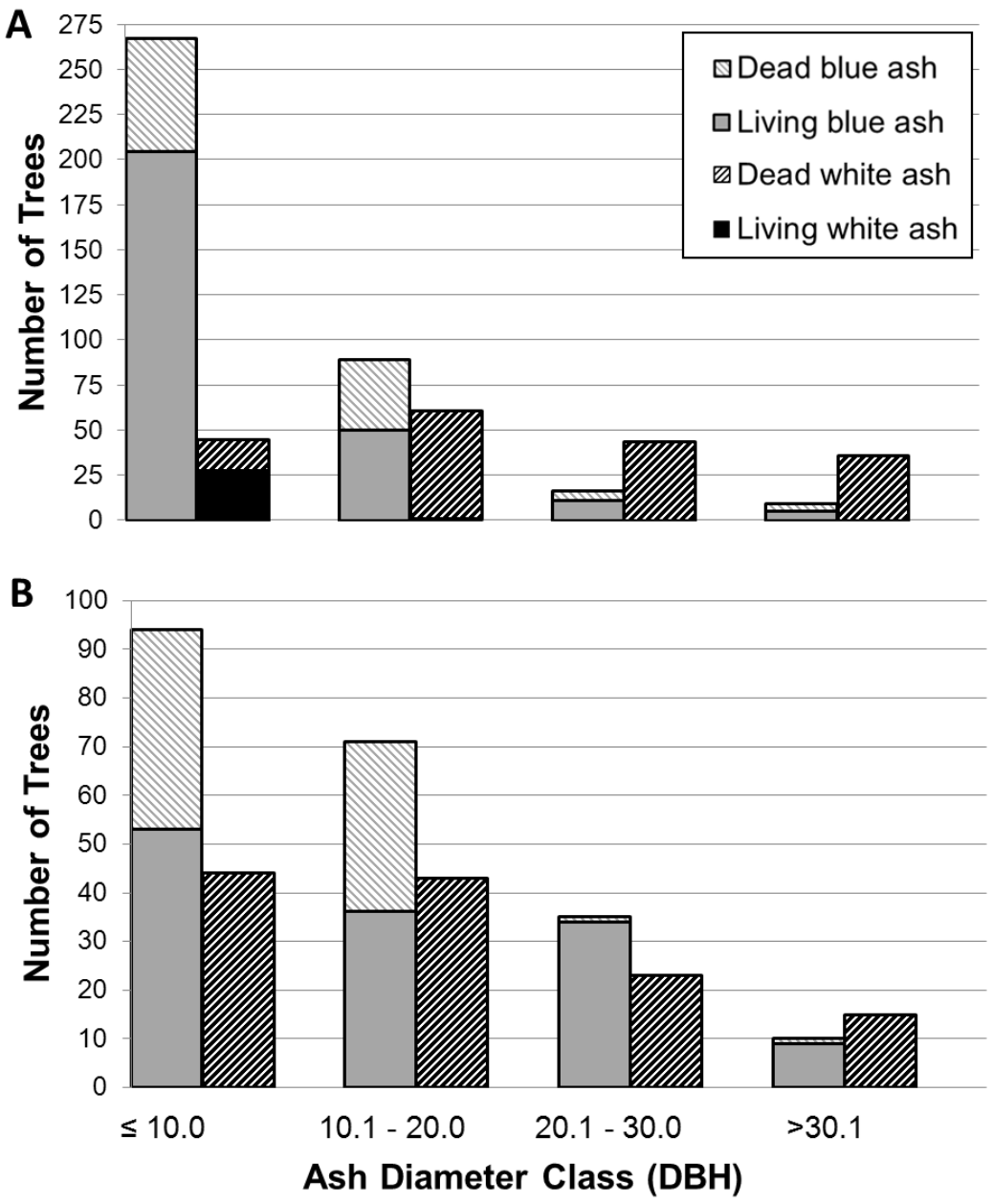


Figure 1.1: Number of live and dead blue ash and white ash trees by diameter class in woodlots in Plymouth (A) and Superior Township (B) Michigan.

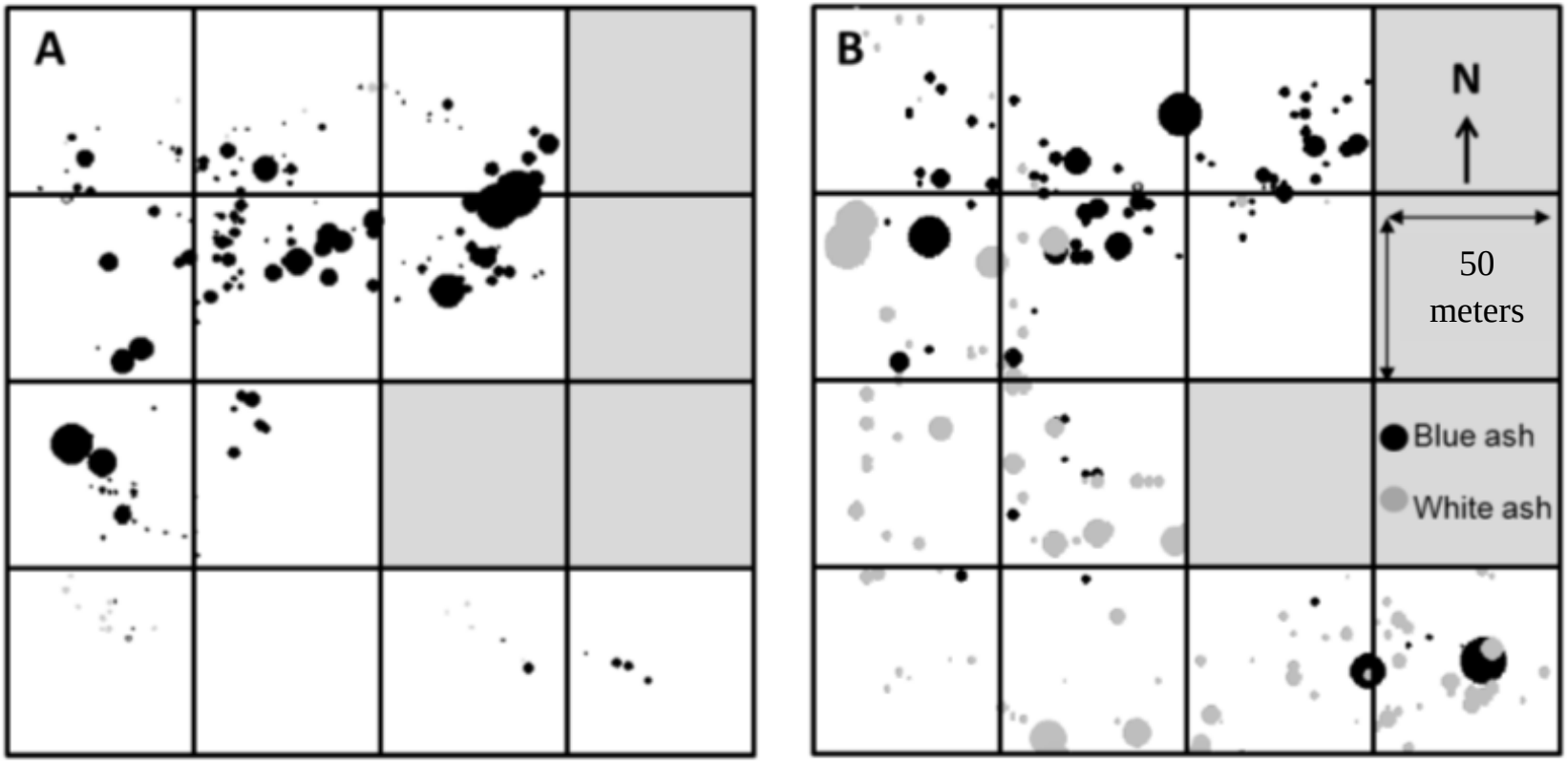


Figure 1.2: Distribution of live (A) and dead (B) blue ash (black points) and white ash (grey points) trees in 50 × 50 m grid cells in a woodlot in Plymouth, Michigan. Point size is proportional to tree DBH (cm). Living blue ash trees ranged from 2.5 - 45.0 cm in DBH. Dead blue ash trees ranged from 2.5 - 50.0 cm in DBH. Living white ash trees ranged from 2.5 - 10.9 cm in DBH. Dead white ash trees ranged from 2.5 - 96.0 cm in DBH. Gray quadrants represent areas where no *Fraxinus* spp. trees were present.

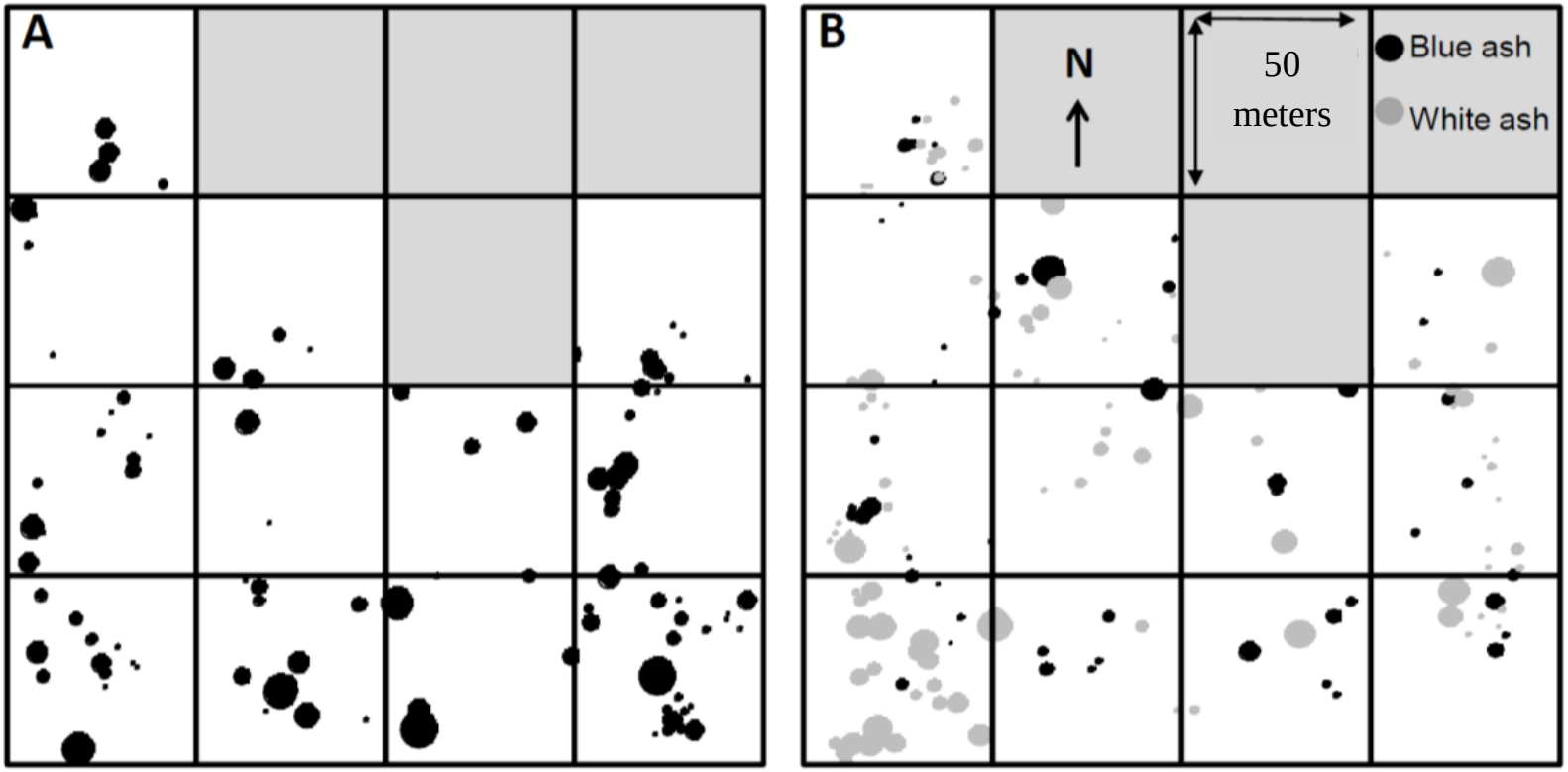


Figure 1.3: Distribution of live (A) and dead (B) blue ash (black points) and white ash (grey points) trees in 50 × 50 m grid cells in a woodlot in Superior Township, Michigan. Point size is proportional to tree DBH (cm). Living blue ash trees ranged from 2.5 - 46.5 cm in DBH. Dead blue ash trees ranged from 2.5 - 31.0 cm in DBH. There were no living white ash trees. Dead white ash trees ranged from 2.5 - 43.5 cm in DBH. Gray quadrants represent areas where no *Fraxinus* spp. trees were present.

CHAPTER TWO

Effects of fertilizer and paclobutrazol on the physiology
and growth of five ash species

ABSTRACT

Ash (*Fraxinus* spp.) trees are widely planted in urban areas across the United States where they experience varying degrees and types of stress. Fertilizer and paclobutrazol (PB), a gibberellin inhibitor, may be applied to enhance tree vigor. Fertilization typically increases leaf area and radial growth but reduces root: shoot ratios, while PB typically reduces leaf area and radial growth but increases root: shoot ratios. In 2010, 105 ash trees in a common garden were assigned to one of three treatments: fertilization (Harrell's Pro blend with Micros, 19-5-10), PB application (Shortstop®, soil drench) or untreated control. Leaves from control or fertilized were similar in size, but they were 19 and 25% larger than leaves from PB trees, respectively. Gas exchange did not vary among species or treatments. Ratios of pre: post treatment radial growth were similar between white ash (*Fraxinus americana*) and Manchurian ash (*F. mandshurica*) trees, but were 39 and 33% higher, respectively, than blue ash (*F. quadrangulata*) tree ratios. Ratios of pre: post treatment radial growth were similar between control and fertilized trees, but were 33 and 43% higher, respectively, than PB tree ratios. Ratios of root: total biomass were 29 and 13% higher in blue ash trees than white ash and Manchurians ash trees, respectively indicating that blue ash trees allocate more growth to their root systems than white ash or Manchurian ash trees. Trees treated with PB had 9 or 10% higher ratios of root: total biomass than control or fertilized trees, respectively. I found PB treatment shifts growth from above to below ground resources, therefore applications could extend the period before trees outgrow available space, enabling trees to persist in confined spaces.

INTRODUCTION

Urban trees are exposed to a variety of environmental stresses. To mitigate stress and enhance vigor, urban trees may be fertilized or treated with paclobutrazol (PB), a growth regulator. Nitrogen (N) fertilizer typically increases shoot growth and leaf production but may reduce root: shoot ratios (Herms 2002, Struve 2002, Scharenbroch and Lloyd 2004, Hasselkus and Schulte 2010). In contrast, PB, a gibberellin inhibitor, typically reduces shoot growth and leaf area but may increase root: shoot ratios (Bai et al 2004, Chaney 2005a,b, Martinez-Trinidad et al. 2011). Both treatments can potentially affect foliar nutrient levels, water potential and gas exchange (Werner 1993, Remphrey and Davidson 1996, Premachandra et al. 1997, Arzani and Roosta 2004, Scharenbroch and Lloyd 2004).

Since the early 20th century, benefits of fertilization for urban trees have been widely disputed (Miller 1998, Herms 2002, Ferrini and Baietto 2006). Fertilizers have been recommended to ensure that urban trees receive adequate nutrients, increase growth and vigor, and maintain or enhance aesthetic qualities (Herms 2002, Scharenbroch and Lloyd 2004, Hasselkus and Schulte 2010). Urban soils may lack nitrogen (N), the most common limiting nutrient for temperate zone trees (Vitousek and Howarth 1991, Aber 1992, Harris 1992), because humans continually alter the N cycle by removing leaf litter and displacing soils during infrastructure development (Scharenbroch and Lloyd 2004). However, many urban substrates contain levels of N and other nutrients adequate to support tree growth (Harris et al. 2008, Watson 2010). In these situations, fertilization is not cost effective and nutrient runoff can be harmful to surrounding environments (Watson 2010). High soil N levels can increase N concentrations in trees which may affect insect performance. High N concentrations in plant tissues (leaves, phloem, etc.) may make them more attractive to insect pests (e.g., aphids) (Kytö 1996). To determine if fertilization is necessary and reduce the potential negative effects of

over-fertilization, ideally, tree care professionals should develop species and site specific fertilizer prescriptions based on tree species, time of year, climate, current tree nutrient status and site conditions (Scharenbroch and Lloyd 2004, Ferrini and Baietto 2006, Harris et al. 2008).

Utility companies began using PB in the late 1970s (Breedlove et al. 1989, Davis and Curry 1991, Redding et al. 1994) to reduce aboveground tree growth and the pruning necessary to ensure lines remained clear of entangling branches (Chaney 2005b). Paclobutrazol has more recently been adopted by the landscape industry to similarly reduce pruning and maintenance costs, particularly for trees growing in confined spaces (e.g., next to buildings) (Chaney 2005a). Application of PB can affect a variety of tree processes, including foliar nutrition (Rieger and Scalabrelli 1990, Yelenosky et al. 1995, Huett et al. 1997, Navarro et al. 2009), gas exchange and water use efficiency (Fletcher and Gilley 2000). In addition, PB reportedly affects tree response to salt and drought stress (Wang and Steffens 1987, Abu El-Kashab et al. 1997, Chaney 2005a,b).

In the Midwestern United States, ash trees often comprise 20-50% of the urban canopy (Schoon 1993, MacFarlane and Meyer 2003, Percival et al. 2006, Poland and McCullough 2006). Abundance of ash in landscapes reflects both the desirable traits of the genus and the previous invasion by Dutch elm disease. In the late 20th century, ash trees became exceedingly popular as replacements for American elm (*Ulmus americana* L.) trees killed by Dutch elm disease (MacFarlane and Meyer 2005, Poland and McCullough 2006). Ash tolerate compacted soils, drought and soil salinity (Dobson 1991, MacFarlane and Meyer 2005, Percival et al. 2006) and perform well in challenging urban locations (e.g., along streets and in parking lot islands and sidewalk cubes) (Schoon 1993, MacFarlane and Meyer 2005, Percival et al. 2006, Poland and McCullough 2006). Ash trees are also easy to transplant and require little maintenance (e.g.,

pruning, irrigation or fertilization) (Dirr 1998). Until the arrival of emerald ash borer (EAB) (*Agrilus planipennis* Fairmaire) in the early 1990s, ash was also popular because it was threatened by relatively few insect pests and is one of few genera not preferred by gypsy moth (*Lymantria dispar* L.) (Liebold et al. 1995).

Several North American ash species are planted in urban areas. Green ash (*Fraxinus pennsylvanica* Marsh.), which has the widest natural distribution of North American species, is also the most prevalent ash in urban landscapes (MacFarlane and Meyer 2003). Cultivars of white ash (*Fraxinus americana* L.), the second most common species (MacFarlane and Meyer 2003), are marketed for their distinct purple fall color and ability to bear snow and ice (uky.edu 2012). Black ash (*Fraxinus nigra* Marsh.) and blue ash (*Fraxinus quadrangulata* Michx.) are also planted in some areas of the United States. In extreme northern climates or sites with poor drainage, black ash and 'Northern Treasure', a hybrid of black ash and Manchurian ash, perform well. Cultivars of blue ash grow slowly, tolerate a variety of soils and are considered relatively drought tolerant (Dirr 1998). Exotic ash species are also used in urban landscapes, including Manchurian ash (*Fraxinus mandshurica* Rupr.) which is native to Asia and Excelsior ash (*Fraxinus excelsior* L.) which is native to Europe. Identification of EAB in 2002 dramatically altered the future of urban ash trees in North America.

Native to Asia, EAB has killed tens of millions of ash trees in 19 states and two Canadian provinces (eab.info Accessed 25 April 2013) and is the most destructive forest insect to invade North America (Aukema et al. 2011, Kovacs et al. 2011). In Asia, *A. planipennis* functions as a secondary pest, typically attacking stressed ash trees (Lyons et al. 2009). In North America, it can colonize both healthy and stressed ash trees (Poland and McCullough 2006), but will preferentially attack stressed trees (McCullough et al. 2009a, 2009b). Interest in ash tree

response to EAB and cultural amendments designed to ameliorate stress related to infestation has subsequently stimulated research on ash tree physiology.

Several studies have assessed effects of fertilization on ash trees both in urban and forest settings, but response is variable. Fertilization did not increase leaf area or stem growth of established urban green ash trees in one study (Watson 2010), while Remphrey and Davidson (1996) found it increased the height of containerized green ash trees at one site but decreased growth at a second site. Fertilization of newly established *F. excelsior* did not affect shoot growth but did increase net photosynthesis and foliar N concentration (Ferrini and Baietto 2006).

Only a few studies have examined effects of PB on ash and results vary, depending on the ash species or growth parameters assessed. Watson (2004) reported that PB application reduced twig growth and leaf area of established green ash trees but on white ash, PB had no effect on lateral shoot growth (Sterrett and Tworkoski 1987, Bai et al. 2004) or suppressed only the central leader (Bai et al. 2004).

Our objective was to evaluate effects of fertilization or PB applications on North American black ash, blue ash, green ash and white ash and Asian Manchurian ash growing in a common location and under otherwise similar conditions. These species were selected because they are planted in urban landscapes, are evolutionarily diverse (Wallender 2008) and have varying levels of inherent resistance to EAB (Cappaert et al. 2005, Anulewicz et al. 2007, Rebek et al. 2008, Pureswaran and Poland 2009, Chen and Poland 2010, Limback 2010, Tanis and McCullough 2012). I measured a range of growth, gas exchange, nutrient concentration and biomass allocation variables to determine if species respond consistently or differently to either fertilizer or PB application.

METHODS

Ash Plantation

On 26 April 2006, 225 ash trees were planted in a randomized complete block design at the Michigan State University Tree Research Center (TRC) in Okemos, Ingham County, Michigan. The plantation consisted of 45 trees each of five species: black ash, blue ash, green ash, white ash and Manchurian ash. Trees arrived from nurseries as bare root liners (black and Manchurian) or balled and burlapped (blue, green and white). Trees were planted 2.4 m (8 feet) apart. Tree size varied among species at the time of planting: caliper diameter (15 cm aboveground) of green ash trees ranged from 6.5 – 4.0 cm (mean (±SE) caliper =6.27 ± 0.06 cm) and white ash trees ranged from 6.0 to 4.1 cm (mean caliper = 5.06 ± 0.06) (Poplar Farms Nursery and Equipment, Waterman, IL, USA), 'Fall Gold' black ash trees ranged from 5.2 to 3.2 cm (mean caliper =4.29 ± 0.08 cm) (Bailey Nurseries, St. Paul, MN, USA), blue ash trees ranged from 6.0 to 3.0 cm (mean caliper =4.62 ± 0.11 cm) (Wirkus Nurseries, Clinton, WI, USA), and 'Mancana' Manchurian ash trees ranged from 2.8 to 2.0 cm (mean caliper =5.06 ± 0.06 cm) (Bailey Nurseries, St. Paul, MN, USA). Sixteen blue ash trees died during the 2008-09 winter and were replaced in May 2009 with 5 cm caliper bare root trees (Connon Nursery, Waterdown, ON, Canada). All trees were shipped from nurseries outside of the known EAB host range and were uninfested when they were planted. Appropriate permits were acquired from state and federal regulatory agencies prior to shipment and planting. Trees were watered twice per week or as needed with drip irrigation.

Trees were fertilized annually from 2006-2009 with a top dressing of Harrel's Pro-Blend with Micros (19-5-10) (Harrell's, Lakeland, FL, USA) at a rate of 70 g (5.5 ounces N) of product per tree. In June 2006 and 2007, trees were protected from EAB with a cover spray of Tempo

SC Ultra® (cyfluthrin, 11.8%, Bayer Environmental Science, Research Triangle Park, NC, USA). In April 2008 and 2009, trees were wrapped with plastic tree wrap (Jobe's TreeWrap Pro, Easy Gardener, Inc., Waco, Texas, USA) overlaid with screen mesh to deter EAB infestation.

Treatments

On 26 May 2010, seven trees of each species (35 trees per treatment) were randomly assigned to one of three treatments: fertilization, PB application or untreated control. Fertilized trees received 350 g of Harrell's Pro-Blend with Micros (19-5-10) (Harrel's, Lakeland, FL, USA) distributed evenly around the base of each tree. ShortStop® (Paclobutrazol 22.3%, Zhejiang Tide Crop Science Co., LTD, Irvine, CA, USA) was applied as a soil drench. Diluted product was applied evenly according to labeled instructions (200 ml diluted product, 4 g a.i., per 2.54 cm DBH) in a shallow trench, approximately 12 cm deep, around the base of each tree. All tree boles and branches larger than 4 cm diameter were again wrapped from May to September with tree wrap overlaid with wire mesh to minimize EAB infestation. On 23 May 2011, trees fertilized in 2010 received a second application. Paclobutrazol, which typically affects tree growth the year after application, was not reapplied in 2011. Tree wrap was not applied in 2011.

Variables Measured

Photosynthesis and transpiration rates of all trees were measured on a mature, sun-exposed leaf using a Li-Cor 6400 portable photosynthesis meter (Li-Cor, Lincoln, Nebraska, USA) on 8 June and 18 August 2010 and 3 June and 9 August 2011 between 11:00 and 16:00. To reduce daily variation in light exposure, the leaf chamber was illuminated with an LED light source set to provide 1,500 µmoles quantum flux $m^{-1}s^{-1}$. Leaf chamber block temperature (°C) was set to correspond to the highest temperature predicted for the day to ensure consistent

temperature across daily measurements. Measurements were recorded when the real time graphical displays indicated photosynthesis and transpiration rates had stabilized.

Relative chlorophyll content was assessed on four sun-exposed lower-canopy leaves from each tree at each of cardinal direction on 8 June and 18 August 2010 and 3 June, 7 July and 7 August 2011 using a handheld Minolta SPAD-502 meter (Spectrum Technologies, Plainfield, IL, USA). Relative chlorophyll content for each tree was determined by averaging the four readings.

To assess individual leaf area and foliar nutrients, four mid-canopy, sun-exposed leaves were removed from each of the cardinal directions on 7 July 2010 and 13 July 2011. Four randomly selected leaflets were removed from petioles and pooled by tree. Individual leaf area was assessed using a flatbed scanner ((Epson Perfection® 4490 Photo, Epson America, Inc., Longbeach, CA) and WinFOLIA® software (Regent Instruments, Quebec, Canada). Individual leaf area was then averaged by tree for each sample date. To assess foliar nutrients, pooled leaves from each tree were oven dried (75°C) for 72 hours, ground and sent to Water's Agriculture Laboratory (Camilla, GA, USA) for foliar macro-nutrient (N, P, K and Ca) and micro-nutrient (Mg, S, B, Zn, Mn, Fe and Cu) analysis.

Biomass Allocation

By August 2011, every black ash and 30 of the 35 green ash trees had been killed by EAB so these species were excluded from evaluations of biomass allocation. Blue ash, white ash and Manchurian ash trees were all still alive in 2011 and were destructively harvested from September to October 2011. Trees were felled 10 cm above graft unions on grafted trees (white ash and Manchurian ash) or approximately 15 cm above the first buttress root on blue ash trees. Leaves and branches were removed from each tree, bagged separately and stored until they could be oven dried.

On 19 and 20 October 2011, a skid steer mounted with a hydraulic tree spade was used to extract the root ball and a consistent quantity of soil surrounding each tree (1.1 m^3). Roots of one fertilized white ash tree could not be removed because the tree spade could not penetrate the soil to the required depth. Between 26 and 28 October 2011, soil was removed from extracted roots by first penetrating the smooth soil surface with a pitchfork, then using a compressor-driven air spade (Air-Spade®, Guardair Corporation, Chicopee, MA, USA). Remaining soil particles were subsequently removed with a garden hose. While air-spades do not typically detach fine roots (Nadezhdina and Čermák 2003), I carefully sifted soil removed by the air-spade to recover roots that might have been disconnected. Intact root masses were stored outdoors between plastic tarps until they could be processed individually into coarse (> 2 mm) or fine roots (≤ 2 mm) using hand pruners. All leaves, twigs, dissected roots, tree boles and root balls were oven dried to constant weight and weighed.

I predicted root, leaf and woody biomass would vary among ash species and treatments. However, I recognize inter- and intra-specific differences in initial, pre-treatment, tree size could skew biomass allocation results. To account for differences in initial tree size among species, I calculated ratios of biomass components (leaves, roots, woody above-ground) to total biomass (the sum of all components), in addition to actual biomass for each tree.

Growth

During harvest, annual growth of the terminal shoot was recorded on the four lowest, living branches and averaged by tree. Leaf scars were used to determine annual shoot growth for two years before and two years after treatment.

Cross sections (1 cm thick) from the base of each tree were cut 10 to 15 cm above ground, oven dried, lightly sanded, sealed with polyurethane (to visually clarify tree rings) and

scanned using a flatbed scanner. Tree ring increments were measured with tree ring analysis programs (Coo-Recorder® and CDendro®, Cybis Elektronik and Data, Saltsjöbaden, Sweden). The four most recent years of radial growth were measured at the top, bottom, right and left of each cross section (16 measurements per tree) and averaged by year. I calculated ratios of pre-treatment (2008 and 2009): post treatment (2010 and 2011) radial growth to standardize measurements.

Statistical Analysis:

Data were analyzed using SAS statistical software (SAS Institute, Inc. 1989). Variables measured in 2010 are presented for all five ash species, while data from 2011 are presented for blue ash, white ash and Manchurian ash trees only because of nearly complete mortality of black ash and green ash trees. Assumptions of normality were tested with residual plots and the Shapiro-Wilk test (Shapiro and Wilk 1965). Leaf area was normalized using a log transformation and foliar nutrient concentrations (N, P, K, Ca, Mg, S, B, Zn, Mn, B, Fe and Cu) were normalized using log (x+2) transformations (Ott and Longnecker 2001). Two-way ANOVA was performed to assess effects of ash species, treatment and interactions between the two factors on shoot and radial growth, leaf area, biomass, relative chlorophyll content, foliar nutrient concentrations and gas exchange rates. Relative chlorophyll content and gas exchange rates were analyzed using repeated measures ANOVA. When ANOVA results were significant ($\alpha \leq 0.05$), Fisher's protected least significant difference (LSD) multi-comparison test with Tukey's adjustment was applied to shoot and radial growth, leaf area, gas exchange and nutrient data sets (Ott and Longnecker 2001).

In 2011, simple linear regression was used to determine if tree caliper was a significant predictor of harvested root biomass. To account for differences in initial tree size, ratios were

calculated for aboveground, leaf, root and total biomass. Biomass allocation data (root: total biomass, aboveground woody: total biomass and canopy: total biomass ratios) were natural log (ln) transformed before computing ratios, standard errors (SE), and ANOVA. Root: total biomass ratios were calculated by dividing total root biomass (fine + coarse root weights) by total biomass (canopy weight + twig weight + bole weight + coarse root weight + fine root weight). Aboveground woody: total biomass ratios were calculated by dividing aboveground woody biomass (twigs + boles) by total biomass. Canopy: total biomass ratios were calculated by dividing leaf weight by total biomass.

Vector analysis of PB or fertilizer effects on total biomass nutrient concentration was performed using methods developed by Haase and Rose (1995). For this analysis, total dry weight (roots, leaves and aboveground woody biomass), nutrient concentration (percentage of each foliar element) and nutrient content (percentage nutrient concentration as a function of tree dry weight) are considered relative values that have been divided by corresponding values from control trees (the mean of control tree variables within species) and multiplied by 100. For example, foliar N levels of blue ash trees treated with PB or fertilizer were compared to the mean foliar N content of blue ash control trees. I then replaced control values with a normalized control always equal to 100, enabling comparisons to be made to a common base. Relative nutrient concentration is thus graphically represented as a function of relative total tree dry weight and relative foliar nutrient content. For presentation purposes, I refer to relative dry weight as "biomass". Relative biomass, nutrient content and nutrient concentration were analyzed using 2-way ANOVA. Where significant for treatment or the species × treatment interaction ($\alpha = 0.05$), Dunnet's adjustment was used in LS means tests to determine significant

differences between treated ash species and the normalized control. If ANOVA results were significant for species only, vector analysis was not performed.

RESULTS

Growth

From 2008-2010, mean (±SE) shoot growth (cm) was different among species (Table 2.1). In 2008 and 2009, shoot growth of blue ash was more than 50% lower than that of white ash and in 2010, blue ash shoots grew 89% less than white ash shoots grew (Table 2.2). In 2008, mean (±SE) shoot growth of blue ash was similar to that of Manchurian ash, but in 2009 and 2010, blue ash shoot growth was 86 and 53% less than white ash and Manchurian ash shoot growth, respectively. In 2008, Manchurian ash shoots grew 77% less than white ash, but in 2009 and 2010, shoot growth was similar between the two species (Table 2.2).

In 2011, two growing seasons after PB treatment, shoot growth and ratios of pre-: post-treatment shoot growth were affected by species, treatment and the interaction (Table 2.2). Manchurian ash trees treated with PB had 74 and 70% less shoot growth than fertilized or control Manchurian ash trees, respectively. In addition, ratios of pre-: post-treatment shoot growth of Manchurian ash trees were 70 and 64% higher for control and fertilized trees, respectively than Manchurian ash PB trees. Blue ash and white ash shoot growth and ratios of pre-: post-treatment shoot growth were unaffected by treatments (Table 2.2).

Interspecific differences in radial growth were apparent during each sample year (2008-2011). In 2008, radial growth of blue ash and white ash trees were similar and both were lower than Manchurian ash (Table 2.4). From 2009-2011, blue ash tree radial growth was at least 50% lower than white ash and Manchurian ash, which were similar (Table 2.4).

Radial growth was unaffected by treatments until 2011 (Table 2.3). Fertilized ash trees had 40% and 20% more radial growth than PB and control trees, respectively (Table 2.4). Ratios of pre: post treatment radial growth were affected by species and treatment but not the

interaction (Table 2.3). Ratios of pre: post treatment radial growth were 39 and 33% higher in white ash and Manchurian ash trees, respectively, than in blue ash but were similar to each other (Table 2.4). Ratios of pre: post treatment radial growth in control and fertilized trees were similar but were 33 and 43% higher than PB trees (Table 2.4).

In 2010, individual leaf area varied among species (F=14.4; df=4,83; $P<0.001$) but treatment ($P=0.16$) and the interaction ($P=0.20$) were not significant. Area of individual white ash leaves averaged 29.2 ± 2.34 cm^2 and were at least 41% larger ($P<0.001$ for all species) than black ash, blue ash and Manchurian ash leaves which averaged 17.2 ± 2.23, 17.2 ± 1.25 and 17.3 ± 0.9 cm^2 respectively. Green ash leaves averaged 12.8 ± 0.97 cm^2 and were 44% smaller ($P<0.001$) than white ash leaves and 26% smaller than leaves on black ash ($P=0.046$), blue ash ($P=0.038$) and Manchurian ash leaves ($P=0.009$).

In 2011, individual leaf area varied among species (F=6.87; df=2, 42; $P=0.003$) and treatments (F=3.10; df=2,42; $P=0.043$) but the interaction was not significant (P=0.31). Area of white ash leaves averaged 29.1 ± 2.57 cm^2 and which was 34% larger ($P<0.001$) than blue ash (19.2 ± 1.10 cm^2) and 23% larger ($P=0.023$) than Manchurian ash leaves (22.6 ± 1.36 cm^2). Blue ash and Manchurian ash leaf area were similar ($P=0.17$). Leaves from trees treated with PB had an average leaf area of 19.8 ± 1.96 cm^2 which was 19% smaller ($P=0.010$) than leaves from control trees (24.5 ± 1.61 cm^2) and 25% smaller ($P=0.003$) than leaves from fertilized trees (26.3 ± 2.32 cm^2). The leaf area of leaves from fertilized and control trees were similar ($P=0.73$).

Biomass Allocation

Root Biomass

Total root biomass was affected by species, but not treatment or the interaction (Table 2.5). Ratios of root: total biomass were affected by species and treatments but not the interaction (Table 2.5). Blue ash trees had the lowest average root biomass (3.98 ± 0.40 kg) but had 29 and 13% higher average ratios of root: total biomass than white ash (average biomass = 8.65 ± 1.46 kg) or Manchurian ash (average biomass = 4.95 ± 0.35 kg) trees, respectively (Fig. 2.1A), indicating blue ash trees allocated more carbon to root systems compared to white ash or Manchurian ash trees. Root biomass extracted was related (R^2=0.432) to tree caliper (Y = -1448.15 + 47.95x) ($F_{[1,46]}$ = 35.03; P < 0.001) (Fig. 2.2).

Blue ash trees had a higher percentage of fine roots than white ash or Manchurian ash trees. Fine root biomass averaged 0.75 ± 0.09 kg per tree for blue ash, 0.64 ± 0.09 kg per tree for white ash and 0.46 ± 0.03 kg per tree for Manchurian ash trees and while the raw data was not variable, ratios of fine: coarse roots varied among species (Table 2.5). The ratio of fine: coarse roots of blue ash averaged 13.2% ± 0.02 which was higher (P <0.001) than the ratios for white ash (5.3% ± 0.01) and Manchurian ash (3.6% ± 0.03) (Table 2.6).

Overall, trees treated with PB had the greatest root biomass (6.13 ± 1.23 kg) and the ratio of root: total biomass was almost 10% higher (P<0.001) than fertilized or control trees (Fig. 2.1B), which averaged 5.92 ± 0.80 kg and 4.67 ± 0.52 kg of root biomass, respectively. The ratio of root: total biomass of fertilized trees was similar (P=0.17) to control trees (Fig 2.1B).

Leaf Biomass

Foliar biomass of blue ash trees averaged 0.87 ± 0.21 kg which was 73% and 59% less than white ash (3.28 ± 0.30 kg) and Manchurian ash (2.11 ± 0.24 kg), respectively (Table 2.6).

Leaf weight varied among species, while ratios of leaf: total biomass were variable among species and treatments (Table 2.5). Blue ash ratio of leaf: total biomass was 6% smaller (P<0.001) than white ash or Manchurian ash. Ratios of leaf: total biomass of white ash and Manchurian ash trees were similar (P=0.88) (Fig. 2.1A).

While the PB trees had a smaller (P=0.010) ratio of leaf: total biomass than fertilized trees, it was only a 2% difference. Ratio of leaf: total biomass for control trees was not different from PB (P=0.13) or fertilized trees (P=0.31) (Fig. 2.1B).

Aboveground Woody Biomass

Given that radial growth was reduced by PB treatment, it is not surprising that trees treated with PB had lower ratios of aboveground woody: total biomass. Aboveground woody biomass was affected by species and the species × treatment interaction and ratios of aboveground woody: total biomass were affected by species and treatments (Table 2.5). White ash trees had the most aboveground woody biomass (14.57 ± 1.11 kg), blue ash trees had the least (3.86 ± 0.73 g), while Manchurian ash trees were intermediate (6.58 ± 0.58 g) (Table 2.6). Consequently, ratios of aboveground woody: total biomass of white ash trees were 13 and 7% higher (P=0.001, P=0.050) than blue ash and Manchurian ash trees, respectively. Ratios of aboveground woody: total biomass for blue ash and Manchurian ash trees were similar (P=0.14) (Fig. 2.1A). Among treatments, ratios of aboveground woody: total biomass of PB trees were 7% lower (P=0.009) than control trees and 10% lower (P =0.050) than fertilized trees. Ratios for fertilized and control trees were similar (P=0.35) (Fig. 2.1B).

Gas Exchange

In 2010, photosynthesis rates were affected by species, the species × treatment and species × date interactions (Table 2.7). Transpiration rates were affected by species, treatment, date, and the species × treatment and species × date interactions (Table 2.7). There were few effects of treatment within species in 2010 (Table 2.8). In June, fertilized and PB blue ash trees had higher photosynthesis rates than blue ash control trees. In August, fertilized and control green ash trees had higher photosynthesis and transpiration rates than green ash PB trees (Table 2.8). However, it is important to note that the majority of PB (71%) green ash and fertilized (71%) and PB (57%) black ash trees failed to leaf out in 2011as a result of EAB infestation. Poor tree health as a result of infestation likely accounts for the low gas exchange rates exhibited by black ash and green ash trees in these treatments in August 2010 (Table 2.8). In 2011, gas exchange rates increased over time but within species, there were no treatment effects (Table 2.8).

Relative Chlorophyll Content

Treatment effects on relative chlorophyll content were variable but generally, chlorophyll content increased between June and August in both growing seasons (Table 2.8). In 2010, chlorophyll content was affected by species, treatment, date and the species × treatment, species × date and treatment × date interactions (Table 2.7). In June 2010, there were no within species treatment effects. In August 2010, fertilized blue ash trees had higher chlorophyll content than blue ash control and PB trees. White ash control and fertilized trees had higher chlorophyll content than white ash PB trees (Table 2.8). There were no differences among treatments for Manchurian ash trees,

In 2011, with the exception of blue ash, treatment effects on chlorophyll content were inconsistent. Mean (±SE) chlorophyll content was affected by species, treatment, date, species × treatment, species × date and treatment × date interactions (Table 2.7). In June, fertilized blue ash trees had higher chlorophyll content than blue ash control and PB trees, which did not differ from each other. No other within-species treatment effects were apparent (Table 2.8). In July, chlorophyll content of fertilized blue ash and white ash trees were higher than blue ash and white ash control and PB trees and Manchurian ash control trees had lower chlorophyll content than PB and fertilized Manchurian ash trees. By August, fertilized blue ash trees still had higher chlorophyll content than blue ash control or PB trees, but no treatment effects persisted among white ash or Manchurian ash trees (Table 2.8).

Nutrient Content

In 2010, foliar concentrations of N, P, K, Mg and Ca in foliage differed among ash species (Table 2.9) and treatment affected N and P concentrations (Table 2.9). Micronutrients, S, Zn, Mn, Fe, B and Cu were not affected by species or treatments. Foliar N concentration was lower in blue ash (1.94% ± 0.086) than all other species (Table 2.10). Blue ash also had lower foliar P concentrations than black, green and white ash and lower foliar K concentrations than white and Manchurian ash (Table 2.10). In contrast, Mg concentration in blue ash foliage averaged 0.65 % ± 0.061, which was two to three times higher than in any other species. Foliar nutrient concentrations in green ash and black ash were similar among N, P, K, Mg and Ca (Table 2.10). White ash foliage had higher N concentration than black ash, while K concentration was lower in white ash than in black ash. Manchurian ash had higher foliar N, P, and K concentrations than blue ash, but lower Mg and Ca concentrations. Magnesium

concentration was higher in Manchurian ash than in black ash, green ash and white ash (Table 2.10).

In 2010, species and treatment affected N and P concentrations (Table 2.9). Nitrogen concentration of fertilized and control tree averaged 2.59% ± 0.077 and 2.68% ± 0.090, respectively, which was 9 and 5% higher than PB trees which averaged 2.45% ± 0.091. Nitrogen concentration was similar between fertilized and control trees. Phosphorous concentrations averaged 1.43% ± 0.036 which was higher than P concentrations for white ash and Manchurian ash trees which averaged 1.33% ± 0.033 and 1.33% ±0.030, respectively. Concentrations of P were not different between blue ash and Manchurian ash trees.

In 2011, blue ash control and PB trees had lower foliar N concentrations than the other ash trees in the study (Table 2.10). Overall, N concentrations were affected by species, treatment and the interaction (Table 2.9). Nitrogen concentrations were higher in fertilized blue ash trees (2.2% ± 0.08) than in blue ash control (1.7% ± 0.12) or PB (1.7% ± 0.13) trees, which were similar. Among white ash and Manchurian ash trees, there were no differences among treatments (Table 2.10). Phosphorous concentration was affected by species and treatment but not the interaction (Table 2.9). Foliar P concentration of Manchurian ash was 15 and 48% higher than in white ash or blue ash foliage, respectively. Among treatments, P concentration was 29 and 20% higher in control tree foliage (0.21% ± 0.019) than in foliage from fertilized (0.15% ± 0.010) or PB (0.17% ± 0.011) trees, respectively. Potassium, Mg, and Ca were affected by species only (Table 2.9). Potassium concentrations were 31% higher in Manchurian ash trees than in blue ash trees but concentrations in Manchurian ash were similar to white ash (Table 2.10). Blue ash foliage had 64% and 44% higher Mg concentration and 45 and 29% higher Ca

concentration than white ash or Manchurian ash foliage, respectively (Table 2.10). As in 2010, S, Zn, Mn, Fe, B and Cu concentrations were not affected by species or treatments.

In 2011, I took advantage of our total tree harvest and used vector analysis to further assess treatment effects on N and P concentrations. In this analysis, percent relative dry weight (the "Z" axis) is consistent across diagrams because the same trees were used for each nutrient. Relative biomass varied among species ($F=7.0$; df=2, 27; $P=0.006$) and treatments ($F=7.2$; df=2, 27; $P=0.005$) but the interaction was not significant ($P=0.19$). When compared to normalized controls, biomass was lowest (- 41%) for blue ash PB trees and highest (+ 25%) for blue ash fertilized trees (Figs. 2.2 and 2.3). White ash PB and fertilized trees had more biomass than normalized controls, while biomass of fertilized and PB Manchurian ash trees was not different from the normalized control (Figs. 2.3 and 2.4).

Relative N concentration (the "Y" axis) was affected by species ($F=5.15$; df=2, 27; $P=0.013$), treatment ($F=7.92$; df=2, 27; $P=0.009$) and the species × treatment interaction ($F=7.21$; df=2, 27; $P=0.003$). Relative N content (the "X" axis) was affected by treatment ($F=5.70$; df=2, 27; $P=0.024$) and the species × treatment interaction ($F=6.33$; df=2, 27; $P=0.006$) but not species ($P=0.25$). The highest relative N concentration was recorded for fertilized blue ash trees with values that ranged from +15 to +38% (Fig. 2.3). Relative N concentrations of all other trees (PB blue ash and PB and fertilized white ash and Manchurian ash trees) were not different from the normalized control (Fig. 2.3). Relative N content was also variable. Blue ash PB trees had 45% less relative N content and blue ash fertilized trees had 57% more relative N content than normalized controls (Fig. 2.3). The lower relative N content in blue ash PB trees, however, is offset by less biomass. In contrast, fertilized blue ash trees had higher relative N content, relative N concentration and biomass than the normalized control. An increase in all

three parameters indicated trees were responding to N deficiency. Manchurian ash and white ash PB and fertilized trees received sufficient N to maintain relative N concentrations.

Vector analysis indicated PB treatment could result in an antagonistic effect on P concentration, whereas fertilization could result in P dilution because of excess growth. Relative P concentration was affected by species ($F=5.85$; df=2, 27; $P=0.008$) and treatment ($F=5.69$; df=2, 27; $P=0.009$) but not the species × treatment interaction ($P=0.09$). Compared to the normalized control, all blue ash and Manchurian ash trees had insufficient relative P concentrations, -11 and -32%, respectively. Relative P content was variable among species and treatments, ranging from -48.4% for blue ash PB trees to +38.5% for white ash PB trees (Fig. 2.4). Blue ash and Manchurian ash PB trees exhibited a decrease in all three relative parameters, indicating the PB might have an antagonistic effect on relative P content. In other words, trees in our plantation were responding phosphorous deficiency induced by PB treatment. In contrast, fertilized blue ash and Manchurian ash trees experienced an increase in biomass, a decrease in relative P concentration and no change in relative P content. The increase in biomass and decrease in P concentration indicated trees were responding to a dilution effect (Fig. 2.4). White ash trees displayed an increase in biomass and relative P content, but there was no change in relative P concentration, indicating that they had sufficient P.

DISCUSSION

Because ash trees are common in urban landscapes and experience varying degrees and types of environmental stress, understanding effects of treatments designed to enhance tree vigor is critical for arborists and landscape professionals. My results indicate that effects of fertilizer and PB varied among ash species which is not surprising given their evolutionary diversity (Wallander 2008) and tendency to occupy diverse ecological niches (USDA plants.gov, Accessed 14 April 2013). While other studies (Bai et al. 2004, Bai et al. 2005) and mine indicate white ash might be relatively unresponsive to PB, I found blue ash and Manchurian ash were sensitive to PB treatment.

I recognize that our study was somewhat limited by the size of the trees. Several studies have assessed the effects of PB on similarly sized/aged ash trees (Sterrett and Tworkoski 1987, Bai et al. 2004, Watson 2004) and even though I could find no studies that examined PB or similar compounds on ash trees larger than the ones used in my study, I suspect that PB treatment effects would likely carry over to larger trees. Most treatment effects of PB are the result of changes in allocation. Stem and radial growth reduction is a well-documented effect of PB (Sterrett and Tworkoski 1986, Watson 2001, Arzani and Roosta 2004, Bai et al. 2004, Yadav et al. 2005, Haas 2012) and has been recorded for a wide variety of plants ranging from turf grass to citrus (Yelenosky et al. 1995) and landscape trees (Chaney 2005 a, b). Growth is reduced by PB at the cellular level and should presumably carry over to large trees. Treated plants generally have the same number of cells in stems and leaves as control plants, but their cells are shorter. Smaller cells reduce shoot and radial growth, as well as leaf area and thickness (Yelenosky et al., 1995, Bai et al., 2004, Yadav 2005). In addition, several studies have shown that PB affects

growth of larger trees in a variety of genera including *Quercus* spp. (Redding et al. 1994, Watson 1996) and *Acer* spp. (Redding et al. 1994, Burch and Wells 1995).

Effects of fertilization in this study were not as dramatic as I might have expected. There is general consensus that fertilization enhances tree growth (Schulte and Whitcomb 1975, Struve 2002, Wang et al. 2012), but not all landscape trees require fertilization (Struve 2002, Ferrini and Baietto 2006, Harris et al. 2008, Watson 2010, Chorbadjian et al. 2011, Wang et al. 2012). I hypothesized initially that fertilization would increase ash shoot growth, but shoot growth of fertilized ash trees in our plantation was similar to control trees. Radial growth was higher in fertilized trees than in control trees in 2011, but differences were small and probably not biologically relevant. Ratios of pre: post treatment shoot and radial growth did not differ between fertilized or control trees, which is not surprising given all trees used in this study were fertilized from 2006-2009. Similarities between control and fertilized trees indicate that our ash trees, like many urban landscape trees, were growing in an environment that provided adequate nutrients. In hindsight, perhaps I should have included a nutrient deficiency treatment in the study (for example, adding a pine bark amendment to the plantation soil (Gilman 2004)).

Urban trees planted in locations with limited space for growth can become stressed from lack of resources when they overgrow their limited space (Chaney 2005a). In this study, PB treatment reduced shoot growth of Manchurian ash trees by 70% and radial growth of ash trees suggesting PB would likely increase the health of ash trees planted in confined, urban areas. Our study illustrates how species within the same genus can react differently to PB treatment. Manchurian ash was the only species to have reduced shoot growth but the 16 blue ash trees planted in 2009 might have been suffering from transplant shock during that year, which could have influenced pre-treatment shoot growth. Other studies also report that white ash trees are

relatively insensitive to PB treatment (Bai et al. 2004, Bai et al. 2005). In a study that compared flurprimidol (a triazole gibberellin inhibitor) and PB trunk injection on growth of black walnut (*Juglans nigra*) and white ash, researchers found that white ash exhibited reduced growth when treated with flurprimidol but not when treated with PB (Sterrett and Tworkoski 1987).

Fertilization often increases relative chlorophyll content but fertilization effects on gas exchange rates are variable among species (Loh et al. 2002, Klooster et al. 2012). In a study using seedling Manchurian ash, Koike and Sanada (1989) found that photosynthesis rates increased when seedlings were fertilized but Klooster et al. (2012) found that net photosynthesis rates of a variety of pot-in-pot hardwood species were unaffected by fertilization. In our study, fertilization did not affect the photosynthesis rates of white ash or Manchurian ash. Fertilized blue ash had higher photosynthesis rates than control trees, but only on one sample date (August 2010). Fertilization consistently increased relative chlorophyll content in blue ash leaves and fertilized white ash and Manchurian ash leaves compared to control white ash and Manchurian ash leaves in July 2011, but the trend had disappeared by August.

Applications of PB generally increase chlorophyll concentration and reduce leaf area (Sharma et al. 2009) but effects on gas exchange rates are variable (Wieland and Wample 1985, Rieger and Scalabrelli 1990, Fletcher and Gilley 2000, Percival and AlBalushi 2007). In a study using English (*Quercus robur* L.*)* and evergreen oak (*Quercus ilex* L.), Percival and AlBalushi (2007) found that chlorophyll content increased, leaf area decreased and photosynthetic efficiency was 60 to 100% higher in PB trees compared to control trees. In our study, PB reduced leaf area but relative chlorophyll content and gas exchange rates were rarely different between PB and control trees within any species.

Because of expense, time constraints and the equipment necessary for excavation, there are few studies that evaluate whole tree coarse-root or fine-root biomass (Vanninen and Mäkelä 1999, Lavigne and Krasowski 2007 Drexhage and Colin 2001). When extracting root systems, a quantity of the fine root system is typically unaccounted for because roots are severed and left behind in the soil. This can lead to standard errors ≥ 99% (Vanninen and Mäkelä 1999). Some researchers correct for this loss by adjusting root biomass with a defined percentage of root loss (for example, Goff et al. 2001 multiplied all root biomass data by 13%), others extract subsamples of the fine root system and use statistics to extrapolate total root biomass (Yin et al. 1989). I recognize that although I did not harvest 100% of the roots from trees in our plantation, I did standardize our study by excavating a consistent volume of soil for each tree.

Fine root biomass generally decreases on sites with nutritious soils and cultural applications of fertilizer can potentially decrease fine root production (Gower and Vitousek 1989, Vanninen and Mäkelä 1999). In this study, root biomass of fertilized and control trees were similar. I could find no reports in the literature of typical root biomass for ash species, but our results were similar to other studies that indicate fine roots generally comprise 2 to 10% of total tree biomass (blue ash trees ratio of fine roots: total biomass (8.7%), white ash (2.4%) and Manchurian ash (3.4%)) (Vanninen and Mäkelä 1999, Hendrick and Pregitzer 1993, Bolte et al. 2004).

When PB reduces shoot and radial growth, shifts in biomass partitioning often result, but effects vary by genera and species (Watson 1996, 2000, 2004, Fletcher and Gilley 2000, Yadav et al. 2005,). Reductions in leaf biomass have been reported for orchard and landscape trees with concomitant increases in root biomass (George and Nissen 2002, Bai et al. 2004). For a biomass allocation shift toward root production to take place, two things must happen. Growth of shoots

and/or leaves must be reduced and the amount of photosynthate produced by trees must increase or remain steady. This creates an excess of available carbohydrates that can be used for root production. In large transplanted green ash trees, root dry weight and length were not affected by PB treatment (Watson 2004). In contrast, PB increased pin oak (*Quercus palustris* Münchh) fine root density in the upper 5 cm of the soil profile by 64% (Watson 1996). In peach, fine root length was reduced with increasing PB concentrations, but root: shoot ratios increased when trees were treated with ≥ 0.10 mg PB per liter (Reiger and Scalabrelli 1990). While other studies have not shown significant effects of PB on ash tree roots (Sterrett and Tworkoski 1986, Watson 2004), in 2011, one year after application, I found that PB increased root biomass. I found that PB reduced aboveground woody material of ash trees by 10% compared to control trees, while photosynthesis rates remained the same. As a result, ratios of root: total biomass of PB trees increased by 10%. Fertilized trees had larger canopies (+2%) and more aboveground woody biomass (+7%) than PB trees but again, photosynthesis rates were not different among treated trees. The decrease of 9% in aboveground biomass of PB trees was therefore reflected in a 9% increase in root biomass.

I assessed effects of species and treatments on foliar micro- and macro-nutrient concentrations and then used vector analysis to assess how treatments were affecting nutrient status and growth response. Vector analysis has been used in several fertilizer and nutrient studies (Imo and Timmer 1997, Labrecque et al. 1998, Isaac et al. 2007) to pin point "dilution effects, nutrient imbalances, and element interactions" (Haase and Rose 1995). In our study, fertilizer effects on foliar nutrient concentrations were most noticeable in blue ash. In 2011, fertilized blue ash trees had higher relative chlorophyll content and N concentrations than control trees. Nutrient vector analysis further indicated that fertilized blue ash trees were responding to

N deficiency. Blue ash occurs naturally on limestone outcropping (plants.gov 2013, Barnes and Wagner 2003) and despite their ability to tolerate poor sites, our study indicated blue ash growth responded positively to fertilization at our site. For the fertilized white ash and Manchurian ash trees, N was not limiting. Vector analysis further indicated that P was limiting for fertilized blue ash and Manchurian ash trees, which were responding to a dilution effect caused by increased biomass. White ash trees received adequate N and P in our study.

PB effects on nutrient concentrations are known to vary among species and genera. In a variety of citrus cultivars and *Arbutus unedo* L. (an evergreen Ericaceous shrub), PB increased N and Ca and some micro-nutrient concentrations (Yelenosky et al. 1995, Navarro et al. 2009). In some peach cultivars, however, PB increased Ca and Mg, but lowered N and P (Rieger and Scalabrelli 1990, Huett et al. 1997). While PB application did not decrease N concentrations in our ash species it did cause an antagonistic effect (PB treatment was reducing P concentration) on P content in both blue ash and Manchurian ash trees. Phosphorous is a key component of the chemical energy produced in chloroplasts which is used for a variety of processes including growth and synthesis of proteins and nucleic acids (Mengel and Kirkby 1982). In ash trees, P deficiency can result in reduced growth, root conductivity and leaf: total biomass ratios (Anderson et al. 1989). In fruit trees, P deficiency can also cause aborted shoot buds, discolored leaves and early leaf senescence (Mengel and Kirkby 1982). While a reduction in growth as a result of P deficiency would not be considered an adverse effect in PB treated trees, discolored leaves and poor root conductivity could potentially reduce urban ash tree health and aesthetics. Further investigation is necessary to evaluate long term effects of PB on P uptake and implications of P deficiency on ash tree health.

I evaluated a variety of growth, physiological and nutrient effects of fertilizer and PB on an evolutionarily diverse group of ash species. Fertilization of ash trees is common in urban areas. Homeowners and tree care companies apply fertilizers to enhance aesthetics and improve tree vigor. Our study site contained adequate nutrition for white ash and Manchurian ash, but blue ash responded positively to additional nutrients. Paclobutrazol is gaining popularity among arborists and is marketed in EAB infested areas to reduce ash tree stress. It is unfortunate that I was unable to determine the effects of PB on green ash as it is the most common ash species in urban areas of the United States. However, I found PB reduces radial growth and shifts biomass allocation from above to below ground resources of ash trees. Therefore, PB application could extend the period before trees outgrow available space, enabling trees to persist in confined spaces.

Table 2.1: Results of analysis of variance to assess effects of treatment on shoot growth and ratios[1] of pre-: post-treatment shoot growth on blue, white and Manchurian ash trees treated with paclobutrazol, fertilizer or left as untreated control.

	Shoot Growth		
	F Value	**Degrees of Freedom**	**P value**
Effect		***2008***	
Species	35.69	2,44	**<0.001***
Treatment	1.43	2,44	0.252
Species × Treatment	1.93	4,44	0.121
		2009	
Species	8.64	2,44	**<0.001***
Treatment	0.95	2,44	0.400
Species × Treatment	0.56	4,44	0.700
		2010	
Species	40.18	2,44	**<0.001***
Treatment	2.18	2,44	0.125
Species × Treatment	0.95	4,44	0.442
		2011	
Species	30.52	2,44	**<0.001***
Treatment	9.92	2,44	**<0.001***
Species × Treatment	4.93	4,44	**0.002***
		Pre-: Post-treatment Ratio	
Species	14.72	2,44	**<0.001***
Treatment	5.34	2,44	**0.008***
Species × Treatment	2.59	4,44	**0.050***

[1]Pre-: post-treatment ratio = (growth 2008 + growth 2009) / (growth 2010 + growth 2011).

Table 2.2: Average (±SE) shoot length (cm) in 2008-2010 and ratios[1] of pre-: post-treatment growth of blue ash, white ash and Manchurian ash trees treated with fertilizer, paclobutrazol or left as untreated controls (2011). Measurements were taken in October 2011 using annual leaf scars. Within years, different letters indicate a significant ($\alpha \leq 0.05$) difference between species (a,b,c). Among average (±SE) shoot length (cm) in 2011 and pre-: post-treatment ratios, different letters indicate a significant ($\alpha \leq 0.05$) difference between treatments within species (x,y).

	Year		
Ash species	2008	2009	2010
Blue	15.0 ± 2.37^{b}	13.4 ± 2.37^{b}	5.2 ± 1.22^{b}
Manchurian	13.1 ± 1.27^{b}	29.1 ± 3.36^{a}	37.0 ± 4.58^{a}
White	30.6 ± 1.76^{a}	31.1 ± 3.07^{a}	44.4 ± 1.80^{a}

Ash Species	**Treatment**	2011	**Pre: Post Treatment Ratio**
Blue	Control	4.4 ± 1.08^{x}	0.51 ± 0.24^{x}
	Fertilizer	7.9 ± 1.15^{x}	0.43 ± 0.13^{x}
	Paclobutrazol	4.0 ± 0.73^{x}	0.43 ± 0.13^{x}
Manchurian	Control	31.8 ± 4.06^{x}	2.54 ± 0.55^{x}
	Fertilizer	37.0 ± 5.84^{x}	2.15 ± 0.43^{x}
	Paclobutrazol	9.7 ± 2.10^{y}	0.76 ± 0.16^{y}
White	Control	25.7 ± 1.26^{x}	1.57 ± 0.36^{x}
	Fertilizer	25.5 ± 2.59^{x}	1.18 ± 0.09^{x}
	Paclobutrazol	21.1 ± 4.30^{x}	0.99 ± 0.18^{x}

[1]Pre-: post-treatment ratio = (growth 2008 + growth 2009) / (growth 2010 + growth 2011).

Table 2.3: Results of analysis of variance to assess effects of treatment on annual radial growth and ratios[1] of pre-:post-treatment growth of blue ash, white ash and Manchurian ash trees treated with paclobutrazol, fertilizer or left as untreated control.

	Annual Increment Growth		
	F Value	**Degrees of Freedom**	**P value**
Effect		***2008***	
Species	3.35	2,44	**0.044***
Treatment	0.10	2,44	0.905
Species × Treatment	1.05	4,44	0.392
		2009	
Species	16.33	2,44	**<0.001***
Treatment	0.16	2,44	0.853
Species × Treatment	0.27	4,44	0.421
		2010	
Species	25.42	2,44	**<0.001***
Treatment	3.07	2,44	0.057
Species × Treatment	1.21	4,44	0.318
		2011	
Species	30.52	2,44	**<0.001***
Treatment	9.92	2,44	**<0.001***
Species × Treatment	4.93	4,44	**0.002***
		Pre-: Post-treatment Ratio	
Species	**17.11**	2,44	**<0.001***
Treatment	**18.53**	2,44	**<0.001***
Species × Treatment	0.78	4,44	0.542

[1]Pre-: post-treatment ratio = (growth 2008 + growth 2009) / (growth 2010 + growth 2011).

Table 2.4: Mean (±SE) radial growth (mm) from 2008-2011 and ratios[1] of pre-: post-treatment radial growth of blue ash, white ash and Manchurian ash trees treated with fertilizer, paclobutrazol or left as untreated controls. Treatment effects were not significant (P>0.05) in 2008-2010. Within years, different letters indicate significant ($\alpha \leq 0.05$) differences between species (a,b,c) and treatments (x,y).

	Year				
Ash species	*2008*	*2009*	*2010*	*2011*	**Pre: Post Treatment Ratio**
Blue	2.44 ± 0.38^{b}	1.94 ± 0.40^{b}	1.76 ± 0.34^{b}	2.34 ± 0.48^{b}	0.92 ± 0.99^{b}
Manchurian	3.51 ± 0.212^{a}	4.06 ± 0.19^{a}	4.42 ± 0.35^{a}	5.49 ± 0.44^{a}	1.37 ± 0.10^{a}
White	3.11 ± 0.265^{b}	3.58 ± 0.305^{a}	4.57 ± 0.29^{a}	4.86 ± 0.30^{a}	1.51 ± 0.09^{a}
Treatment					
Control	NS	NS	NS	4.01 ± 0.56^{y}	1.29 ± 0.11^{x}
Fertilizer	NS	NS	NS	5.04 ± 0.41^{x}	1.52 ± 0.08^{x}
Paclobutrazol	NS	NS	NS	3.05 ± 0.54^{y}	0.87 ± 0.10^{y}

[1]Pre-: post-treatment ratio = (growth 2008 + growth 2009) / (growth 2010 + growth 2011).

Table 2.5: Results of analysis of variance to assess effects of species and treatment on leaf, aboveground woody, fine root, coarse root, total root and total biomass and leaf, aboveground woody, and ratios of root biomass: total biomass and fine: coarse root of blue ash, white ash and Manchurian ash trees treated with paclobutrazol, fertilizer or left as untreated control. Trees were harvested in October 2011.

Effect	F Value	Degrees of Freedom	*P* Value
Leaves			
Species	25.54	2, 41	**<0.001***
Treatment	1.15	2, 41	0.329
Species × Treatment	1.30	4, 41	0.285
Aboveground Woody			
Species	32.77	2, 41	**<0.001***
Treatment	0.98	2, 41	0.385
Species × Treatment	2.75	4, 41	**0.040***
Fine Roots			
Species	1.91	2, 41	0.162
Treatment	1.15	2, 41	0.326
Species × Treatment	1.30	4, 41	0.287
Coarse Roots			
Species	11.92	2, 41	**<0.001***
Treatment	1.78	2, 41	0.182
Species × Treatment	2.01	4, 41	0.112
Total Roots			
Species	10.54	2, 41	**<0.001***
Treatment	1.64	2, 41	0.207
Species × Treatment	2.11	4, 41	0.097
Total Biomass			
Species	38.18	2, 41	**<0.001***
Treatment	1.06	2, 41	0.358
Species × Treatment	2.71	4, 41	**0.043***

	F Value	Degrees of Freedom	*P* Value
Leaf: Total Biomass			
	25.94	2, 41	**<0.001***
	3.68	2, 41	**0.034***
	1.95	4, 41	0.110
Aboveground Woody: Total Biomass			
	4.77	2, 41	**0.014***
	3.42	2, 41	**0.042***
	1.02	4, 41	0.569
Total Roots: Total Biomass			
	5.64	2, 41	**0.007***
	17.46	2, 41	**<0.001***
	0.88	4, 41	0.480
Fine Roots: Coarse Roots			
	15.72	2,41	**<0.001***
	0.97	2, 41	0.430
	1.04	4, 41	0.350

Table 2.6: Mean (±SE) biomass (kg) of leaves, total roots, fine roots, coarse roots aboveground woody and total biomass of blue ash, white ash and Manchurian ash trees treated with fertilizer, paclobutrazol or left as untreated controls. Biomass was harvested in October 2011. Among plant parts, different letters indicate a significant ($\alpha \leq 0.05$) difference between species (leaves, total roots, fine roots and coarse roots) (a,b,c) or within species and treatments (aboveground woody and total biomass) (x,y). Treatment effects on leaves, total roots, fine roots and coarse roots were not significant (ns).

	Biomass Allocation (kg)			
Ash Species	*Leaves*	*Fine Roots*	*Coarse Roots*	*Total Roots*
Blue	0.87 ± 0.21^{c}	0.75 ± 0.09^{a}	3.23 ± 0.41^{b}	3.983 ± 0.40^{b}
White	3.28 ± 0.28^{a}	0.64 ± 0.09^{a}	4.54 ± 0.36^{b}	8.65 ± 1.46^{b}
Manchurian	2.11 ± 0.23^{b}	0.46 ± 0.03^{a}	7.51 ± 1.13^{a}	4.95 ± 0.35^{b}
Treatment				
Control	1.96 ± 0.32^{ns}	0.58 ± 0.09^{ns}	4.09 ± 0.54^{ns}	4.67 ± 0.52^{ns}
Fertilizer	2.21 ± 0.32^{ns}	0.69 ± 0.08^{ns}	5.23 ± 0.78^{ns}	5.92 ± 0.80^{ns}
Paclobutrazol	1.60 ± 0.36^{ns}	0.57 ± 0.07^{ns}	5.12 ± 0.88^{ns}	6.13 ± 1.23^{ns}

Ash Species	**Treatment**	*Aboveground Woody Biomass*	*Total Biomass*
Blue	Control	4.21 ± 1.78^{x}	9.22 ± 3.33^{x}
	Fertilizer	5.18 ± 1.26^{x}	11.27 ± 2.02^{x}
	Paclobutrazol	2.03 ± 0.25^{y}	5.32 ± 0.47^{x}
White	Control	9.90 ± 3.24^{y}	20.23 ± 2.63^{x}
	Fertilizer	14.61 ± 1.68^{x}	26.75 ± 4.51^{x}
	Paclobutrazol	16.74 ± 0.91^{x}	31.67 ± 3.38^{x}
Manchurian	Control	7.29 ± 1.02^{x}	14.09 ± 1.75^{x}
	Fertilizer	6.60 ± 1.13^{x}	13.78 ± 1.27^{xy}
	Paclobutrazol	5.55 ± 0.73^{x}	12.84 ± 1.42^{y}

Table 2.7: Results of analysis of variance of effects of species, treatment and date on photosynthesis and transpiration rates and relative chlorophyll content of black, blue, green, white and Manchurian ash trees (2010) or blue ash, white ash and Manchurian ash trees (2011) treated with paclobutrazol, fertilizer or left as untreated control. Trees were sampled in June and August 2010 and 2011.

		2010			2011		
			Degrees of Freedom			Degrees of Freedom	
	Effect	F Value	(num, den)	*P* Value	F Value	(num, den)	*P* Value
Photosynthesis	Species	8.28	4,124	**<0.001***	26.93	2, 106	**<0.001***
	Treatment	0.24	2, 124	0.384	0.53	2, 106	**0.588**
	Species × Treatment	2.95	8,124	**0.005***	0.55	4, 106	0.702
	Date	2.89	1, 124	0.099	25.44	1, 106	**<0.001***
	Species × Date	4.83	4, 124	**0.001***	3.05	2, 106	**0.051**
	Treatment × Date	0.70	2,124	0.711	2.27	2, 106	0.108
	Species × Treatment × Date	1.13	8, 124	0.708	1.29	4, 106	0.278
Transpiration	Species	12.41	4,124	**<0.001***	23.31	2, 106	**<0.001***
	Treatment	3.67	2, 124	**0.028***	0.91	2, 106	0.407
	Species × Treatment	3.35	8,124	**0.002***	0.25	4, 106	0.911
	Date	10.47	1, 124	**0.002***	121	1, 106	**<0.001***
	Species × Date	4.54	4, 124	**0.002***	10.72	2, 106	**<0.001***
	Treatment × Date	1.06	2,124	0.469	2.56	2, 106	0.082
	Species × Treatment × Date	0.40	8, 124	0.939	0.57	4, 106	0.682
Relative Chlorophyll Content	Species	99.89	4,124	**<0.001***	119.4	2, 106	**<0.001***
	Treatment	7.24	2, 124	**<0.001***	52.61	2, 106	**<0.001***
	Species × Treatment	3.33	8,124	**<0.001***	18.85	4, 106	**<0.001***
	Date	60.82	1, 124	**<0.001***	339.57	1, 106	**<0.001***
	Species × Date	7.24	4, 124	**<0.001***	12.23	2, 106	**<0.001***
	Treatment × Date	4.94	2,124	**0.008**	4.1	2, 106	**0.003***
	Species × Treatment × Date	0.57	8, 124	0.739	0.78	4, 106	0.624

Table 2.8: Mean (±SE) photosynthesis rates (μmol CO^2 m^{-2} s^{-1}), transpiration rates (mmol H_2O m^{-2} s^{-1})[1] and relative chlorophyll content[2] of black ash, blue ash, green ash, white ash and Manchurian ash trees (2010) or blue ash, white ash and Manchurian ash trees (2011) treated with fertilizer, paclobutrazol (PB) or left as untreated controls. Measurements were taken in June and August of 2010 and 2011. Within species, different letters indicate a significant (α=0.05) difference between treatments.

		2010					
		Photosynthesis		Transpiration		Relative Chlorophyll Content	
Ash Species		June	August	June	August	June	August
Black	Control	9.5 ± 2.44	5.0 ± 2.77	0.17 ± 0.062	0.17 ± 0.075	38.1 ± 1.62	45.6 ± 1.46
	Fertilizer	7.8 ± 0.04	3.3 ± 1.65	0.18 ± 0.038	0.11 ± 0.04	30.9 ± 2.50	44.1 ± 2.62
	PB	4.5 ± 3.0	3.3 ± 1.20	0.07 ± 0.024	0.10 ± 0.026	33.2 ± 1.60	39.3 ± 1.78
Blue	Control	6.9 ± 0.93	**3.87 ± 1.38[a]**	0.23 ± 0.042	0.10 ± 0.054	37.0 ± 1.02	**34.2 ± 1.54[a]**
	Fertilizer	10.3 ± 0.65	**8.3 ± 2.51[b]**	0.25 ± 0.029	0.23 ± 0.083	35.9 ± 0.83	**37.3 ± 1.56[b]**
	PB	12.4 ± 1.72	**6.6 ± 2.14[ab]**	0.21 ± 0.047	0.18 ± 0.060	36.1 ± 0.91	**34.1 ± 1.65[a]**
Green	Control	10.3 ± 1.79	**16.8 ± 1.31[a]**	**0.35 ± 0.012[a]**	**0.49 ± 0.053[a]**	43.8 ± 1.12	47.8 ± 1.49
	Fertilizer	9.0 ± 1.99	**15.3 ± 1.02[a]**	**0.36 ± 0.061[a]**	**0.47 ± 0.052[a]**	46.9 ± 1.04	51.6 ± 1.59
	PB	7.2 ± 2.85	**6.9 ± 3.33[b]**	**0.09 ± 0.048[b]**	**0.21 ± 0.102[b]**	43.6 ± 1.02	45.3 ± 1.61
Manchurian	Control	10.3 ± 1.01	12.5 ± 1.02	0.27 ± 0.025	0.40 ± 0.053	31.1 ± 0.88	36.1 ± 1.15
	Fertilizer	10.7 ± 1.52	12.2 ± 1.32	0.28 ± 0.033	0.42 ± 0.069	30.5 ± 1.18	36.9 ± 1.04
	PB	9.9 ± 1.30	12.8 ± 0.90	0.18 ± 0.036	0.48 ± 0.022	32.5 ± 1.01	36.6 ± 0.76
White	Control	7.8 ± 1.47	9.0 ± 1.44	0.16 ± 0.006	0.31 ± 0.072	43.3 ± 0.72	**45.9 ± 0.87[a]**
	Fertilizer	11.0 ± 1.50	10.9 ± 1.68	0.27 ± 0.020	0.36 ± 0.059	42.6 ± 0.80	**45.9 ± 0.72[a]**
	PB	11.8 ± 2.31	11.7 ± 0.46	0.28 ± 0.053	0.42 ± 0.040	41.4 ± 0.75	**41.6 ± 1.27[b]**

Table 2.8 (cont'd)

		2011						
		Photosynthesis		Transpiration		Relative Chlorophyll Content		
		June	August	June	August	June	July	August
Blue	Control	11.3 ± 0.75	14.0 ± 0.84	0.29 ± 0.022	0.40 ± 0.018	**27.2 ± 0.63[a]**	**31.1 ± 1.01[a]**	**31.9 ± 1.12[a]**
	Fertilizer	12.5 ± 0.99	13.1 ± 1.08	0.30 ± 0.028	0.40 ± 0.040	**31.8 ± 0.36[b]**	**39.0 ± 0.59[b]**	**39.3 ± 0.66[b]**
	PB	12.5 ± 0.64	11.8 ± 1.54	0.30 ± 0.017	0.34 ± 0.062	**28.4 ± 0.58[a]**	**31.3 ± 0.95[a]**	**32.1 ± 1.08[a]**
Manchurian	Control	15.7 ± 0.56	17.1 ± 0.72	0.38 ± 0.021	0.61 ± 0.018	26.9 ± 0.34	**33.1 ± 0.73[a]**	36.3 ± 0.43
	Fertilizer	14.2 ± 1.31	20.1 ± 0.71	0.33 ± 0.035	0.65 ± 0.015	27.7 ± 0.34	**35.7 ± 0.40[b]**	37.0 ± 0.55
	PB	16.4 ± 0.96	17.9 ± 0.66	0.39 ± 0.037	0.59 ± 0.023	28.7 ± 0.33	**36.2 ± 0.63[b]**	37.8 ± 0.42
White	Control	11.8 ± 1.16	16.5 ± 0.62	0.27 ± 0.032	0.55 ± 0.021	29.9 ± 0.66	**37.5 ± 1.10[a]**	41.3 ± 0.61
	Fertilizer	11.7 ± 1.09	16.2 ± 0.96	0.22 ± 0.025	0.54 ± 0.053	31.1 ± 0.57	**42.2 ± 0.62[b]**	42.9 ± 0.61
	PB	11.5 ± 1.55	13.9 ± 2.13	0.24 ± 0.052	0.45 ± 0.085	31.3 ± 0.89	**39.3 ± 1.11[a]**	40.9 ± 1.04

[1]Photosynthesis and transpiration rates were measure with a Li-Cor 6400 portable photosynthesis meter.

[2]Relative chlorophyll content was measured with a handheld Minolta SPAD-502 meter.

Table 2.9: Results from analysis of variance of effects of ash species and treatment on macro nutrients (percentage dry weight) in foliage of black ash, blue ash, green ash, white ash and Manchurian ash trees sampled in July 2010 or July 2011.

		F Value	df (num, den)	*P* Value
Nutrient	**Effect**		**2010**	
Nitrogen	Species	**28.00**	4, 75	**<0.001***
	Treatment	**3.94**	2, 75	**0.024***
	Species × Treatment	0.46	8, 75	0.877
Phosphorous	Species	**10.58**	4, 75	**<0.001***
	Treatment	**6.58**	2, 75	**0.002***
	Species × Treatment	2.02	8, 75	0.055
Potassium	Species	**14.96**	4, 75	**<0.001***
	Treatment	1.34	2, 75	0.268
	Species × Treatment	0.76	8, 75	0.636
Calcium	Species	**32.71**	4, 75	**<0.001***
	Treatment	1.33	2, 75	0.270
	Species × Treatment	1.01	8, 75	0.437
Magnesium	Species	**29.17**	4, 75	**<0.001***
	Treatment	0.77	2, 75	0.466
	Species × Treatment	1.09	8, 75	0.380

Table 2.9 (cont'd)

		2011		
Nitrogen	Species	**43.34**	2, 41	**<0.001***
	Treatment	**3.29**	2, 41	**0.047***
	Species × Treatment	**3.50**	4, 41	**0.015***
Phosphorous	Species	**26.48**	2, 41	**<0.001***
	Treatment	**6.53**	2, 41	**0.003***
	Species × Treatment	1.93	4, 41	0.123
Potassium	Species	**8.26**	2, 41	**<0.001***
	Treatment	0.89	2, 41	0.418
	Species × Treatment	0.26	4, 41	0.904
Calcium	Species	**32.88**	2, 41	**<0.001***
	Treatment	0.22	2, 41	0.807
	Species × Treatment	0.72	4, 41	0.586
Magnesium	Species	**31.09**	2, 41	**<0.001***
	Treatment	0.10	2, 41	0.909
	Species × Treatment	0.18	4, 41	0.946

Table 2.10: Mean (±SE) percentage of macronutrients in foliage of black ash, blue ash, green ash, white ash and Manchurian ash trees treated with paclobutrazol, fertilizer or left as untreated control sampled in July 2010 and July 2011. Different letters indicate significant ($\alpha \leq 0.05$) differences in nutrient concentration among species (a,b,c) or treatments (x,y).

Ash Species	Nitrogen	Phosphorous	Potassium	Magnesium	Calcium
			2010		
Black	2.95 ± 0.125^{a}	0.28 ± 0.027^{ab}	0.95 ± 0.122^{c}	0.30 ± 0.131^{b}	1.76 ± 0.120^{bc}
Blue	1.94 ± 0.086^{c}	0.17 ± 0.020^{c}	1.01 ± 0.163^{c}	0.65 ± 0.061^{a}	3.12 ± 0.165^{a}
Green	2.87 ± 0.066^{ab}	0.33 ± 0.031^{a}	1.35 ± 0.104^{bc}	0.23 ± 0.015^{b}	1.60 ± 0.068^{c}
White	2.59 ± 0.050^{b}	0.23 ± 0.014^{bc}	1.61 ± 0.076^{ab}	0.22 ± 0.013^{b}	1.69 ± 0.070^{c}
Manchurian	2.69 ± 0.063^{ab}	0.27 ± 0.014^{ab}	1.99 ± 0.045^{a}	0.29 ± 0.017^{b}	2.11 ± 0.093^{b}
			2011		
Blue		0.13 ± 0.006^{c}	1.07 ± 0.115^{b}	0.59 ± 0.047^{a}	2.53 ± 0.126^{a}
Control	1.72 ± 0.128^{x}				
Fertilizer	2.19 ± 0.085^{y}				
Paclobutrazol	1.66 ± 0.120^{x}				
White		0.17 ± 0.006^{b}	1.32 ± 0.041^{ab}	0.20 ± 0.013^{b}	1.39 ± 0.052^{b}
Control	2.43 ± 0.113^{x}				
Fertilizer	2.42 ± 0.061^{x}				
Paclobutrazol	2.46 ± 0.030^{x}				
Manchurian		0.22 ± 0.015^{a}	1.56 ± 0.062^{a}	0.33 ± 0.019^{b}	1.63 ± 0.088^{b}
Control	2.56 ± 0.061^{x}				
Fertilizer	2.53 ± 0.145^{x}				
Paclobutrazol	2.55 ± 0.061^{x}				

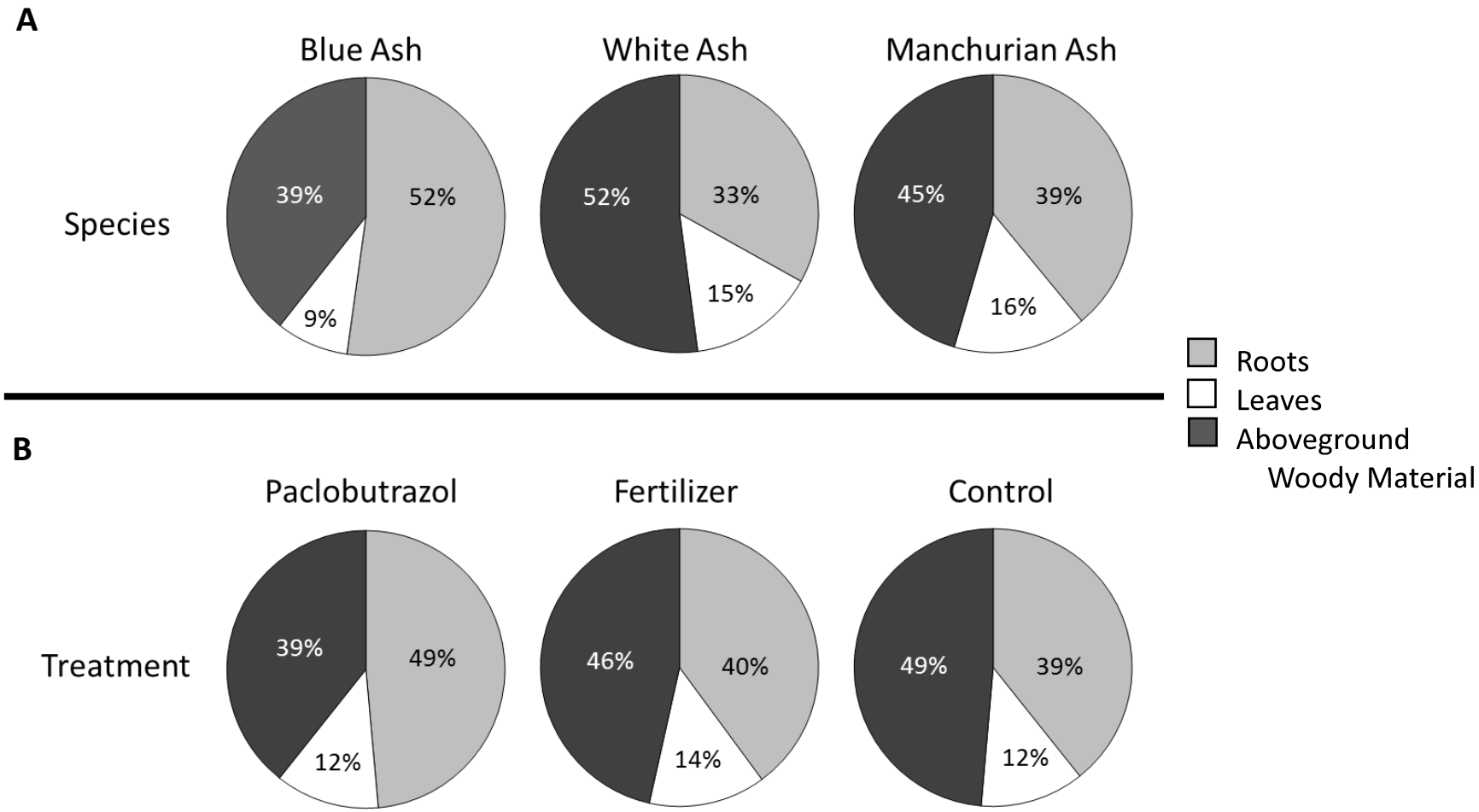


Figure 2.1: Percentage of biomass allocated to roots, leaves and aboveground woody material (shoots and bole) in blue ash, white ash and Manchurian ash trees (A) and ash trees treated with paclobutrazol, fertilizer or left as untreated controls (B).

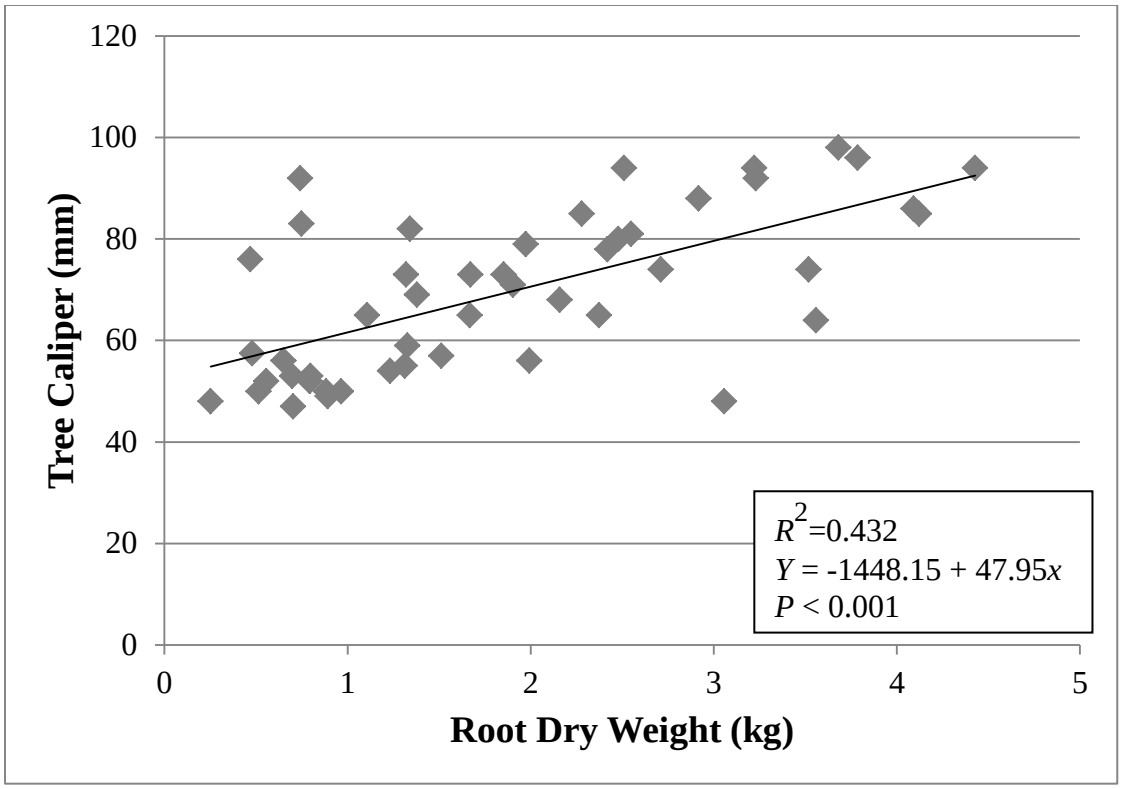


Figure 2.2: Relationship between root biomass (kg) and tree caliper (mm) of blue ash, white ash and Manchurian ash trees harvested in October 2012 from a plantation in Okemos, Michigan.

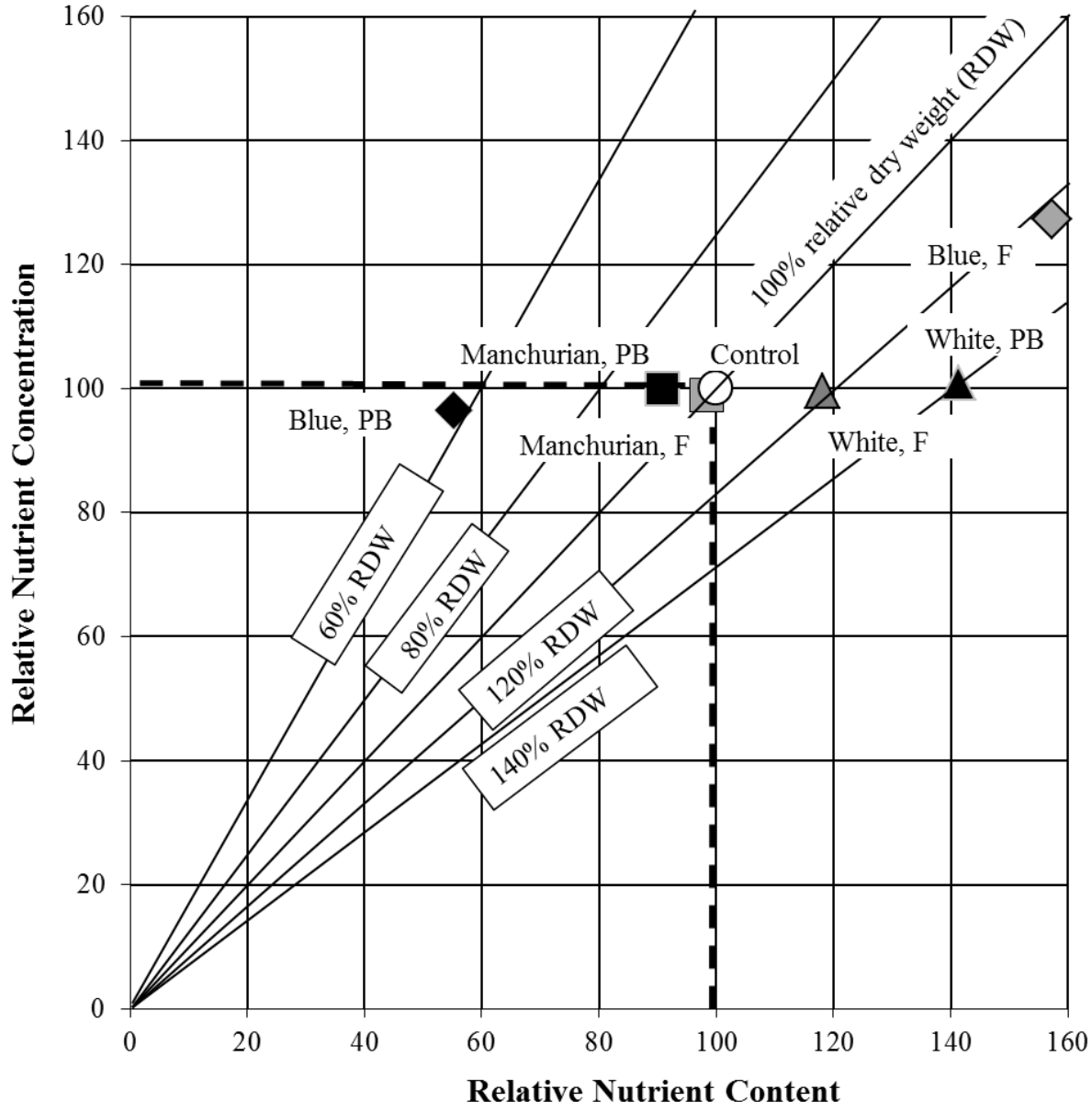


Figure 2.3: Biomass vector analysis of nitrogen for blue ash (diamonds), white ash (triangles) and Manchurian ash (squares) trees treated with paclobtrazol (black, PB) or fertilizer (gray, F). Dry weight is the dry weight of all leaves, twigs, boles and roots.

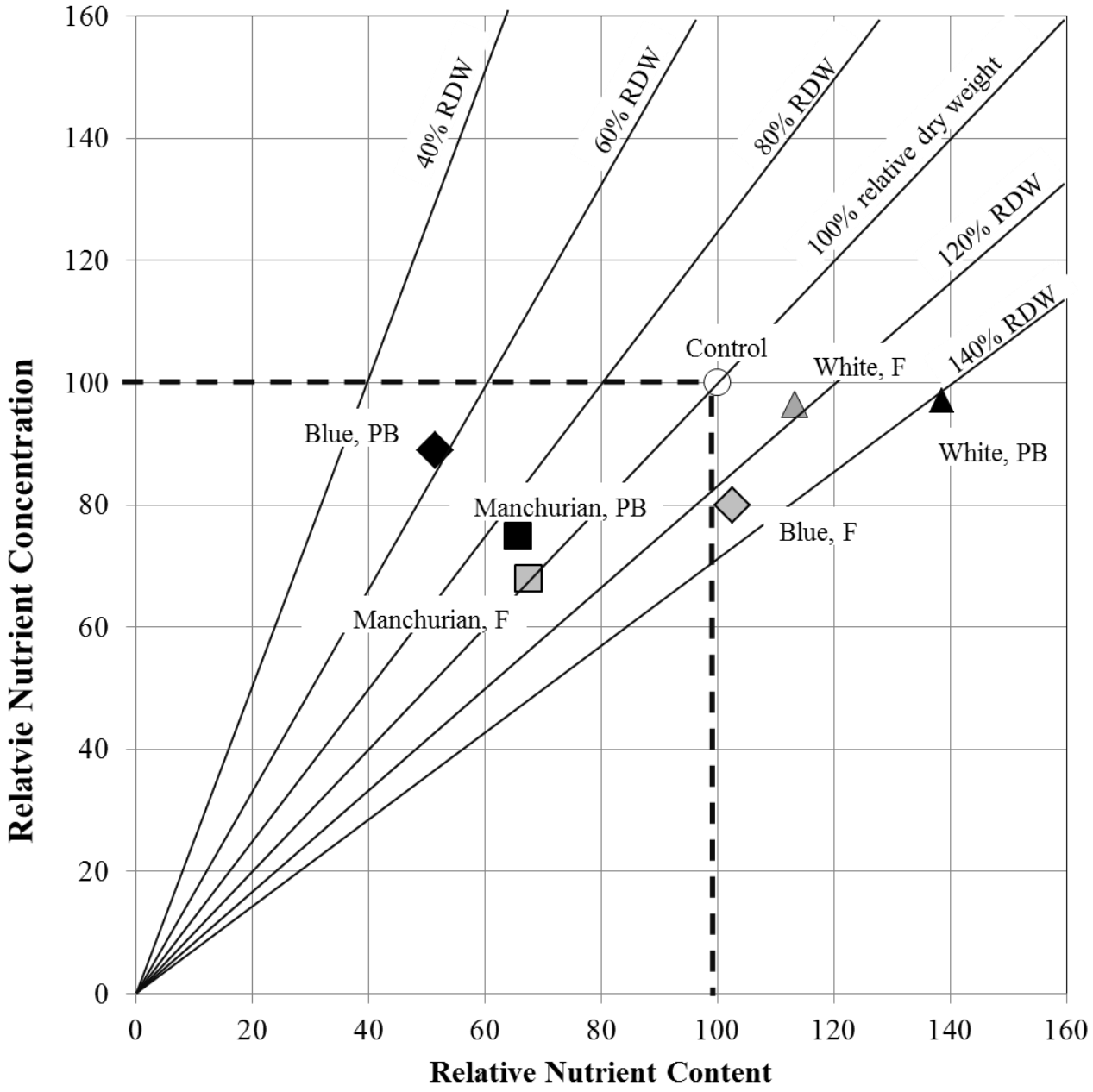


Figure 2.4: Biomass vector analysis of phosphorous for blue ash (diamonds), white ash (triangles) and Manchurian ash (squares) trees treated with paclobtrazol (black, PB) or fertilizer (gray, F).

CHAPTER THREE

Effects of paclobutrazol and fertilization on host resistance and suitability of five *Fraxinus* species to *Agrilus planipennis*

ABSTRACT

Agrilus planipennis Fairmaire is the most destructive forest insect to ever invade North America. All *Fraxinus* spp. (ash) assessed to date are suitable hosts, but *A. planipennis* exhibits interspecific and intraspecific host preferences. In conjunction with insecticide applications, fertilizer and paclobutrazol (PB) treatments may be recommended to ensure ash trees remain healthy. I assessed differences in host preference and suitability among 1) four North American (*Fraxinus nigra* Marsh., *F. quadrangulata* Michx., *F. pennsylvanica* Marsh. and *F. americana* L.) and one Asian ash species (*F. mandshurica* Rupr.) and 2) trees treated with PB, fertilizer or left as untreated controls. Species effects overshadowed most treatment effects. In 2010, adult beetle survival was highest when beetles were caged with black ash (53%), intermediate for beetles caged with green, white and Manchurian ash (30%, 32%, or 30%, respectively) and lowest (14%) for beetles caged with blue ash. When beetles were caged on intact blue ash and white ash leaves, they consumed 42 and 31% more leaf area per beetle day than beetles caged on excised blue ash and white ash leaves but survival was similar within species. In contrast, leaf area consumed per beetle day was similar between beetles caged on intact or excised Manchurian ash leaves, but survival was 11% higher for beetles caged on excised Manchurian ash foliage than for beetles caged on intact leaves from the same tree. In 2011, larval gallery density (galleries per m^2) was highest on black (235.9±36.41) and green ash (220.1±39.77), intermediate on white ash (40.7±11.61), and lowest on blue and Manchurian ash (2.0±0.98 and 1.5±0.67, respectively). Results indicate that blue ash and Manchurian ash trees are more resistant to *A. planipennis* than black ash, green ash or white ash.

INTRODUCTION

Even though it is a secondary pest in its native range (Yu 1992, Gao et al. 2004), *Agrilus planipennis* Fairmaire (emerald ash borer) is the most destructive forest insect to ever invade North America (Aukema et al. 2011, Kovacs et al. 2011). Tens of millions of ash trees in Michigan alone have been killed by *A. planipennis* since it was identified in 2002 and to date, infestations have been found in 19 states and two Canadian provinces (Cappaert et al. 2005, EAB.info 2013). An estimated 8 billion ash trees occur in forested settings across the United States (EAB.info 2013). Annualized economic impacts of *A. planipennis* were recently projected to exceed 900 billion USD by 2020 (Aukema et al. 2011, Kovacs et al. 2011). The cost of treating or removing even 50% of the landscape ash trees in affected US cities will likely exceed 10.5 billion USD by 2019 (Kovacs et al. 2011).

Ash species are evolutionarily diverse (Wallender 2008) and have varying levels of inherent resistance to *A. planipennis* (Rebek et al. 2008, Duan et al. 2012, Tanis and McCullough 2012). The Asian ash species, Manchurian ash (*Fraxinus mandshurica* Rupr.) and Chinese ash (*Fraxinus chinensis* Roxb.), are relatively resistant to *A. planipennis* and are attacked only when stressed (Liu et al. 2003, Duan et al. 2012). In contrast, North American species, lack a co-evolutionary history with *A. planipennis* (Bryant et al. 1994, Lieutier 2008, Gandhi and Herms 2010, Nielsen et al. 2011). This lack of evolved tree resistance and the absence of native congeners (Drooz 1985) and natural enemies likely accounts for the widespread ash mortality associated with *A. planipennis* (EAB.info 2012). While all ash species assessed to date can be colonized, *A. planipennis* prefer some species over others (Anulewicz et al. 2007, Rebek et al. 2008, Limback 2010, Tanis and McCullough 2012). In northeast China, *A. planipennis* preferentially attacked two North American species, green ash (*Fraxinus pennsylvanica* Marsh.)

and velvet ash (*Fraxinus velutina* Torrey) rather than native Manchurian or Chinese ash (Duan et al. 2012). In a common garden in southeast Michigan, white ash (*Fraxinus americana* L.), green ash and a black ash (*Fraxinus nigra* Marshall) × Manchurian ash hybrid ('Northern Treasure') all succumbed to *A. planipennis* infestation before Manchurian ash (Rebek et al. 2008). Among North American ash species, black ash and green ash are highly preferred by *A. planipennis* (Cappaert et al. 2005, Limback 2010) while white ash is considered moderately preferred (Anulewicz et al. 2007). To date, blue ash (*Fraxinus quadrangulata* Michx.) appears to be the most resistant North American species (Anulewicz et al. 2007, 2008, Pureswaran and Poland 2009). In areas where blue ash and white ash co-occurred in southeast Michigan, 60-70% of blue ash of all diameter classes survived the *A. planipennis* invasion compared to <5% of white ash (Tanis and McCullough 2012).

Intraspecific *A. planipennis* host preference is also apparent (McCullough et al. 2009a, 2009b). In Asia, *A. planipennis* functions as a secondary pest, typically attacking stressed ash trees (Lyons et al. 2009). In North America, it can colonize both healthy and stressed ash trees (Poland and McCullough 2006), but will preferentially attack stressed trees (McCullough et al. 2009a, 2009b). Larval development in stressed trees takes one year, but larvae require two years to develop on healthy trees (Siegert et al. 2010, Tluczek et al. 2011).

Since the North American discovery of *A. planipennis* in 2002, researchers have been working to identify or improve control options for valuable ash landscape trees. Systemic insecticides, applied via trunk injection or soil applications are most commonly used (Herms et al. 2009, McCullough et al. 2011). Some researchers and tree care professionals also recommend fertilization to ensure ash trees remain healthy and receive adequate nutrients (Rebek and Smitley 2005). Although enhanced vigor could enable trees to resist and/or tolerate *A.*

planipennis infestation, there have been no studies to assess the effects of fertilizer on *A. planipennis* host preference or host suitability.

Effects of fertilization on urban trees have been widely disputed since the early 1920's (Miller 1998, Marshall et al. 2003, Herms 2002, Ferrini and Baietto 2006). Fertilization has been promoted as a means to increase tree resistance (reviewed by Waring and Cobb 1992), while Raupp et al. (2010) stated "fertilization almost always decreases tree resistance to herbivores". Fertilizer application can affect growth rates, volatile production, and in some plants, the production of chemical defenses (Gouingené and Turlings 2002). Growth and development of phytophagous insects are often limited by nitrogen (N) availability (Scriber and Slansky 1998) and most insects prefer N rich over N limited foliage (Reviewed by Herms 2002, Schoonhoven et al. 2005). However, fertilization may also enhance defensive chemical production and decrease herbivore survival or reproduction (Coley et al. 1985, McCullough and Kulman 1991). Whether fertilization affects ash resistance or *A. planipennis* host selection behavior has not been examined.

Many tree care companies promote plant growth regulator treatments to reduce tree growth and ameliorate stress (Chaney 2005a, 2005b). Paclobutrazol (PB), a triazole gibberellin inhibitor, is considered one of the most potent growth inhibitors in its class (Yadav 2005). It reduces aboveground plant growth by inhibiting sterols (Dalziel and Lawrence 1984) and blocks the gibberellin biosynthesis pathway (Raese and Burts 1983). Changes in gibberellin production alter source - sink relationships within trees by reducing aboveground growth and increasing stored carbohydrates (Ramina and Tonutti 1985, Davis and Shankhla 1987). Paclobutrazol, commonly applied to landscape trees as a soil drench (Chaney 2005a, 2005b), can persist in soil up to three years (Yadav 2005) and there is typically a one season lag between application and

measurable changes in growth (Bai et al. 2004). Although it was originally used by utility companies to reduce pruning and maintenance of trees and shrubs in right of ways (Mann 1995, Chaney 2005a, 2005b), PB is commonly marketed in the landscape industry as a means to increase tree vigor (Chaney 2005a, 2005b). In addition to reducing growth, PB increases tolerance of cold (Haas 2012), drought (Davis and Shankhla 1987, Marshall et al. 2000, Percival and AlBalushi 2007) and salt (Haas 2012); chlorophyll concentration (Yadav 2005); stored carbohydrates (Fletcher et al. 2000); root: shoot ratio; cell division (not cell elongation) (Wiesman and Lavee, 1994); and fine root biomass (Fletcher et al. 2000, Chaney 2005a, 2005b, Haas 2012).

In a concurrent study, I found radial growth, biomass allocation, relative chlorophyll content and nutrient concentrations were affected by fertilization and PB treatments. One year after application, trees treated with PB had reduced radial growth and biomass allocation shifted from aboveground to belowground (Tanis, Chapter 2). Blue ash and Manchurian ash trees were relatively sensitive, and responded to PB treatment in almost every parameter assessed. In contrast, white ash was relatively unaffected, which is consistent with previous reports (Sterrett and Tworkoski 1987, Bai et al. 2004). Trees rarely responded to fertilization with the exception of blue ash; fertilized trees had higher relative chlorophyll content and nutrient concentrations than control or PB trees (Tanis, Chapter 2).

Although inter- and intraspecific *A. planipennis* host preference is apparent, it is not clear whether the susceptibility of ash species is driven by adult beetles, that select trees for feeding and oviposition, or by the ability of larvae to feed and develop, or both. In this study, I hypothesized that blue ash and Manchurian ash trees will be less suitable hosts for adult *A. planipennis* than black ash, green ash and white ash and that *A. planipennis* larval densities will

reflect host suitability or adult beetle host preference. I also hypothesized trees treated with PB will be more resistant to *A. planipennis* infestation than fertilized or control trees. In oak, trees that have moderate to high starch reserves (and theoretically more energy for the production of defensive chemicals) tend to resist attack by two-lined chestnut borer (*Agrilus bilineatus* Weber) (Dunn et al. 1987), a North American *A. planipennis* congener. Our concurrent study indicated that PB shifts biomass allocation from aboveground to belowground which suggests PB could increase resources available for defense against *A. planipennis*.

METHODS

Ash Plantation

In 2007, 225 ash trees were purchased from commercial nurseries and planted (2.4 m apart) in a randomized complete block design at the Michigan State University Tree Research Center (TRC) in Okemos, Michigan, Ingham County. The plantation consisted of 45 trees of each species: black ash, blue ash, green ash, white ash and Manchurian ash. Each block consisted of one tree of each species. Trees were grown as either bare root (black ash and Manchurian ash) or balled and burlapped stock (green ash, white ash and blue ash). Tree size varied among species (Tanis, Chapter 2). All trees were shipped from outside of *A. planipennis* quarantine zones in cooperation with state and federal agencies and were not infested when they were planted. During the 2008-09 winter, 16 blue ash trees died from non- *A. planipennis* causes and were replaced with bare root trees in spring 2009. Throughout the experiment, trees were watered twice per week or as needed with drip irrigation. Trees were fertilized in July (2006-2009) with a top dressing of Harrel's Pro-Blend with Micros (19-5-10) (Harrell's, Lakeland, FL, USA) at a rate of 70 g (5.5 ounces N) per tree.

In May 2006 and 2007, trees were sprayed with a foliar application of Tempo® (Bayer Environmental Science, New Jersey, USA) to prevent *A. planipennis* infestation. In May 2008 - 2010 rather than apply insecticide, tree boles were wrapped with tree wrap (Jobe's TreeWrap Pro, Easy Gardener, Inc., Waco, Texas, USA) over laid with screen mesh to deter *A. planipennis* infestation. Tree wrap was removed every September.

On 26 May 2010, seven trees of each species were randomly assigned to one of the following treatments: control, fertilizer or PB (35 trees per treatment). Control trees received no chemical applications. Fertilized trees received 350 g (a higher rate than above) of Harrell's Pro-

Blend with Micros (19-5-10) (Harrel's, Lakeland, FL, USA) sprinkled at the base of each tree. ShortStop® (Paclobutrazol 22.3%, Zhejiang Tide Crop Science Co., LTD, Irvine, CA, USA) was applied as a soil drench. Diluted product was applied evenly according to label instructions (200 ml diluted product, 4 grams a.i., per 2.54 cm DBH) in a shallow trench, approximately 12 cm deep, around the base of each tree. All trees were protected with wrap during the summer of 2010 as described above.

Trees fertilized in 2010 received a second application on 23 May 2011. Because PB applications typically persist in soils for two to three years, PB trees did not receive additional treatments. In 2011, trees were left exposed to wild *A. planipennis* populations.

Adult *A. planipennis*

Adult *A. planipennis* used for this study were reared in the laboratory from infested ash logs harvested in autumn 2009 or 2010. Logs were cut to 60 cm, sealed with Bailey's® seal (Bailey's, Laytonville, CA USA) and placed into coolers maintained at 4º C. Logs were removed from cold-storage approximately three weeks before experiments began and placed in rearing tubes (ambient temperature). After two weeks, rearing tubes were checked daily. As beetles emerged, they were collected, separated by sex and placed directly into bioassays.

Leaf feeding bioassays

In July 2010 and 2011, I assessed effects of ash species and treatments on adult *A. planipennis* foliage consumption and survival using excised (2010 and 2011) or intact (2011 only) leaves from each tree. In June 2010, a single sun-exposed leaf was removed from each tree, sealed in a plastic bag, placed in a cooler and brought back to the laboratory. Upon arrival, a section with 4-5 leaflets still attached to the petiole was removed (whole leaves were too large to fit into petri dishes), weighed and scanned with a flatbed scanner (Epson Perfection® 4490

Photo, Epson America, Inc., Longbeach, CA) to determine pre-feeding leaf weight and area. Leaf area was assessed using WinFOLIA® software (Regent Instruments, Quebec, Canada). After obtaining initial measurements, leaf petioles were inserted into a water pick, to slow desiccation, and placed in a Petri dish.

Four newly emerged beetles (two males and two females) were immediately added to each Petri dish. Petri dishes with beetles were maintained in growth chambers with 16:8 hr light: dark, 75% humidity and 25°C. Beetles were allowed to feed for four days and the number of surviving *A. planipennis* was recorded daily. On the fourth day, the leaf was removed from the Petri dish, re-weighed and scanned to quantify leaf weight and area consumed. Leaf weight and area consumed were standardized using "beetle days". On day four, beetle days were calculated for each sample by tallying the number of surviving beetles from each day. For example, if all beetles survived in the Petri dish, the number of beetle days would be 16 (four beetles alive on each of four days). Leaf weight and area consumed per beetle day were calculated by dividing the leaf weight (g) or area consumed (cm^2) by the number of beetle days. Bioassays were repeated in June 2011.

In spring 2011, I developed an inexpensive, novel cage that allowed me to cage beetles with intact leaves still attached to trees. Cages consisted of two foam dessert plates (15 cm diameter, Gordon Food Service, Wyoming, MI, USA). To allow airflow and sunlight to penetrate the cage, I removed a 10 cm diameter section from the center of each plate and glued a piece of screen mesh over the center to prevent beetles from escaping. I then stapled two plates together, top to top. A small opening, approximately 10 cm long, was left to allow for the addition of beetles and to enable me to move cages from leaf to leaf. Cages were attached to

undamaged leaves, beetles were added and the opening along the edge was sealed with three small binder clips. Cages were secured to trees with twine to reduce wind damage.

In July 2011, a cage containing four newly emerged beetles (two males and two females) was placed on a single undamaged sun-exposed leaf of each tree. The number of surviving beetles was recorded daily for four days then the leaf was removed, sealed in a plastic bag, returned to the lab and scanned with a flatbed scanner. Each leaf scan was printed twice. One copy was used to quantify pre-feeding leaf area, the other to quantify post-feeding leaf area. To determine pre-feeding area, I carefully filled in the area of the leaf that was missing as a result of *A. planipennis* feeding. Post feeding images were not altered. Both images were re-scanned with WinFOLIA® software and area consumed was determined by subtracting post-feeding area (cm^2) from pre-feeding area (cm^2). Leaf area consumed per beetle day was recorded.

Mortality Bioassays

After emergence, adult *A. planipennis* typically feed for two weeks before they begin mating. In 2010 and 2011, bioassays from (excised and intact) were continued for 10 additional days to determine how many beetles would survive the maturation period. After the first four days, beetles in Petri dishes were given a new excised leaf from the same tree every other day for the 10 remaining days (14 days total). Beetles and cages in the plantation (2011 only) were moved to a new leaf on the same tree every other day for 10 additional days (14 days total). Number of surviving beetles was recorded when foliage was changed or cages were moved.

Larval Gallery Density

Local populations of *A. planipennis* were very high and despite our efforts to protect trees from infestation, 66% of black ash trees and 71% of green ash trees were killed in 2010. By August 2011, all but one of the remaining black ash and green ash trees had severe canopy

dieback (>80%). I harvested and debarked dead black ash (14 of 21) and green ash (15 of 21) trees in May 2011 to ensure they did not rot over the summer. Remaining trees were left exposed to the local wild *A. planipennis* populations during the 2011 growing season, then were harvested and debarked in the fall. For each tree, I assessed galleries made by the 2010 and 2011 *A. planipennis* larval cohorts.

On trees debarked in spring 2011, I counted the number of exit holes created by larvae from the 2009 cohort, but because trees were dead and many were starting to desiccate, I could not record with confidence the number or state of larvae from the 2009 cohort. Larval condition (dead or alive), larval stage (first, second and third instar larvae were classified as early instars, fourth instar and prepupae larvae were classified as late instars) were recorded for larvae from the 2010 cohort. I also assessed woodpecker or parasitoid attacks on the 2010 larval cohort. Parasitoid attacks were recorded when an *Atanycolus* spp. cocoon was present in the *A. planipennis* larval gallery or when an *Atanycolus* spp. larva was attached to an *A. planipennis* larva. All of the larvae recorded from trees debarked in the spring of 2011 were from the 2010 larval cohort.

For trees debarked in fall 2011, I distinguished between galleries made by the 2010 and 2011 cohorts using methods described by Tluzcek et al. (2011). Larvae from the 2010 cohort were assessed for larval condition and larval stage as described above. Holes made by woodpeckers removing EAB larvae in 2010 were differentiated from holes made in 2011 by the state of the bark surrounding the hole. Holes created by birds in 2011 were still green and fresh when trees were harvested. *Atanycolus* spp. cocoons from 2010 contained an adult emergence hole, whereas 2011 cocoons still contained an *Atanycolus* spp. pupae. Larval cohort was

assigned for parasitized larvae or larvae removed by woodpeckers based on the appearance of the gallery (Tluzcek et al. 2011).

Statistical Analysis

All data were analyzed using SAS statistical software (SAS Institute, Inc. 2003). Results from 2010 are presented for all five ash species, while 2011 results are presented for blue ash, white ash and Manchurian ash only. Assumptions of normality were tested with residual plots and the Shapiro-Wilk test (Shapiro and Wilk 1965). Leaf weight and leaf area consumed per beetle day were normalized using log transformations (Ott and Longnecker 2001). Two-way ANOVA was performed to evaluate effects of ash species, treatments and interactions between the two factors on changes in leaf weight (2010 and 2011) and leaf area consumed per beetle day (2010). In 2011, three-way ANOVA was used to evaluate effects of ash species, treatments, leaf type (intact or excised) and interactions of the three factors on leaf area consumed per beetle day. In 2011, repeated measures ANOVA was used to evaluate effects of ash species, treatments, leaf type, days after treatment and the interactions on beetle survival.

The number of exit holes, parasitoid and woodpecker attacks, density of living and dead larvae, and total gallery density, could not be normalized using transformations. Friedman's nonparametric two-way ANOVA was used to analyze all gallery data (number of total, alive and dead larvae, adult *A. planipennis* exit holes, woodpecker attacks and parasitized larvae). When species or treatment effects were significant ($\alpha \leq 0.05$), I used Fisher's non-parametric protected least significant difference (LSD) multi-comparison test with Tukey's adjustment to determine differences among means (Ott and Longnecker 2001).

RESULTS

Leaf Area and Weight Consumed per Beetle Day

2010 – Excised Leaves

Area consumed per beetle day of excised leaves (2010 and 2011) varied among ash species (Figures 3.1A). In 2010, area of excised leaves consumed per beetle day was affected by species ($F=14.33$; df=4,89; $P<0.0001$) but not treatment ($P=0.63$) or the interaction ($P=0.31$). In 2010, area of excised leaves consumed per beetle day of white ash and Manchurian ash was over twice as high as the area of excised leaves consumed per beetle day of blue ash (Fig. 3.1A). Leaf weight consumed per beetle day (excised leaves) did not differ among species ($P=0.54$), treatments ($P=0.43$) or their interactions ($P=0.81$).

2011 – Excised and Intact Leaves

In 2011, I caged beetles on intact or excised foliage for the feeding and survival bioassays. Leaf area consumed per beetle day varied by species ($F=45.01$; df=2, 111; $P<0.0001$), leaf type (excised or intact) ($F=52.31$; df=1; 111; $P<0.001$) and the species × leaf type interaction ($F=9.87$; df=2, 111; $P=0.001$) but not treatment ($P=0.06$). Species × treatment ($P=0.38$), treatment × leaf type ($P=0.17$) and species × treatment × leaf type ($P=0.19$) interactions were also not significant. Beetles caged with excised leaves from blue ash trees consumed less foliage than beetles caged with any other foliage type (Fig. 3.1B). Beetles consumed 42 and 31% more ($P<0.001$) leaf area when caged with intact white ash and blue ash leaves, respectively, than beetles caged with excised white ash and blue ash leaves (Fig. 3.1B). Leaf area consumed per beetle day was similar ($P=0.07$) for excised and intact Manchurian ash foliage (Fig. 3.1B). As in 2010, leaf weight consumed per beetle day (excised leaves only) did not differ among species ($P=0.66$) treatments ($P=0.36$), or the interaction ($P=0.87$).

Adult *A. planipennis* Mortality

In 2010, beetle survival was affected by species, day, species × treatment and species × day interactions (Table 3.1). In 2011, I caged beetles on intact, as well as, excised leaves. Beetle survival was affected by species, day and species × day, species × leaf type (excised or intact), treatment × leaf type and species × treatment × leaf type interactions (Table 3.1).

In 2010, at least 38% more beetles survived for 14 days when caged with excised black ash leaves than beetles caged with excised leaves from the other four ash species (Table 3.2). Species effects were first apparent on day six when 48 and 38% of beetles caged with excised blue ash or Manchurian ash leaves were dead, respectively, compared to 18, 35 and 23% of beetles caged with excised black ash, green ash or white ash foliage (Fig. 3.2A). At the end of the 14 day trial, survival was highest (53%) for beetles caged with excised black ash, intermediate for beetles caged with excised green ash (30%), white ash (32%) or Manchurian ash (30%) foliage, and lowest (15%) for beetles caged with excised blue ash foliage (Table 3.2).

In 2011, six days after emergence, 85% of beetles caged with white ash and Manchurian ash foliage (intact + excised) were alive compared to 65% of beetles caged with blue ash foliage. This pattern was consistent for the remaining eight days of the bioassays (Fig. 3.2B). Throughout the 14 day trial, survival rates were similar between beetles caged with white ash and Manchurian ash leaves (Table 3.3). At least 73% of beetles caged with white ash or Manchurian ash foliage survived the two week maturation period. In contrast, only 33% of beetles caged with blue ash foliage survived 14 days (Fig. 3.2B).

2010 – Excised Leaves

Within species, treatment effects on survival of beetles caged with excised foliage were variable (Table 3.4). There were no treatment effects on survival when beetles consumed

excised black ash leaves. For beetles caged with excised blue ash leaves, survival was highest on leaves from control trees (72%) and lowest on leaves from fertilized (55%) or PB trees (53%) (Table 3.4). Among beetles caged with excised green ash or Manchurian ash leaves, survival was higher for *A. planipennis* caged with leaves from PB trees (74% and 74%) than beetles caged with leaves from fertilized (63% and 69%) or control (73% and 58%) green ash or Manchurian ash trees, respectively (Table 3.4). Beetles caged with excised white ash leaves had higher survival when caged with leaves from fertilized (81%) or PB (76%) trees than beetles caged with leaves from control (60%) trees, respectively.

2011 - Excised Leaves

Treatment effects on beetle survival were evident when beetles were caged with excised foliage, but results varied among species (Table 3.4). Survival was unaffected by treatment when beetles were caged with excised blue ash or Manchurian ash leaves (Table 3.4). However, when beetles were caged with excised leaves from white ash PB trees, survival was 11% higher than when beetles were caged with leaves from white ash control or fertilized trees (Table 3.4).

2011 – Intact Leaves

Treatment and species effects on survival were also variable among beetles caged on intact leaves (Table 3.4). When beetles were caged on intact leaves on blue ash PB and control trees, survival was 15 and 18% higher, respectively, than when beetles were caged on intact leaves on fertilized blue ash trees. In contrast, survival of beetles caged with intact leaves on PB white ash trees was 11% lower than survival of beetles caged on intact leaves on control trees. Beetle survival on intact white ash leaves from fertilized trees (85%) did not differ from beetle survival on intact leaves from PB (78%) or control (88%) white ash trees. Survival was

unaffected by treatment when beetles were caged on intact leaves from Manchurian ash trees (Table 3.4).

2011 - Excised vs. Intact Leaves

Beetle survival was similar between beetles caged with excised foliage from blue ash control and PB trees and intact foliage from control or PB blue ash trees (Table 3.4). Among beetles caged with white ash foliage, survival was higher for beetles caged with excised foliage from PB trees than for beetles caged with all other types of white ash foliage (Table 3.4). Across all three treatments, beetles caged with excised Manchurian ash foliage had higher survival than beetles caged with intact Manchurian ash foliage (Table 3.4).

Larval Gallery Density

A total of 4,016 larval galleries were examined in this study when trees were debarked in 2011. Only, 14% of the galleries produced an adult *A. planipennis* as evidenced by an adult emergence hole. Among all ash species, 22% of galleries contained live larvae, 8% of these were early instar larvae (first, second or third instars) and 92% were late instar larvae (fourth instar or prepupae). Only 11% of galleries contained dead larvae, 61% of these were early instars and 39% were late instars. Across all species, 54% of larvae were killed by either woodpeckers or *Atanycolus* spp. I also assessed the densities of dead and living larvae, adult *A. planipennis* exit holes and evidence of woodpecker and parasitoid attacks by species (Table 3.5).

Larval density varied among species (χ^2=67.53; df=4,104; P<0.001) but not treatments (P=0.72) or the interaction (P=0.93). Black ash and green ash trees were heavily infested (Fig. 3.3) and had larval densities so high (235.9 ± 36.41 and 220.1 ± 39.77, respectively) that 18 black ash and 11 green ash trees failed to leaf out in 2011. Larval densities on blue ash and Manchurian ash trees were significantly lower than on black ash or green ash and ranged from 0

to 15 and 0 to 13 galleries per m^2, respectively (Fig. 3.3). Fifteen blue ash and 13 Manchurian ash trees had no *A. planipennis* galleries. Larval density on white ash trees was moderate (Fig. 3.5) ranging from 0 to 178 galleries per m^2. Three white ash trees contained > 89 larvae per m^2, a density high enough to result in tree death (McCullough and Siegert 2007), but five trees had <10 galleries per m^2 and two trees had no evidence of *A. planipennis* infestation.

2009 Larval Cohort

Black ash and green ash trees were infested by the 2009 larval cohort. Adult emergence holes from the 2009 larval cohort comprised 15 and 6% of the total larval galleries found on black ash and green ash trees, respectively (Table 3.6).

2010 Larval Cohort

Among black ash trees, 5% of galleries made by the 2010 larval cohort were associated with an adult emergence hole, 21% contained live larvae and 74% were associated with dead larvae. Woodpecker predation and *Atanycolus* spp. parasitism accounted for 62% of the dead larvae (Table 3.6). There were no larvae from the 2010 cohort on blue ash trees (Table 3.6). Among green ash trees, 14% of galleries made by the 2010 larval cohort contained an adult emergence hole, 17% contained live larvae and 69% were associated with dead larvae. Among the dead larvae, 56% were killed by either woodpeckers or *Atanycolus* spp. (Table 3.6). White ash trees had 79% fewer galleries from the 2010 larval cohort than black ash or green ash trees. Among the 175 galleries found on white ash trees, 7% contained an exit hole, 28% contained live larvae and 65% were associated with dead larvae. While woodpecker predation accounted for 91% of the dead larvae on white ash trees, there was no evidence that *Atanycolus* spp. parasitized

any of the larvae from the 2010 cohort (Table 3.6). Manchurian ash trees contained no larvae from the 2010 larval cohort (Table 3.6).

2011 Larval Cohort

In 2011, only seven black ash and six green ash trees remained in the plantation for infestation by the 2011 larval cohort. Among the 304 galleries examined on black ash trees, 8% contained live larvae and 92% were associated with dead larvae. Of the dead larvae, 96% were killed by woodpeckers or *Atanycolus* spp. (Table 3.6). Among blue ash trees, 41% of larval galleries contained live larvae and 59% were associated with dead larvae. Woodpecker predation and parasitism by *Atanycolus* spp. accounted for 50% of the dead larvae (Table 3.6). Among the 952 galleries found on green ash trees, 39% contained live larvae and 61% were associated with dead larvae, of these, 85% were killed by woodpeckers or *Atanycolus* spp. (Table 3.6). The number of larval galleries on white ash trees increased 45% from 2010 to 2011. Of the 319 galleries assessed, 38% contained live larvae and 62% contained dead larvae. Contrary to 2010 when parasitism by *Atanycolus* spp. did not occur, 79% of the dead larvae from the 2011 cohort were parasitized by *Atanycolus* spp. and 21% were killed by woodpeckers (Table 3.6). Among the 10 galleries found on Manchurian ash trees, 40% were alive and 60% were dead. Neither woodpeckers nor *Atanycolus* spp. killed any of the larvae on Manchurian ash trees (Table 3.6).

DISCUSSION

In this study, ash species was the main driver behind *A. planipennis* adult host suitability and larval gallery density. Several studies indicate *A. planipennis* adults prefer some ash species over others, but the mechanisms underlying preference are not fully understood. In a study using excised leaves and caged beetles, *A. planipennis* spent less time on and consumed less area of Manchurian ash and blue ash leaves when given a choice between them and black ash, green ash and white ash leaves (Pureswaran and Poland 2009). In my no choice bioassays, *A. planipennis* caged with Manchurian ash leaves consumed as much or more leaf area as beetles caged with black ash, green ash or white ash leaves. In a concurrent study, I determined that foliar N concentration of Manchurian ash was similar to black ash, green ash and white ash (Tanis, Chapter Two). Results of previous studies suggest foliar N concentrations might influence *A. planipennis* adult host selection (Pureswaran and Poland 2009, Chen et al. 2011) because N is often the limiting nutrient for folivorous insects (Scriber and Slansky 1981). Although *A. planipennis* adults may vacate Manchurian ash leaves in favor of other available species (Pureswaran and Poland 2009), our foliar nutrition data (Tanis, Chapter 2) suggests they are probably not reacting to N levels in Manchurian ash foliage. In contrast to Manchurian ash, blue ash foliar N concentrations were consistently lower than in the other four ash species (Tanis, Chapter 2), but *A. planipennis* adults did not exhibit a compensatory feeding response as might be expected (Simpson and Simpson 1990). Beetles caged with blue ash foliage consumed less leaf area and died at higher rates than beetles caged with the other ash species. This pattern suggests blue ash foliage may contain compounds that are toxic or trigger an *A. planipennis* antifeedant response.

Induced foliar defenses can be activated in a variety of ways including leaf excision (Schmelz et al. 2001) and insect feeding (Crook and Maestro 2010). Previous assessments of adult *A. planipennis* feeding, host preference and survival have typically used captive *A. planipennis* caged with excised foliage (Chen and Poland 2009a, 2009b, Pureswaran and Poland 2009). Removing ash leaves from trees, however, could trigger localized defensive responses within leaves or short-circuit inducible defenses in trees responding to *A. planipennis* feeding. In 2011, beetles caged with excised leaves consumed less blue ash and white ash leaf area than beetles caged with intact leaves on blue and white ash trees. In my feeding bioassays, some excised leaves became desiccated even though they were kept in a carefully controlled environment. I suggest leaf area consumed per beetle day was low when beetles were caged with excised leaves simply because leaf quality and moisture were low. Effects of leaf excision on beetle survival were highly variable among species and treatments and no consistent patterns were apparent. Leaf area consumed was similar between beetles caged with excised Manchurian ash leaves and beetles caged on intact Manchurian leaves, but *A. planipennis* survival was consistently higher across treatments when beetles were caged on intact Manchurian ash leaves than when caged with excised Manchurian ash leaves from the same tree.

In North America, *A. planipennis* attack only ash trees (Anulewicz et al. 2007, 2008, Rebek et al. 2008). To locate suitable hosts, *A. planipennis* process a complex combination of visual and olfactory cues that likely act as both attractants and repellents (Rodriguez-Saona et al. 2006, Pureswaran and Poland 2009). Host tree attributes (stress and species) not only influence adult behavior, but can also have important impacts on larval distribution, development and survival. For example, most larvae require two years to develop on healthy trees while larvae

develop in one year on stressed or heavily infested trees (McCullough and Siegert 2007, Tluczek et al. 2011).

A combination of biotic and abiotic factors influence host suitability and subsequently female feeding and oviposition preferences. In my study, *A. planipennis* adults consumed less leaf area and fewer beetles survived (14% in 2010, 33% in 2011) when caged with blue ash leaves (excised or intact) than when caged with black ash, green ash, white ash or Manchurian ash leaves. This is consistent with a study by Pureswaran and Poland (2009) that indicated *A. planipennis* will avoid blue ash leaves when given a choice between blue ash and black ash, green ash or white ash leaves. Given the unsuitability of blue ash leaves as an adult food source, it is perhaps not surprising that 71% of blue ash trees in our plantation had no larval galleries. In fact, larval density on blue ash, a North American species, was similar to that of Manchurian ash, an Asian ash species that shares an evolutionary history with *A. planipennis*.

While host suitability might explain why larval densities on blue ash trees were low (2.0 ± 0.98 galleries per m^2), it does not explain differences in gallery density among the other species where relationships among leaf area consumed, beetle survival and larval gallery densities were inconsistent. In 2010, survival was highest for beetles caged with excised black ash leaves (53%). Beetles caged with excised white ash and Manchurian ash leaves consumed more leaf area than beetles caged with black ash leaves, but survival was 42% lower. In 2011, beetles caged with intact white ash leaves consumed more leaf area than beetles caged with intact Manchurian ash leaves (I did not perform bioassays on black ash or green ash trees in 2011 due to poor tree health) but beetle survival was high (at least 73%) regardless of whether beetles were caged with white or Manchurian ash leaves. If oviposition choice were driven by adult host suitability, I might expect larval densities to be similar among black ash, green ash, white ash

and Manchurian ash because adult survival was relatively similar among these host species. Instead, gallery densities on black ash and green ash trees were at least 82 and 99% higher than gallery densities on white ash and Manchurian ash trees, respectively. While this does not negate the hypothesis that female host preference is driving larval densities, it does imply that females are making oviposition choices unrelated to the suitability of ash species as a food source.

When removing the bark from ash trees, I was extremely careful to examine individual larvae and galleries. On black ash and green ash trees, larval densities were so high (235.9 ± 36.41 and 220.1 ± 39.77 galleries per m^2, respectively) that bark fell away from the bole when I was removing trees from the plantation. In contrast, only 17 larvae (41% alive, 29% dead) were found on the blue ash trees, and 10 larvae (40% alive, 60% dead) were found on the Manchurian ash trees. During the debarking process, I examined the outer sections of bark and phloem to determine if neonate larvae were dying before they were able to penetrate the cambium. I found three first instar larvae on Manchurian ash that had died before they reached the cambium but the two early instar larval cadavers found on blue ash trees had died as third instars. It is important to note I was unable to find evidence (eggs, tissue discoloration or neonate cadavers) that female *A. planipennis* oviposit on blue ash and Manchurian ash trees at rates similar to green ash and black ash. I therefore suspect *A. planipennis* females are avoiding blue ash and Manchurian ash trees for oviposition. Among the dead larvae, I was unable to discern the cause of death but there was no evidence of parasitism or woodpecker predation. It is possible neonate larvae were killed by toxins as they began to chew into tree bark, but there were so few larvae on any of the Manchurian ash trees that further assessment using artificially positioned eggs would be necessary to confirm or negate this hypothesis.

There were no indications that any of the ash species responded to infestation by callusing over tunneling larvae, a mechanism healthy North American paper birch (*Betula papyrifera* Marsh.) trees use to defend phloem from a native *A. planipennis* congener, bronze birch borer (*Agrilus anxius* Gory) (Nielsen et al. 2011, Miller et al. 1991). In contrast, Duan et al. (2010) reported 32.0 to 41.1% and 17.5 to 21.5% of experimentally established and wild *A. planipennis* larvae, respectively, were killed by callus tissue produced by healthy green ash trees. While they report tree resistance in the form of callusing over was the "major source of (*A. planipennis* larval) mortality" in their study, it was not observed on any of the 4,016 galleries examined in my study. Perhaps it was not observed because my trees were smaller than the ones used in their study, but McCullough et al. have debarked hundreds of ash trees for a variety of studies and callusing over of larvae has not been observed in any of the healthy ash trees they have examined (D.G.M. unpublished data).

Adult *A. planipennis* often prefer to attack open grown trees (McCullough et al. 2009a, 2009b). Trees in our plantation were evenly spaced and most received full sun, as a result, tree location within the plantation did not appear to hinder or encourage *A. planipennis* infestation. Blue ash and Manchurian ash trees with zero galleries were located adjacent to black ash or green ash trees with 35 to 125 galleries per tree. Within individual blocks (five trees, one of each species), there was not a single case where black ash or green ash trees did not have higher gallery densities than blue ash, white ash and Manchurian ash trees.

Use of PB to enhance resistance to insect pests has been evaluated for several insects on an assortment of host plants. Enhanced insect resistance is typically attributed to an array of secondary responses that reflect altered growth patterns, but the exact mechanisms of resistance are rarely identified and resistance effects are variable and generally unpredictable. In paper

birch (*Betula papyrifera* Marshall), PB reduced radial growth and height and increased specific leaf mass and foliar tannin concentrations the year after PB application but not the following year. White-marked tussock moth (*Orgyia leucostigma* Smith) and gypsy moth (*Lymantria dispar* Linnaeus) survival, however, was reduced two years after treatment, but not the year that specific leaf mass and tannin concentrations increased (Chobadjian et al. 2011). In pear (*Pyrus* spp.), PB reduced pear psylla (*Cacopsylla bidens* Sulc.) oviposition and survival and development of nymphs. The authors originally hypothesized that differences in psylla performance would result from changes in foliar N concentrations, PB treatment, however, had no effect on foliar N concentrations, indicating that some other mechanism was acting on the psylla populations (Shaltiel-Harpaz et al. 2010).

In my study, ash species effects on *A. planipennis* host preference and host suitability overshadow most treatment effects. In my concurrent study, I showed that PB reduces aboveground growth which in theory might have provided additional resources for defensive chemical production but the results of my adult *A. planipennis* feeding and mortality bioassays were highly variable and no clear patterns emerged.

The majority of black ash and green ash trees in the plantation were heavily infested the first summer of our study and nearly all of them had died by August 2011. When I harvested the majority of black ash and green ash trees in spring 2011, they had 240 and 89 galleries per m^2, respectively, made by the 2010 larval cohort. Because larval densities were so high during the summer of PB application (2010), and because effects of PB typically do not become apparent during the year of application, there were likely no PB treatment effects on *A. planipennis* larval gallery densities for black ash and green ash. It is unfortunate that green ash trees were so heavily attacked before PB effects could become apparent as green ash is the most popular ash

tree in North American landscapes and urban areas (MacFarlane and Meyer 2003). The opposite scenario occurred with blue ash and Manchurian ash trees. Gallery densities on these species were so low that treatment effects would have been irrelevant.

Even without the burden of *A. planipennis* infestation, urban ash trees experience an array of environmental stresses (Poland and McCullough 2006). They are planted in confined areas (parking lot islands and sidewalk cubes) and subjected to drought, salt stress, soil compaction and nutrient deficiency (MacFarlane and Meyer 2005). Treating trees with PB in confined locations could reduce growth and increase root mass (Tanis, Chapter Two) and ultimately extend the period before trees outgrow available space. Healthy ash trees are less attractive to *A. planipennis* adults (McCullough et al. 2009a, 2009b) and presumably, trees treated with PB might have enhanced resistance to *A. planipennis* simply because they are healthier than their untreated counterparts.

Trees in our plantation were not treated with insecticide for two years prior to and the two years during our experiments, and yet almost 30% had no evidence of *A. planipennis* infestation. Many blue ash (43%) and Manchurian ash (37%) trees were completely free of *A. planipennis* galleries and 95% had <5 galleries (two blue ash trees had six galleries). In contrast, surrounding black ash and green ash trees were heavily infested and dying despite efforts to carefully protect them with physical barriers. My study confirms reports of blue ash and Manchurian ash inherent resistance (Rebek et al. 2007, Anulewicz et al. 2007, Tanis and McCullough 2012), but further investigation is necessary to identify resistance mechanisms.

Table 3.1: Results of repeated measures analysis of variance to assess effects of species, treatment and day (2010) and species, treatment, day and leaf type (2011) on survival when *Agrilus planipennis* adults were caged on excised black, blue, green, white or Manchurian ash trees (2010) and excised or intact blue, white and Manchurian ash trees (2011) treated with paclobutrazol, fertilizer or left as untreated control.

Effect	F Value	Degrees of Freedom	*P* Value
		2010	
Species	**23.03**	4, 874	**<0.001***
Treatment	0.11	2, 874	0.894
Species × Treatment	**10.94**	8, 874	**0.001***
Day	**114.79**	9, 874	**<0.001***
Species × Day	**1.91**	36, 874	**0.001***
Treatment × Day	0.86	18, 874	0.630
Species × Treatment × Day	0.76	72, 874	0.929
		2011	
Species	**126.13**	2, 999	**<0.001***
Treatment	2.36	2,999	0.095
Species × Treatment	0.27	4, 999	0.899
Day	**62.11**	8, 999	**<0.001***
Species × Day	**10.66**	16, 999	**<0.001***
Treatment × Day	0.42	16, 999	0.978
Species × Treatment × Day	0.17	32, 999	1.000
Leaf Type	0.18	1, 999	0.675
Species × Leaf Type	**26.32**	2, 999	**<0.001***
Treatment × Leaf Type	**4.20**	2, 999	**0.015***
Species × Treatment × Leaf Type	**6.32**	4, 999	**<0.001***
Day × Leaf Type	0.58	8, 999	0.796
Species × Day × Leaf Type	0.99	16, 999	0.460
Treatment × Day × Leaf Type	0.59	16, 999	0.893
Species × Treatment × Day × Leaf Type	0.45	32, 999	0.997

Table 3.2: *P* values generated by repeated measures least significant difference tests used to compare mean number of live *Agrilus planipennis* caged with leaves from black, blue, green, white or Manchurian ash leaves over a 14 day period. Bioassays were performed in June 2010. An "*" denotes significant differences in beetle survival (α=0.05) between host species.

		***P* Values**			
Ash Species	**Day**	**Blue**	**Green**	**White**	**Manchurian**
Black	1	0.868	0.869	0.760	0.869
	2	0.856	0.742	0.988	0.988
	3	0.820	0.610	0.610	0.844
	4	0.237	0.683	0.572	0.925
	6	**<0.001***	**0.028***	0.369	**0.007***
	8	**<0.001***	**0.012***	**0.031***	**0.003***
	10	**<0.001***	**<0.001***	**0.015***	**<0.001***
	12	**<0.001***	**<0.001***	**0.021***	**<0.001***
	14	**<0.001***	**0.005***	**0.008***	**0.005***
Blue	1		1.000	0.630	1.000
	2		0.878	0.867	0.867
	3		0.452	0.452	0.666
	4		0.107	**0.076***	0.196
	6		0.081	**0.002***	0.218
	8		0.120	**0.047***	0.297
	10		0.478	**0.013***	0.821
	12		**0.041***	**0.001***	0.060
	14		**0.020***	**0.013***	**0.020***
Green	1			0.634	1.000
	2			0.751	0.751
	3			1.000	0.751
	4			0.874	0.751
	6			0.179	0.616
	8			0.691	0.616
	10			0.081	0.634
	12			0.153	0.874
	14			0.874	1.000
White	1				0.634
	2				1.000
	3				0.751
	4				0.634
	6				0.064
	8				0.365
	10				**0.030***
	12				0.112
	14				0.874

Table 3.3: *P* values generated by repeated measures least significant difference tests comparing mean number of live *Agrilus planipennis* caged with leaves from blue, white or Manchurian ash leaves over a 14 day period. Bioassays were performed in June 2011. An "*" denotes significant differences in beetle survival (α=0.05) between host species.

		***P* values**	
Ash Species	**Day**	White	Manchurian
Blue	1	0.781	0.781
	2	1.000	0.487
	3	0.266	0.165
	4	0.331	0.126
	6	**<0.001***	**<0.001***
	8	**<0.001***	**<0.001***
	10	**<0.001***	**<0.001***
	12	**<0.001***	**<0.001***
	14	**<0.001***	**<0.001***
White	1		0.578
	2		0.487
	3		0.781
	4		0.578
	6		0.677
	8		0.890
	10		0.211
	12		0.165
	14		0.071

Table 3.4: Mean (±SE) number of surviving *Agrilus planipennis* adults caged with excised black, blue, green, white or Manchurian ash leaves in June 2010 or excised or intact blue, white or Manchurian leaves in June 2011. There were two male and two female *A. planipennis* per cage. Letters indicate differences among species and treatments (α=0.05).

Ash Species	Control	Fertilizer	Paclobutrazol
Black	3.4 ± 0.16^{a}	3.4 ± 0.10^{a}	3.1 ± 0.15^{a}
Blue	2.9 ± 0.16^{a}	2.2 ± 0.18^{b}	2.1 ± 0.19^{b}
Green	2.9 ± 0.18^{a}	2.5 ± 0.18^{b}	3.0 ± 0.15^{a}
White	2.4 ± 0.18^{b}	3.3 ± 0.14^{a}	3.0 ± 0.15^{a}
Manchurian	2.3 ± 0.22^{b}	2.7 ± 0.16^{ab}	2.9 ± 0.13^{a}

Ash Species		Control	Fertilizer	Paclobutrazol
Blue	Excised	2.8 ± 0.15^{de}	2.9 ± 0.16^{de}	2.7 ± 0.17^{de}
	Intact	2.7 ± 0.15^{e}	2.3 ± 0.20^{f}	2.8 ± 0.17^{de}
White	Excised	3.4 ± 0.09^{bc}	3.4 ± 0.11^{bc}	3.8 ± 0.06^{a}
	Intact	3.5 ± 0.09^{b}	3.4 ± 0.11^{bc}	3.1 ± 0.10^{c}
Manchurian	Excised	3.3 ± 0.10^{bc}	3.3 ± 0.11^{bc}	3.4 ± 0.11^{bc}
	Intact	3.9 ± 0.04^{a}	3.7 ± 0.06^{a}	3.8 ± 0.05^{a}

Table 3.5: Mean (±SE) number of *Agrilus planipennis* adult exit holes, parasitized larvae, woodpecker attacks, living larvae, dead larvae and total galleries per m^2 on five species of ash trees (N=21 trees per species) and *P* values generated by least significant difference tests comparing mean densities. An "*" denotes significant differences between species (α=0.05).

		***P* Values**			
		Blue	Green	White	Manchurian
Ash Species	**Mean (±SE)**	**Adult Exit Holes**			
Black	40.1 ± 8.61	**<0.001***	1.000	**<0.001***	**<0.001***
Blue	0.0 ± 0.00		**<0.001***	1.000	1.000
Green	30.4 ± 8.04			**<0.001***	**<0.001***
White	1.0 ± 0.39				1.000
Manchurian	0.0 ± 0.00				
		Parasitized Larvae			
Black	96.5 ± 21.3	**<0.001***	**0.043***	**<0.001***	**<0.001***
Blue	0.4 ± 0.27		**0.038***	0.963	1.000
Green	46.5 ± 15.64			0.205	**0.043***
White	11.3 ± 6.77				0.962
Manchurian	0.0 ± 0.00				
		Woodpecker Attacks			
Black	26.6 ± 4.91	0.229	**0.013***	0.733	0.245
Blue	0.2 ± 0.20		**<0.001***	0.922	1.000
Green	61.9 ± 14.90			**<0.001***	**<0.001***
White	10.2 ± 4.93				0.924
Manchurian	0.0 ± 0.00				
		Live Larvae			
Black	38.9 ± 9.38	0.115	0.544	0.404	0.126
Blue	1.0 ± 0.60		**0.001***	0.971	1.000
Green	63.9 ± 23.65			**0.013***	**0.002***
White	10.3 ± 3.02				0.970
Manchurian	0.6 ± 0.53				

Table 3.5 (cont'd)

		Dead Larvae (cause unknown)			
Black	30.5 ± 11.37	**0.002***	0.353	**0.006***	**0.003***
Blue	0.4 ± 0.29		0.286	0.999	1.000
Green	14.6 ± 5.78			0.438	0.329
White	2.1 ± 0.56				1.000
Manchurian	0.6 ± 0.36				
		Total Galleries			
Black	235.9 ± 36.41	**<0.001***	0.821	**<0.001***	**<0.001***
Blue	2.0 ± 0.98		**<0.001***	**<0.001***	0.681
Green	220.1 ± 39.77			**<0.001***	**<0.001***
White	40.7 ± 11.61				**<0.001***
Manchurian	1.5 ± 0.67				

Table 3.6: Number of *Agrilus planipennis* emerged adults from 2009 and 2010 larval cohorts and total larvae from the 2010 and 2011 larval cohorts including live and dead early instar larvae (first, second and third), total late instar larvae (fourth and prepupae) including those parasitized by *Atanycolus* spp. or killed by woodpeckers found on five *Fraxinus* species in a plantation, N=21 trees per species. Larvae from the 2010 cohort were progeny from *A. planipennis* adults that emerged in 2009. Larvae from the 2011 cohort were progeny from adults that emerged in 2010. Trees were harvested before larvae from the 2011 cohort could pupate (Fall 2011).

	Black Ash	**Blue Ash**	**Green Ash**	**White Ash**	**Manchurian Ash**
Phloem Area (m^2)	7.6	9.9	13.6	15.3	8.8
Emerged Adults, 2009 larval cohort	***191***	***0***	***135***	***0***	***0***

	Live	**Dead**	**Live**	**Dead**	**Live**	**Dead**	**Live**	**Dead**	**Live**	**Dead**
Larvae, 2010 cohort	**175**	**614**	**0**	**0**	**206**	**830**	**49**	**114**	**0**	**0**
Instars 1-3	*7*	*104*	*0*	*0*	*0*	*38*	*0*	*9*	*0*	*0*
4th Instars + Prepupae	*168*	*510*	*0*	*0*	*206*	*792*	*49*	*105*	*0*	*0*
Parasitized		345		0		170		0		0
Woodpecker Predation		145		0		505		104		0
Emerged Adults, 2010 larval cohort		***37***		***0***		***170***		***12***		***0***

	Live	**Dead**	**Live**	**Dead**	**Live**	**Dead**	**Live**	**Dead**	**Live**	**Dead**
Larvae, 2011 cohort	**23**	**281**	**7**	**10**	**367**	**585**	**122**	**197**	**4**	**6**
Instars 1-3	*0*	*29*	*4*	*2*	*8*	*71*	*54*	*19*	*0*	*3*
4th Instars + Prepupae	*23*	*252*	*3*	*8*	*359*	*514*	*68*	*178*	*4*	*3*
Parasitized		220		3		293		155		0
Woodpecker Predation		21		2		205		15		0

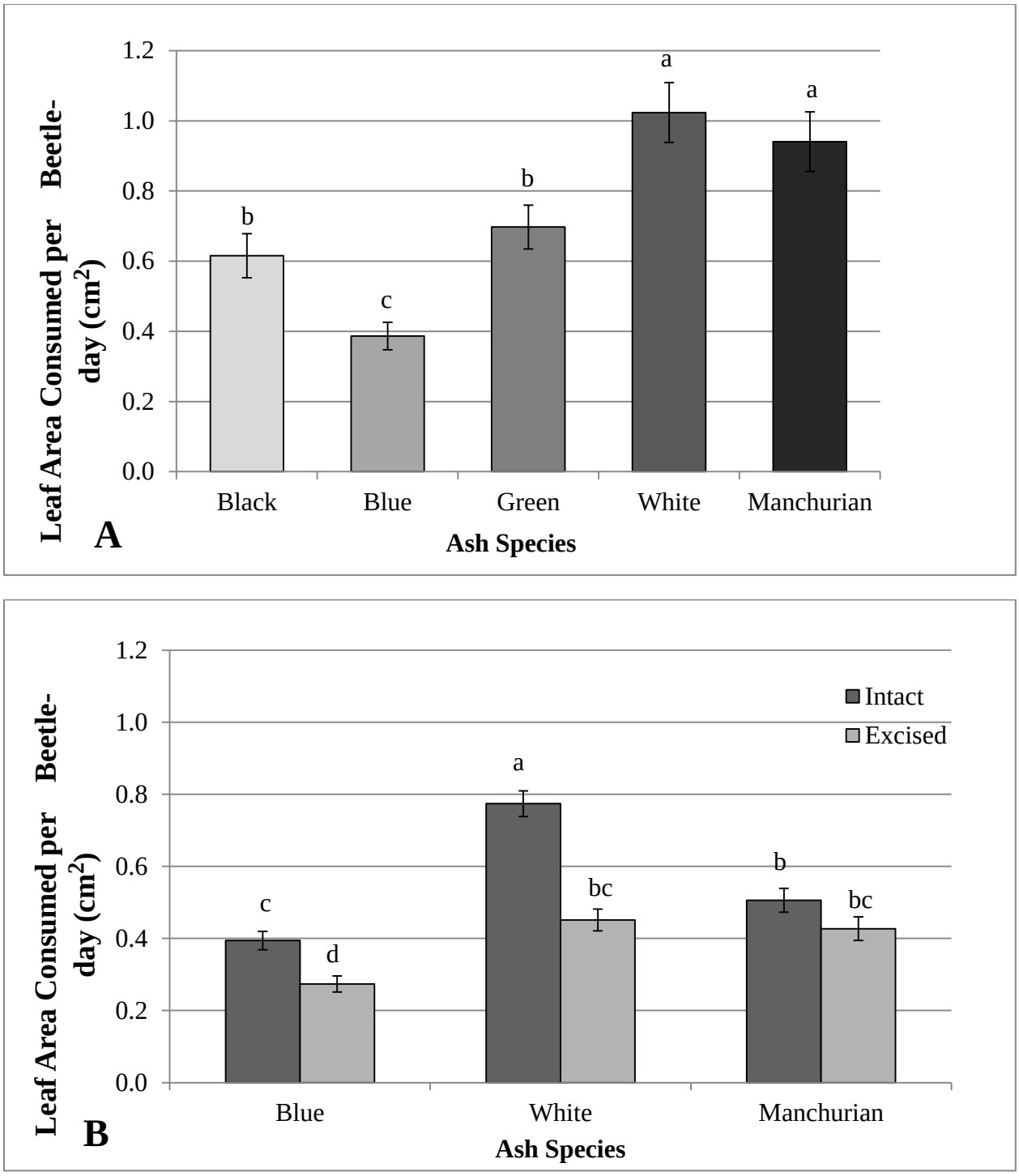
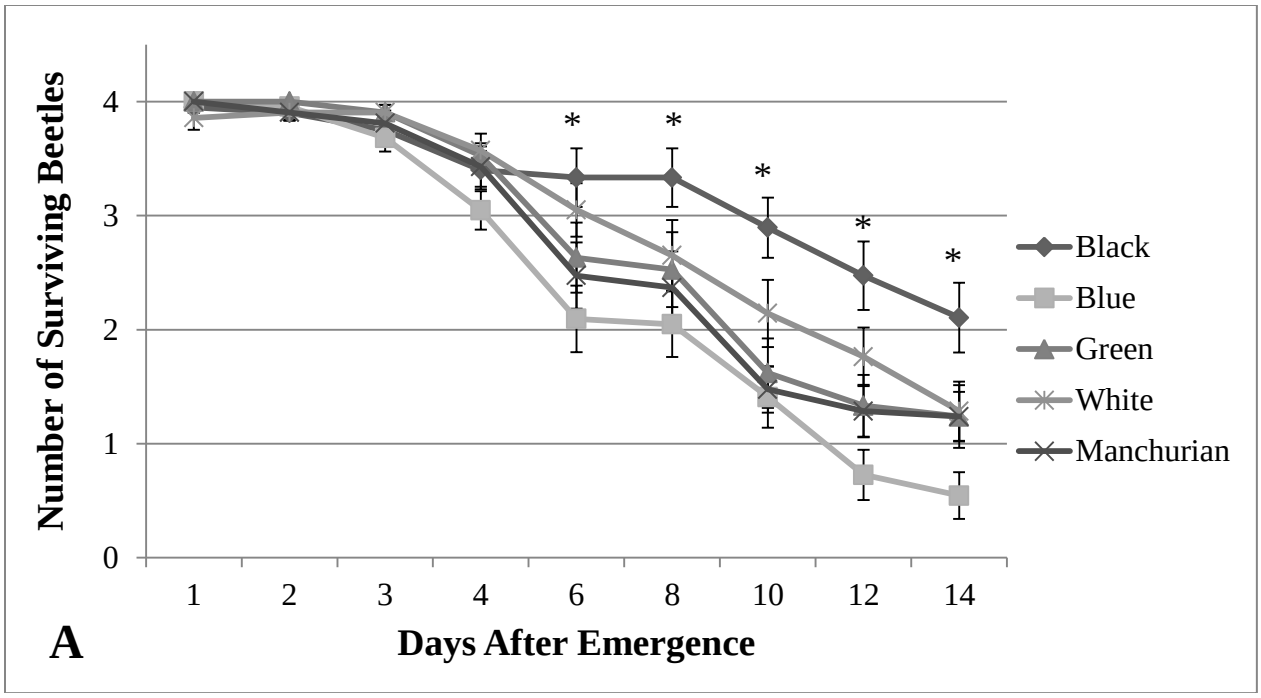


Figure 3.1: Mean (±SE) leaf area (cm^2) consumed per beetle day by *Agrilus planipennis* caged with (A) excised leaves from black, blue, green, white or Manchurian ash trees in 2010 or (B) excised or intact leaves from blue, white or Manchurian ash in 2011. Letters indicate differences among species and leaf types (α=0.05).



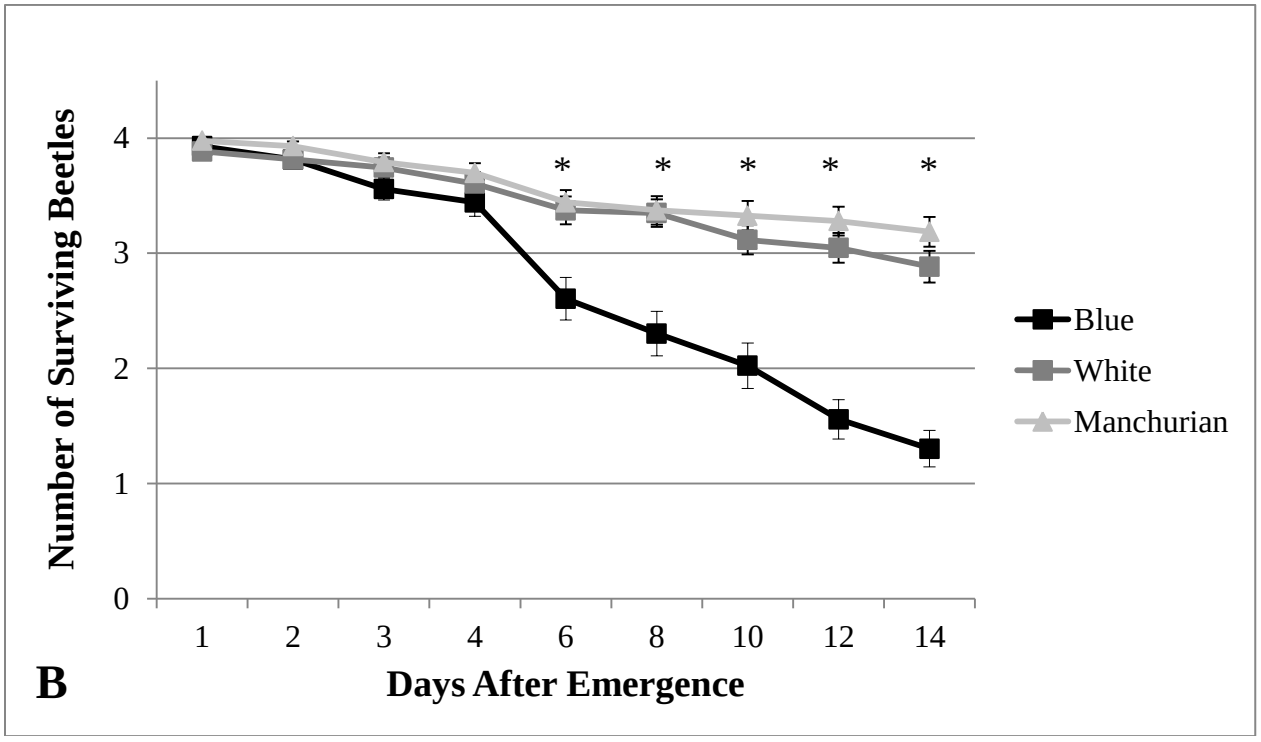


Figure 3.2: Mean (±SE) number of surviving *Agrilus planipennis* adults caged with (A) black, blue, green, white or Manchurian ash leaves in 2010 or (B) blue, white or Manchurian ash in 2011. An "*" denotes significant differences among species on that day (α=0.05).

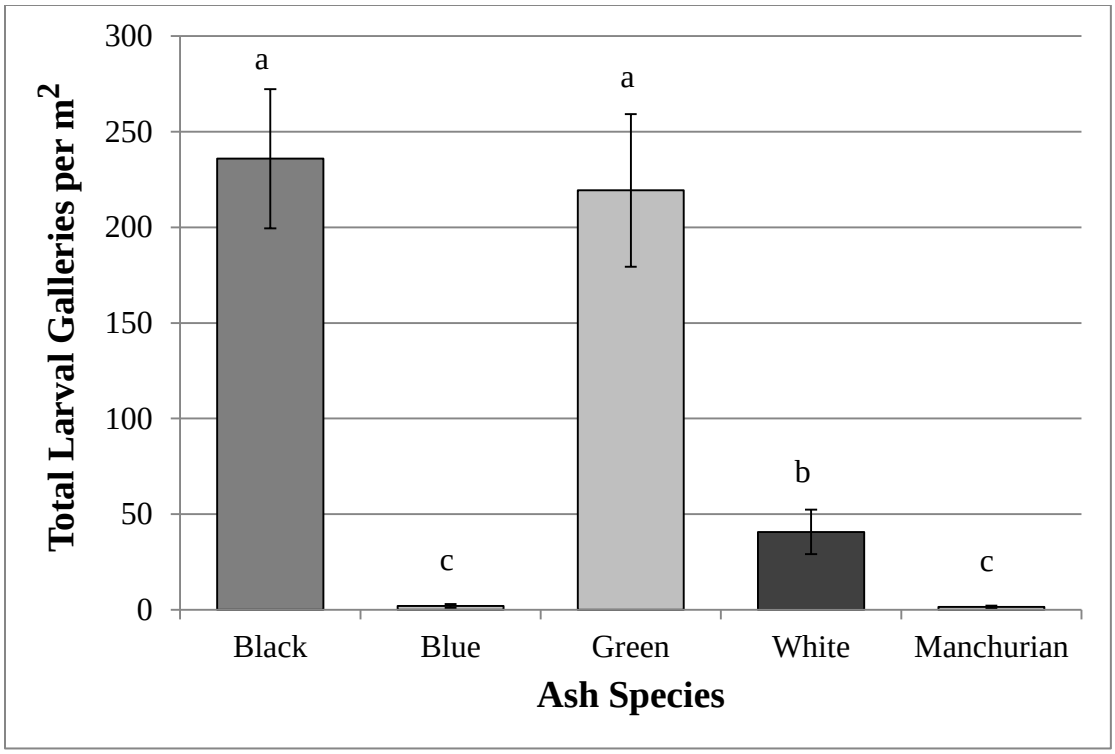


Figure 3.3: Mean (±SE) number of *Agrilus planipennis* larval galleries per m^2 of debarked phloem on black, blue, green, white or Manchurian ash trees harvested in 2011. Letters indicate differences among species (α=0.05).

CHAPTER FOUR

Evaluation of ash xylem discoloration and architecture associated with macro-injections of a systemic insecticide

ABSTRACT

Emerald ash borer (EAB, *Agrilus planipennis* Fairmaire) (Coleoptera: Buprestidae) was first identified near Detroit, Michigan in 2002. It has killed tens of millions of ash (*Fraxinus*) trees in 19 states and two Canadian provinces. Systemic insecticides applied as trunk injections are often used to protect ash trees from EAB, but wounds and injury are a concern. I assessed bark cracks and wound closure on 22 green ash (*Fraxinus pennsylvanica* Marsh.) and 24 white ash (*Fraxinus americana* L.) trees macro-injected with either a low or medium-high rate of emamectin benzoate in either 2008 or 2008+2009. Of the 233 injections sites assessed, only 12 (5%) injection sites had associated bark cracks and there was no evidence of pathogen infection. On 39 (85%) of the 46 trees, new xylem was healing over injection sites. Ash species, insecticide application rates and injection frequency had little effect on incidence of bark cracks or wound closure. Xylem discoloration occurred around all injection sites, but it was likely caused by a marker dye and was not indicative of tissue damage. In a related study, I examined xylem anatomy in blue ash (*Fraxinus quadrangulata* Michx.) and green ash trees (caliper diameter=77.3±2.73 and 81.0±2.52 mm, respectively). Trunk injected insecticides can experience friction created by passing through xylem lumen or bordered pits. I assessed these structures with CLSM to determine if xylem anatomy could influence insecticide uptake. Cross sectional area of earlywood lumen was 55% larger and hydraulic conductivity was 67% greater in green ash than blue ash, however, densities of bordered pits were similar between the two species.

INTRODUCTION

Emerald ash borer (EAB, *Agrilus planipennis* Fairmaire) (Coleoptera: Buprestidae), a phloem boring insect pest from Asia, was first identified near Detroit, Michigan in 2002 (Cappaert et al. 2005). Since its arrival, EAB has killed over 40 million ash (*Fraxinus* spp.) trees in southeast Michigan alone and tens of millions in 18 additional states and two Canadian provinces (EAB.info 2013). A variety of systemic insecticides are available to protect ash trees from EAB, including products with azadirachtin, dinotefuran, emamectin benzoate and imidacloprid as their active ingredients (Herms et al. 2009, McKenzie et al. 2010). Logistical constraints, environmental concerns and cost typically limit use of these products to high-value, urban ash trees (Mercader et al. 2011).

Most products used to protect ash trees from EAB are applied systemically as soil drenches or injections, basal bark sprays or trunk injections (Herms et al. 2009). Ash trees are ring porous (Dickison 2000) and systemic products are translocated upward through the xylem tissues in the outer sapwood to the canopy (Herms et al. 2009; Mota-Sanchez et al. 2009; Choat et al. 2007). Soil applications must be applied annually and contain the neonicotinoids imidacloprid or dinotefuran. Dinotefuran can also be applied as a basal trunk spray, but annual treatment is needed (Herms et al. 2009). Environmental contamination from soil and basal bark applications can be a concern, however, especially near open water or sites with high water tables. Neonicotinoids can persist in aquatic environments and potentially harm aquatic invertebrates (Young-Song et al., 1997; Tišler et al. 2009). Systemic products that are injected directly into the base of tree trunks eliminate insecticide drift and minimize non-target or environmental effects (Sanchez-Zamora and Escobar 2000, Sur and Stork 2003, Herms et al. 2009, Hahn et al. 2012). Emamectin benzoate, sold as TREE-äge® (4.0% emamectin benzoate,

Syngenta Crop Protection, Inc., Greensboro, NC, USA), an avermectin product, has become especially popular in many EAB infested areas (Smitley et al. 2010; McCullough et al. 2011; Mercader et al. 2011). In a large scale study, TREE-äge applied in the spring provided >99% EAB control for at least two years (Smitley et al. 2010; McCullough et al. 2011).

Potential wounds from trunk injections, however, can be a concern for homeowners and tree care professionals (Doccola et al. 2011). Wounding from the holes made during the application process can sever conductive tissues and may provide an entry point for pathogens (Shigo 1977; Lawson and Dahlsten 2003; Smith and Lewis 2006). Two types of trunk injections are generally used to apply insecticides: micro-injections are applied by drilling holes ≤ 0.5 cm (3/16") in diameter whereas macro-injections are applied by drilling holes ≥ 1.0 cm (3/8") in diameter (Costonis 1981). Micro-injections generally deliver 1 to 3 ml of product per injection site and have been used for many years (Costonis 1981). In contrast, macro-injections like those performed by the TREE IV Micro-Infusion® system (Arborjet, Inc. Woburn, MA, USA), are relatively new. Macro-injection systems were designed to inject high rates and volumes of product into large trees. While they require larger drill holes, the higher volume injections improve within tree product distribution (Doccoloa and Wild 2012).

Pressure associated with trunk injection varies among application systems and can potentially cause tree damage. The Wedgle Direct-Inject® micro-injection system (ArborSystems, Omaha, Nebraska, USA), does not require drilling but the injection pressure created during injection can be high enough ≥ 334 kPa (≥50 PSI) to physically separate the bark from the cambium (Smith and Lewis 2006). The J.J. Mauget Company (Arcadia, CA, USA) uses a closed system, micro-injection capsule that is pre-loaded with an imidacloprid (Imicide®,

10% imidacloprid, J.J. Mauget Co., Arcadia, CA, USA). These capsules are inserted into 0.3 cm (1/8") diameter holes drilled into the base of trees. Insecticide is injected into the sapwood under approximately 41 kPa (6 PSI) of pressure (Mauget.com, Accessed 14 April 2013). TREE-äge is typically injected with a TREE IV Micro-Infusion®, or a QUIK-jet® system (Arborjet, Inc. Woburn, MA, USA). Holes are drilled around the base of trees with a 0.95 cm drill bit (3/8") and Arbor plugs® are inserted to prevent leaking and seal the drill hole (Arborjet.com, Accessed 14 April 2013). Insecticides are injected with a needle into the sapwood under approximately 310 kPa (45 PSI) or 1379 kPa (200 PSI) of pressure for the TREE IV or QUIK-jet, respectively. TREE-äge is efficacious for up to three years, which means substantially fewer injection sites compared to products that must be applied annually (Smitley et al. 2010; Doccola et al. 2011; McCullough et al. 2011).

In addition to wounds made by drilling, trunk injected insecticides can also cause internal tissue discoloration in multiple genera, including ash (Shigo et al. 1977, Smith and Lewis 2006, Mota-Sanchez et al. 2009, Doccola et al. 2011, Tanis et al. 2012). When maple and ash trees were injected with imidacloprid, discoloration in tissues adjacent to injection wounds was considered a wound compartmentalization response (Smith and Lewis 2006). If tissue discoloration is indicative of compartmentalization, insecticide distribution through the tree could be compromised because barrier zone tissues rarely translocate (Shigo 1977). Whether discoloration is indicative of wounding, however, is questionable. In a study where ^{14}C-imidacloprid (Imicide®, J.J. Mauget Co., Arcadia, CA, USA) was injected into small ash trees (Mean DBH = 4.5 cm), discolored areas adjacent to injection sites had 75 to 300 times higher imidacloprid equivalent concentrations than adjacent unstained tissues (Mota-Sanchez et al.

2009, Tanis et al. 2012). Whether cells were viable, however, is unknown. Tissue discoloration was also observed in four green ash (*Fraxinus pennsylvanica* Marsh.) trees (mean DBH = 27.6 cm) treated with TREE-äge but Doccola et al. (2011) reported discolored areas remained "firm" and no symptoms of infection or deterioration were observed four years after the injections. While their study indicated that discoloration was not indicative of tree damage, their sample size was small and the integrity of xylem cells was not evaluated.

Although trunk injection systems and insecticide formulations have been continually refined, potential effects on hydraulic conductivity and tree response to macro-injection remain relatively unevaluated. Understanding the effects of macro-injections on ash trees is especially important given the increasingly wide use of TREE-äge for EAB protection. Objectives of our study were to determine if 1) TREE-äge trunk injections created external bark cracks or necrosis in green ash or white ash trees; 2) discoloration in the xylem was indicative of tree injury and 3) whether wounding varied between ash species, application rates or frequency of TREE-äge injections

Success of trunk injections can also be affected by an array of physical parameters (tree health, size, previous injury), environmental factors (air temperature, soil moisture) and cultural practices (application timing, delivery method) (Smith and Lewis 2005, Doccola et al. 2006, Mota-Sanchez et al. 2009, Tanis et al. 2012). Uptake and translocation of systemic products is further influenced by root, xylem and crown anatomy (Tatter et al., 1998, Young, 2002, Mota-Sanchez et al. 2009, Tanis et al. 2012).

Interspecific variability may also affect the rate at which systemic products move into trees. Among individual ash trees, injection efficiency and efficacy can be highly variable among trees depending on soil moisture and weather related factors that affect transpiration and

uptake (Mota-Sanchez et al. 2009; Tanis et al. 2012). Blue ash (*Fraxinus quadrangulata* Michx.) trees, for example, are reportedly difficult to trunk inject compared to green ash or white ash trees. Xylem architecture is known to play an important role in the translocation of insecticides within ash trees (Tanis et al. 2012), therefore, I hypothesize that interspecific differences in xylem anatomy might affect trunk injected insecticide movement in trees. Trunk injected products are subject to two sources of friction: 1) resistance created by vertical passage through xylem lumen, the hollow portion of the xylem vessel elements and 2) resistance resulting from lateral passage in the xylem through the bordered pits (Choat et al. 2007). Our second goal was to measure blue ash and green ash earlywood lumen area and bordered pit densities using confocal laser scanning microscopy (CLSM).

METHODS

Trunk Injection Potential Injury

I evaluated injection sites on 46 ash trees growing in four locations in Ionia, Genesee and Clinton Counties in central Michigan. The 22 green ash trees ranged in size from 4.3 to 11.5 cm diameter at breast height (DBH, 1.4 meters aboveground) (mean DBH= 7.13 ± 0.45 cm) and the 24 white ash ranged in size from 4.2 to 8.9 cm DBH (mean DBH = 6.90 ± 0.24 cm).

Between 21 and 23 May 2008 all 46 trees were injected with TREE-äge (4.0% emamectin benzoate, Syngenta Crop Protection, Inc., Greensboro, NC, USA). A total of 24 trees were treated using the lowest label application rate (EB-low) (0.1 g a.i. per 2.5 cm DBH) applied with a QUIK-jet system (Arborjet, Inc. Woburn, MA, USA). The other 22 were treated with a medium-high rate (EB-high) (0.4 g a.i. per 2.5 cm DBH) plus an equal amount of water applied with a TREE IV Micro-Infusion system (Arborjet, Inc. Woburn, MA, USA). The number of injections sites was determined by tree DBH, according to label directions. Injection sites were evenly spaced and care was taken to avoid placing injection sites near injured or dead tissue. Injection sites were drilled into the base of each tree with a 0.95 cm drill bit (3/8"). Arbor plugs (#4, 0.95 cm (3/8"), Arborjet, Inc. Woburn, MA, USA) were carefully inserted with a set tool and plastic mallet, according to label directions.

On 2-4 June 2009, 22 trees, including 11 EB-high and 11 EB-low trees were re-treated. Holes were drilled as above, approximately 5 cm above and offset from 2008 injections, according to label directions. The remaining 24 trees, including 11 EB-high and 13 EB-low trees were not re-treated. Trees were felled between 10 October and 29 December 2009. After felling, the basal 50 cm of each bole was brought to the laboratory and cut into 5.1 cm thick

cross sections. Cross sections were reassembled after cutting and reference points were established to ensure proper orientation throughout the experiment.

I examined a total of 239 injection sites on 301 cross sections from the 46 trees. Six injection sites had no associated discoloration (one 2008 EB-high, one 2008 EB-low tree, four 2008+2009 EB-high trees) because the applicator determined plugs were set improperly and no insecticide was injected. These six injection sites were excluded from further analyses. Of the 233 injection sites examined, 50 were from the 11 EB-high trees treated in 2008, 77 were from the 11 EB-high trees treated in 2008 + 2009, 42 were from the 13 EB-low trees treated in 2008 and 64 were from the 11 EB-low trees treated in 2008 + 2009. I assessed discoloration depth, width and height for each injection site. Discoloration depth (mm) was the longest distance between the outside edge of discolorations (near the bark) to the inside edge (near the central pith) (Fig. 4.1). Discoloration width (mm) was the widest distance between left and right edges of the discoloration associated with a single site (Fig. 4.1). Discoloration height (cm) was the vertical distance discoloration persisted above and below the basal cross sections. I also measured bark thickness at two opposite points on each cross section.

I visually assessed all injection sites to evaluate their condition (Fig 4.1). I assumed xylem produced during the same year trees were injected was latewood (trees were injected in May) while xylem produced the year after injections was both earlywood and latewood (Dickison 2000). I visually examined the cross sections and recorded external bark cracks, internal xylem necrosis and evidence of pathogen infection.

Transverse tissue samples (approximately 5 × 3 mm) were removed from the discolored/unstained tissue interface (Fig. 4.1) of randomly selected injection sites on 20 different trees using a razor blade. I removed 5 to 10 samples from each injection site to ensure

that I would have quality samples. After removal, samples were immediately placed into fixing solution (50% ethanol, glycerol, formaldehyde, 18:1:1) for at least 72 hours. After fixation, samples were placed in a solution of safranin orange dye (1% safranin, Carolina Biological Supply Company, Burlington, NC, USA) for 15 minutes, then dehydrated through 30, 50, 75, 90 and 100% ethanol (Decon Labs, Inc., King of Prussia, PA, USA), then rehydrated backward through the same solutions. Samples remained in each alcohol solution for 10 minutes. Solutions were changed multiple times during each cycle to remove excess dye. After rehydrating, samples were placed in deionized water for 15 minutes then processed through 25, 50 and 75% glycerol solutions (1 hour each with frequent decanting) and finally left overnight in 100% glycerol. Samples were viewed with an Olympus Fluoview 1000® confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan) the following day.

I used confocal laser scanning microscopy (CLSM) to evaluate sapwood integrity. All tissue samples were illuminated with argon (λ=543 nm) and helium-neon (λ=488 nm) lasers. Single channel z-scan collections were recorded (Hutzler et al. 1989). A 10× dry objective was used to magnify transverse samples removed from the discolored/unstained interface of trees trunk injected with TREE -äge. Total slice depth ranged from 10.2 to 14.5 µm and 7 to 21 slices per sample were obtained depending on optimal image acquisition parameters.

Xylem Architecture

I assessed xylem architecture in young blue ash and green ash trees growing in a common garden setting at the Michigan State University Tree Research Center (MSU TRC) in Okemos, Michigan (Ingham County). Trees which arrived as whips (≤1cm diameter) were planted in April 2006 and protected from EAB colonization using a cover spray in 2006-2007 and by physical barriers from 2008-2012). Trees were watered twice per week or as needed with drip

irrigation and fertilized annually with a top dressing of Harrel's Pro-Blend with Micros (19-5-10) (Harrell's, Lakeland, FL, USA).

On 12 July 2012, three blue ash (mean (±SE) caliper diameter=77.3 ± 2.73 mm) and three green ash trees (81.0 ± 2.52 mm) (all trees were 7 years old) were injected with safranin orange dye (1% safranin, Carolina Biological Supply Company, Burlington, NC, USA) through two injection sites on opposite sides at the base of each tree using a 0.95 cm drill bit (3/8"), 20 cm above the ground. Arbor plugs (#4) were carefully inserted into each injection site as described above. I used the QUIK-jet system (Arborjet, Inc. Woburn, MA, USA) to inject 15 ml of safranin into each injection site (30 ml per tree). I was unable to inject dye into the sites on two of the blue ash trees, so I drilled two new injection sites on each tree, 5 cm above and offset from the original injection sites and successfully injected the dye. After injections were complete, trees were left untouched for 24 hours, they were then felled, returned immediately to the lab and dissected to assess within tree translocation. I also removed two cross sections (1 cm thick, each) from each bole, 10 cm above the injection sites.

Each cross section was dissected with a chisel to isolate wood stained by the injected safranin. Because earlywood vessel size can vary from year to year depending on environmental conditions (Dickison 2000), these stained portions were further dissected to isolate stained xylem produced in 2011. I removed several thin (approximately 200 µm thick) transverse and tangential cross sections with a razor blade. After removal, samples were dehydrated with alcohol and cleared with glycerol as described above.

Samples were viewed with the Olympus Fluoview 1000® confocal laser scanning microscope the following day. Transverse samples taken from trees injected with safranin were magnified with a 10× dry objective. Slice depth ranged from 5.0 to 11.5 µm and the number of

slices ranged from 14 to 22 per sample. Blue ash and green ash xylem elements on tangential samples were magnified with a 20× dry objective. Total slice depth was 1.0 to 2.0 µm and the number of slices ranged from 38 to 62 slices per sample. Within these samples, bordered pits were further magnified with a 1.8× zoom, slice depth was 1.0 µm for all samples and the number of slices ranged from 44 to 95 per sample.

I used CLSM to determine the cross sectional area of all earlywood lumen that were larger than 30 µm in each sample (these were the vessels that translocated a majority of the dye). I examined 48 transverse samples (six trees, two injections per tree, two cross sections per tree, two transverse samples from each injection stain on each cross section) from earlywood produced in 2011. I used Olympus Fluoview® software (FV10-ASW Viewer 3.0, Olympus Corporation, Tokyo, Japan) to determine the length (µm) and width (µm) of large earlywood vessels in each image (image size = 1.6 mm^2). I then calculated lumen cross-sectional area using the formula ellipse area = $\pi \times (\text{length} / 2) \times (\text{width} / 2)$ (Figs. 4.2 and 4.3). Total cross sectional area of large earlywood lumen was obtained by summing the cross sectional areas.

To assess average density of bordered pits, I identified 48 vessels (six trees, two injections per tree, two cross sections per tree, one tangential sample per injection on each cross section, two vessels within each tangential sample) and assessed the number of bordered pits within two 50 × 50 µm sections of each vessel (Figs. 4.4 and 4.5). I calculated the bordered pit density per vessel by averaging the number of bordered pits within the two 50 × 50 µm sections.

Statistical analysis

Data were analyzed using SAS statistical software (SAS Institute, Inc. 1989). Assumptions of normality were tested with residual plots and the Shapiro-Wilk test (Shapiro and Wilk 1965). Width and length of discolored tissue on cross sections from trees treated with

TREE-äge were normalized using log transformations (Ott and Longnecker 2001), while all other variables met assumptions of normality. Three-way ANOVA was performed to assess effects of ash species (green or white), TREE-äge rate (EB-low or EB-high) and number of applications (2008 only or 2008 + 2009) and their interactions on the presence of xylem laid over injection sites, presence of bark cracks or necrosis and discoloration depth, width and height. When ANOVA results were significant ($\alpha \leq 0.05$), Fisher's protected least significant difference (LSD) multi-comparison tests were applied with Tukey's adjustment for unbalanced data sets (Ott and Longnecker 2001) to determine differences between means. For the blue ash and green ash trees injected with dye, I used Student's *t* tests to determine if earlywood lumen cross sectional area, cross sectional area of all earlywood lumen (>30 µm) per image, density of earlywood vessels or density of bordered pits differed between blue ash and green ash trees.

RESULTS

Trunk Injection Potential Injury

Diameter at breast height of green ash and white ash trees used for this study ranged from 10.7 to 29.2 cm but DBH was similar among treatment combinations (EB low or EB high trees treated in 2008 or 2008+2009) (P=0.65). The number of injections on EB-high and EB-low trees treated only in 2008 was similar and averaged 4.6 ± 0.53 and 3.3 ± 0.24 injections per tree, respectively. The number of injection sites on EB-high and EB-low trees treated in 2008 + 2009 was also similar and averaged 7.4 ± 0.54 and 5.8 ± 0.54 injections per tree, respectively. Number of injections did not vary between species (P=0.75) nor was the species × insecticide rate interaction significant (P=0.37).

Tree species, insecticide application rate, injection frequency and the interactions of the factors did not affect whether or not xylem was growing over drilled injection sites (Table 4.1). Xylem was healing over 134 (58%) of the injection sites, but 42% of the injection sites were still completely open. Among the 46 trees examined in this study, 39 (85%) had new xylem growing over at least one injection site and 37 trees (80%) had new tissue growing over multiple injection sites.

I assessed 12 trunk injuries near injection sites on six trees (13%), the remaining 40 trees had no evidence of injury. All of the injuries were vertical bark cracks (six bark cracks on green ash trees, six bark cracks on white ash trees). Among the 13 EB-low trees that were treated in one year (2008), three trees had bark cracks, but no trees had internal necrosis or cracking. Among the 11 EB-low trees that received two treatments (2008 + 2009), one tree had bark cracks but none of the trees had internal necrosis or cracking. Among the 11 EB-high trees treated in one year (2008), one tree had bark cracks but no trees had internal necrosis or cracking. Among

the 11 EB-high trees treated in two years (2008 + 2009) one tree had bark cracks, but no trees had internal necrosis or cracking. The number of bark cracks was higher (Table 4.1) in trees that received only one treatment in 2008 (nine bark cracks) than trees that received treatments in 2008+2009 (three bark cracks). There were no significant interactions among species, insecticide rate or number of injections (Table 4.1). One white ash EB-low tree treated in 2008 had seven injections sites, four of which had associated bark cracks.

Depth and width of discolored xylem were not affected by species, treatment rates, number of applications or their interactions (Table 4.1). Average (±SE) discoloration depth was 31.6 ± 3.25 mm while average (±SE) discoloration width was 13.7 ± 1.22 mm. Discoloration height was 63% higher (P<0.001) in EB-high trees (32.3 ± 2.4 cm) than in EB-low trees (12.1 ± 0.96 cm) (Table 4.1) but was similar among species and between trees treated in 2008 or 2008 + 2009. No interactions were significant (Table 4.1). At least 67% of trees had discolored tissue below the basal felling cut, which was at ground level.

There was no evidence that tissue damage was associated with discoloration among any of the samples examined with CLSM (Fig. 4.6). Cell lysis was not present in any of the samples and there were no visible symptoms of tissue damage or infection. I scrutinized areas where discolored/unstained tissue interfaces were well-defined, as these areas might be indicative of a barrier zone (Shigo 1984), but I saw no evidence that trees attempted to compartmentalize around discolored tissues (Fig. 4.6). When preparing tissues for examination with CLSM, much if not all of the discoloration disappeared from the xylem tissue. In addition, discoloration associated with 2008 injection sites or 2009 injection sites overlapped and were continuous through cross sections indicating tissues discolored in 2008 were translocating product in 2009.

Xylem Architecture

The safranin orange dye injected in July 2012 was translocated primarily through large earlywood vessels (> 30µm) formed in 2011 and 2012. Dye was present in the terminal leader and lateral branches of all six trees within 24 hours of injection. Cross sectional area of large earlywood lumen was 55% smaller in blue ash trees than green ash trees ($t_{[1,689]} = 13.48$; $P < 0.001$). Cross sectional area of earlywood lumen in blue ash averaged 0.14 ± 0.02 mm^2 per lumen compared to 0.31 ± 0.02 mm^2 in green ash. There were more ($t_{[1,47]} = 11.52$; $P < 0.001$) earlywood vessels per image in the blue ash images (19 ± 1.02 per image) than in green ash images (12.4 ± 0.57 per image), but the total cross sectional area occupied by all of the large earlywood vessels in an image was lower ($t_{[1,47]} = 7.41$; $P < 0.001$) in blue ash (141.3 ± 20.0 μm^2) than in green ash (308.6 ± 19.2 μm^2).

The density of bordered pits was similar between species ($P = 0.13$) and averaged 76.3 ± 3.9 and 70.5 ± 2.2 pits per 2,500 μm^2 for blue ash and green ash, respectively. I observed that helical thickening was present in all blue ash vessels (Fig. 4.7) but only 45% of green ash vessels. The degree of helical thickening was also much more pronounced in blue ash (Fig. 4.7) than in green ash (Fig. 4.8).

DISCUSSION

Over the next ten years, approximately 37.9 million ash trees in urban areas across the United States are projected to be within areas infested by EAB (Kovaks et al. 2010). When EAB was first discovered in 2002, many landscape ash trees were removed. Treatment success was variable and the logistics and cost of annual treatment were impractical (Herms et al. 2009). Registration of TREE-äge in 2010 and evidence of highly effective control, however, have reduced the costs and logistical constraints associated with treatment. As a result, large mature ash trees, and the ecosystem services they provide, are being preserved in EAB infested areas (Smitley et al 2010; Doccola et al. 2011; McCullough et al. 2011; McCullough and Mercader 2012).

Our study indicates that damage to ash trees is rare when trees are trunk injected with TREE-äge using QUIIK-jet or TREE IV Micro-Infusion systems. Among the 233 injection sites on the 46 white ash and green ash trees assessed in this study, only 5% of injection sites had evidence of bark cracks and none had disease symptoms. Our results are consistent with those of Doccola et al. (2011) who found no "cracking, oozing or decay" on 4 green ash trees (DBH 21.5 to 36.3 cm DBH; mean = 27.6 cm) injected with emamectin benzoate (the four trees were treated in 2005, and two of these were retreated in 2008) (TREE-äge, injected with a TREE IV Micro-Infusion system). Unlike imidacloprid products that require annual applications, TREE-äge is efficacious for up to three years (Smitley et al. 2010; McCullough et al. 2011). Reduced application frequency means fewer opportunities for wounding.

Many studies have reported the existence of tissue discoloration adjacent to trunk injection sites, but the integrity of discolored tissue has not been previously assessed (Smith and Lewis 2006; Mota-Sanchez et al. 2009; Doccola et al. 2010; Tanis et al. 2012). In this study,

multiple lines of evidence show that discoloration surrounding injection sites was not indicative of tissue damage. Results from observations with CLSM determined the integrity of xylem tissues immediately surrounding injection sites was sound regardless of ash species, insecticide rate or the number of injections. In addition, when I prepared samples for examination with CLSM, much, if not all, of the discoloration was removed when samples were soaked in alcohol. If discoloration was indicative of wounding, I would have expected it to persist. I also found discolored tissues from 2008 injections effectively translocated products injected in 2009, which would not have been possible if discoloration represented barrier zone formation (Shigo 1977). There were also six "blank" injection sites (no insecticide was injected) with no associated discoloration further suggesting the insecticide formulation was the source of discoloration. If wounding had been the cause, tissues around these blank sites should have also been discolored. In hindsight, it would have been advantageous to inject water to assess the wound response exclusively associated with drilling and injection pressure. In other macro-injected ash trees, insecticide concentration in discolored tissues was much higher than in adjacent unstained tissues (Mota-Sanchez et al. 2009, Tanis et al. 2012) and a previous study on green ash trees, discolored tissues were sound even four years after injection (Doccola et al. 2011).

Tissue discoloration in trunk injected trees is most likely caused by a "marker dye". Marker dyes are typically inert and are used to color pesticides so that applicators can see and safely handle the products they are applying (Pepling et al 1997). The identity of the blue marker dye used in the TREE-äge formulation is protected by Arborjet's proprietary patent.

In my study and others, tissue discoloration diminished with tree height above the injection site (Mota-Sanchez et al. 2009; Tanis et al. 2012). As I followed discoloration upward through the tree cross sections, discolored areas became smaller and paler. This is likely because

the volume of translocated insecticide decreased with tree height. Previous studies indicate that a reservoir of trunk injected insecticide can persist for several years around injection sites but these studies used high concentrations of imidacloprid a product that may have low solubility. This suggests that not all of the injected product is actually translocated into the canopy in a given year (Mota-Sanchez et al. 2009). In our study, EB-high trees received at least 2× more liquid than EB-low trees. As a result, discoloration height was over twice as high in EB-high trees as it was in EB-low trees, even though EB-low trees were likely injected under greater pressures. This suggests vertical discoloration was a function of the volume of insecticide that was translocated to the canopy. In contrast, discoloration width and length did not vary between EB-high and EB-low trees. This suggests the lateral movement of insecticides in the lower 50 cm of the trees was unaffected by volume or the pressure applied during application.

I identified interspecific variations in blue ash and green ash xylem anatomy that could influence uptake of trunk injected insecticides. While blue ash appears relatively resistant to EAB compared to other North American ash species (Tanis and McCullough 2012), blue ash trees will likely require insecticide treatments during peak densities of EAB invasion. Arborists have noted the difficulty of injecting blue ash relative to other ash species and I similarly experienced this difference. I was unable to inject dye into four of our original six injections sites on blue ash trees and had to create additional injection sites to apply the dye. After investigating the internal anatomy of blue ash and green ash xylem, multiple factors likely contribute to injection efficiency.

During translocation, trunk injected products are subject to friction created by passage through the lumen and pit fields (Choat et al. 2007). North American ash trees are ring porous, straight grained and in spring, when hydraulic pressure is low, ash trees transport water through

large earlywood vessels (Dickison 2000). As the growing season progresses, production of the number of large vessels drops and trees produce smaller vessels (latewood) that can transport water under high hydraulic pressure (Choat et al. 2007). The Hagen-Poiseuille equation states that hydraulic conductivity, the ease with which water can flow through a plant, is proportional to the sum of vessel radii raised to the fourth power. Xylem conductivity therefore is a function of lumen diameter and frequency (Choat et al. 2007, Kiten et al. 2010). Applying the Hagen-Pousille equation to determine the hydraulic conductivity of earlywood vessels in our study (Figs. 4.2 and 4.3) indicates green ash hydraulic conductivity was 67% greater than that of blue ash, despite the fact that the density of large earlywood vessels in blue ash was twice as high as green ash. Earlywood vessel size can be influenced by tree age, environment and genetic variability (Dickison 2000). Our trees were all the same age (seven years old), received the same cultural amendments, and samples were removed from tissues produced in the same year (2011), indicating differences in cross sectional area of lumen were species dependent.

While lumen transport water vertically, bordered pits, the gateway between xylem conduits, play a critical role in controlling the lateral movement of water (Zwieniecki et al. 2001, Kiten et al. 2004, 2009 Domec et al. 2006, Choat et al. 2007). I originally hypothesized that density of bordered pits in blue ash would be substantially less than in green ash because bordered pits can account for 50% of the resistance undergone by injected insecticides (Choat et al. 2007). This was not the case, however, densities of bordered pits were similar between the two species.

I observed that earlywood vessel elements in blue ash had a greater incidence of helical thickening than green ash vessels. Helical thickenings are lignin deposits that develop in earlywood vessels. When thickening crosses back and forth across xylem elements, as seen in

this study, the term helical or spiral thickening is used (Toole and Toole 2004). The extent of thickening, and the patterns that appear as a result of thickening, can differ among species. Helical thickening generally increases with tissue age (Bailey 1944) and can block water movement (Kiten et al. 2004). In our study, the extent of helical thickening in blue ash earlywood vessels was striking. In some cases, blue ash vessels appeared completely blocked. Differences in helical thickening between blue ash and green ash samples could not be attributed to environment or tissue age. The abundance of helical thickening in blue ash xylem likely contributes to the difficulty of blue ash trunk injection.

Injection of systemic insecticides for EAB control generally occur in the spring, allowing time for products to translocate to the canopy of ash trees where EAB adults are feeding on leaves (Herms et al. 2009). I injected dye in July to assess the anatomy of xylem tissues that were involved in translocation. While the timing of our dye injection was later than what is typically recommended for optimal EAB control (Herms et al. 2009; McCullough et al. 2011) I examined lumen size and bordered pit density in mature xylem produced by trees in 2011

Given the continued spread of EAB across North America, understanding effects of trunk injection systems and products on ash tree health is important. In this study, I found green ash and white ash trees were rarely harmed by the trunk injection process. Internal discoloration of xylem tissues after trunk injection was previously assumed to be indicative of an ash tree compartmentalization response (Smith and Lewis 2005). Our results indicate discoloration was likely caused by inert dyes found in the TREE-äge formulation. It is also important to understand how ash tree anatomy can affect the uptake of trunk injected insecticides. I found xylem characteristics of blue ash and green ash trees likely contribute to interspecific differences in uptake of trunk injected insecticide. Trees of many genera receive trunk injections, including

Acer, *Populas* and *Quercus*, perhaps further investigation of interspecific differences in xylem anatomy in these species could advance our general understanding of the trunk injection process.

Table 4.1: Results of analysis of variance to assess differences between green ash and white ash, insecticide rate (low rate = 0.1 g a.i. per 2.5 cm DBH or medium-high rate = 0.4 g a.i. per 2.5 cm DBH), injection frequency (2008 or 2008 + 2009) and their interactions on the number of trunk injection sites with new wood growing over them or secondary wounds and tissue discoloration depth, width and height.

	New Wood		**Secondary Wounds**	
Effect	**F Value**	**P value**	**F Value**	**P value**
Species (Spp)	1.10	0.300	1.18	0.285
Insecticide Rate (Ins Rate)	1.66	0.205	1.47	0.233
Spp × Ins Rate	0.05	0.817	0.00	0.950
Injection Frequency (Inj Freq)	1.97	0.169	**5.57**	**0.024***
Spps × Inj Freq	0.05	0.828	1.18	0.285
Ins Rate × Inj Freq	0.51	0.481	1.47	0.233
Spp × Ins Rate × Inj Freq	0.04	0.839	0.00	0.950

	Discoloration Depth		**Discoloration Width**		**Discoloration Height**	
Effect	**F Value**	**P value**	**F Value**	**P value**	**F Value**	**P value**
Species (Spp)	0.33	0.571	1.20	0.281	0.27	0.605
Insecticide Rate (Ins Rate)	0.01	0.934	3.09	0.087	**61.66**	**<0.001***
Spp × Ins Rate	0.37	0.548	0.49	0.487	0.26	0.616
Injection Frequency (Inj Freq)	0.60	0.445	0.05	0.821	0.34	0.561
Spps × Inj Freq	0.59	0.447	1.16	0.289	0.80	0.377
Ins Rate × Inj Freq	1.20	0.280	0.66	0.422	0.03	0.875
Spp × Ins Rate × Inj Freq	0.32	0.576	1.20	0.281	0.21	0.649

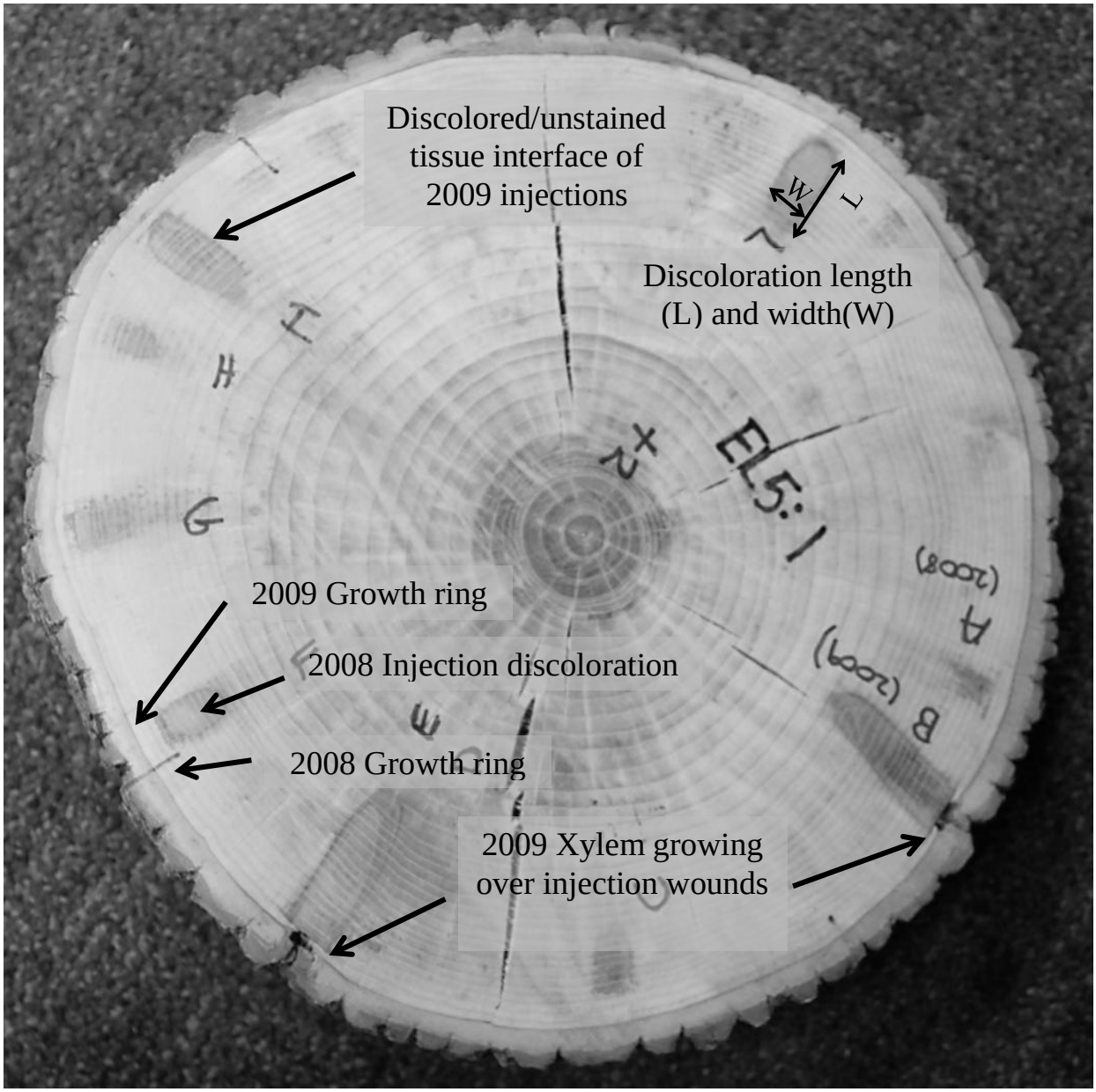


Figure 4.1: A cross section of a *Fraxinus pennsylvanica* tree injected in 2008 + 2009 with a low rate (0.1g a.i. per 2.54 cm at diameter at breast height) of emamectin benzoate (TREE-äge®) applied with a QUIK-jet® system. The cross section was cut 10.2 cm above 2008 injection sites. Discoloration from 2008 and 2009 injections, and injection wounds from 2009 are visible. Xylem is present around wounds and discolored tissues. Cracking in the cross secti+on was caused by drying, not the injection process.

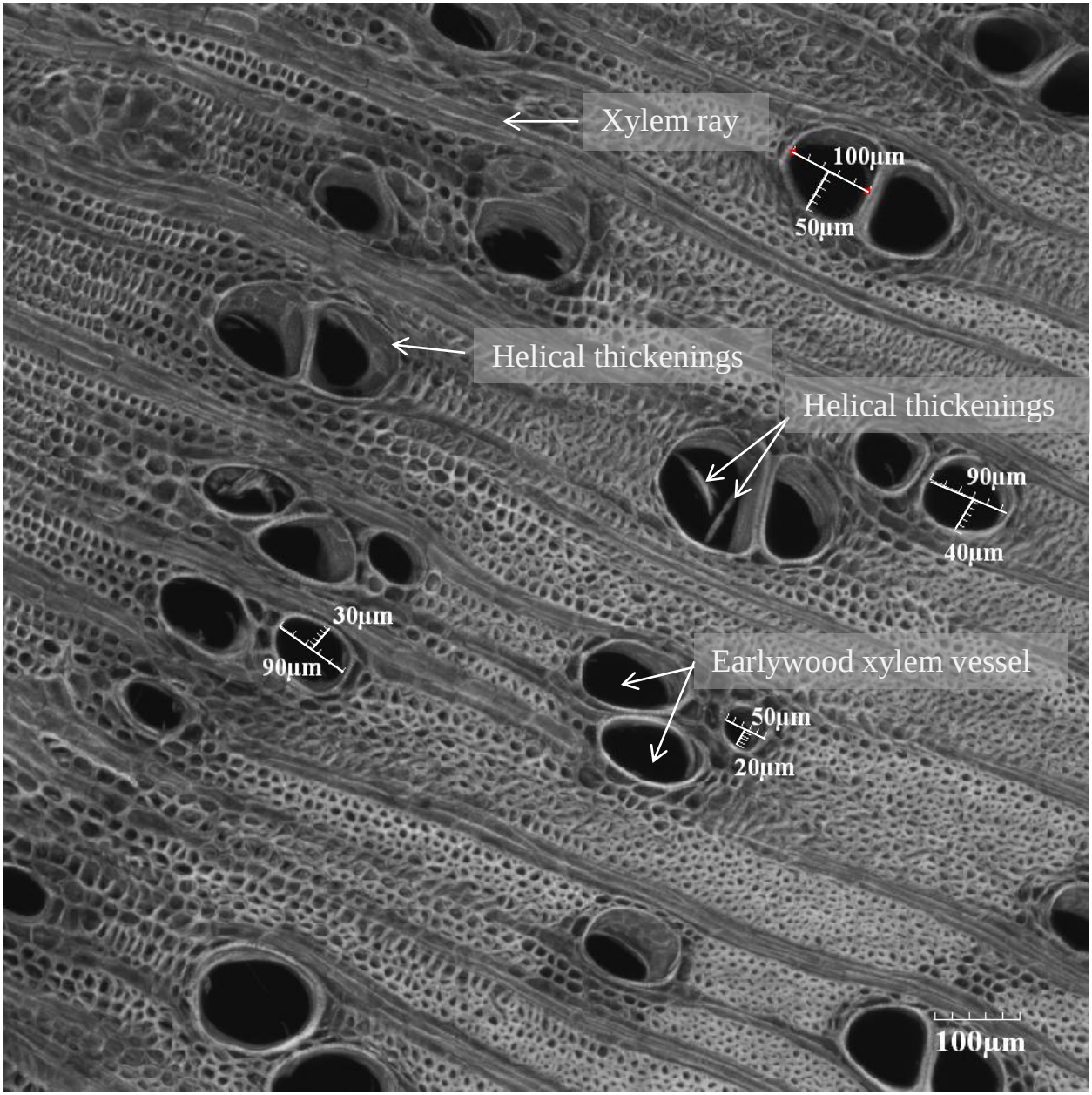


Figure 4.2: Transverse cross section of *Fraxinus quadrangulata* earlywood xylem tissue dyed with safranin orange. The image was obtained with a confocal laser scanning microscope equipped with helium-neon (λ=543 nm) and argon (λ=488 nm) lasers and a 10× dry objective for magnification. The three-dimensional image consists of 19 slices obtained 11.5 µm apart.

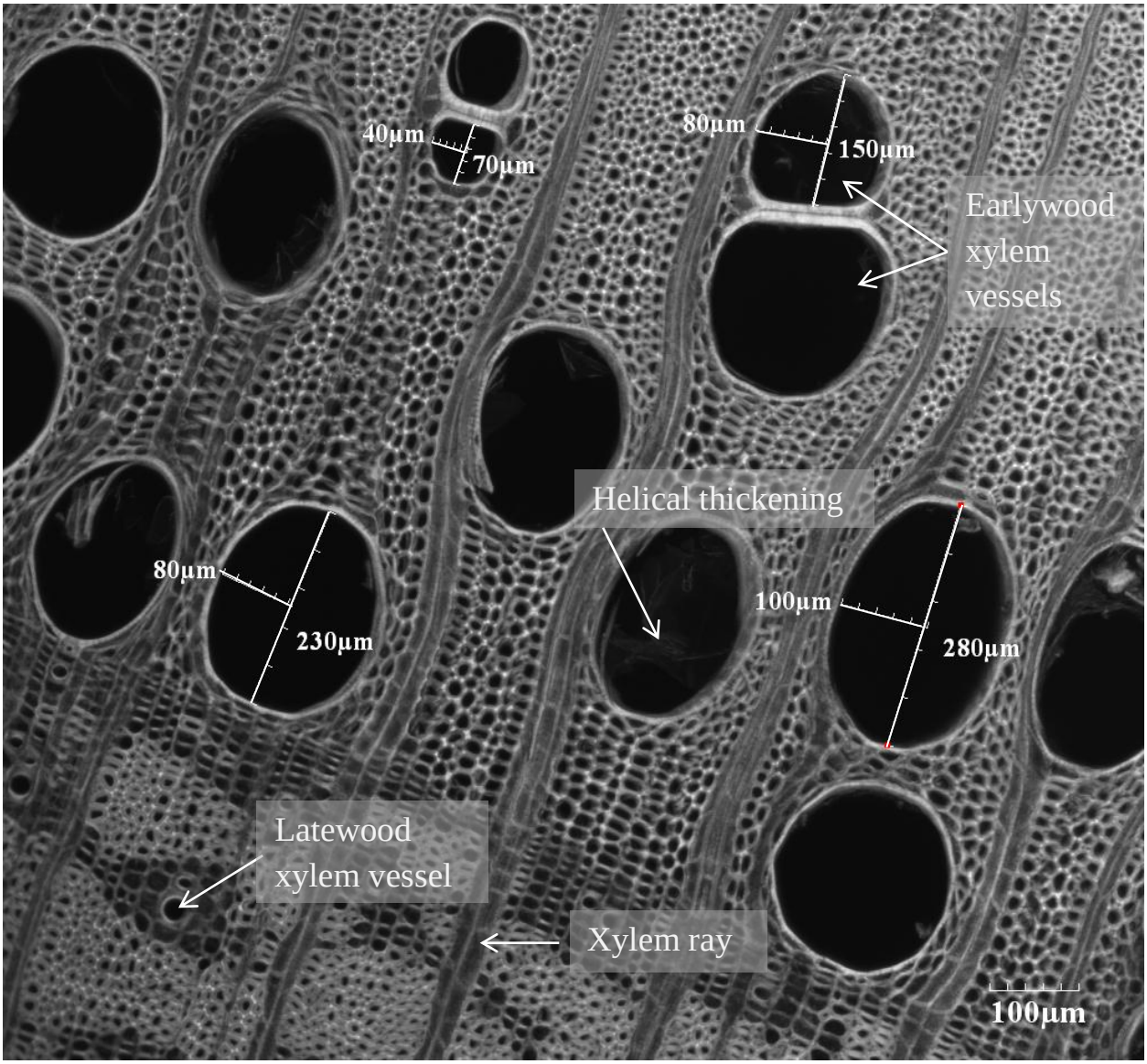


Figure 4.3: Transverse cross section of *Fraxinus pennsylvanica* earlywood xylem tissue. The image was obtained with a confocal laser scanning microscope equipped with helium-neon (λ=543 nm) and argon (λ=488 nm) lasers and a 10× dry objective for magnification. The three-dimensional image consists of 15 slices obtained 5.0 µm apart.

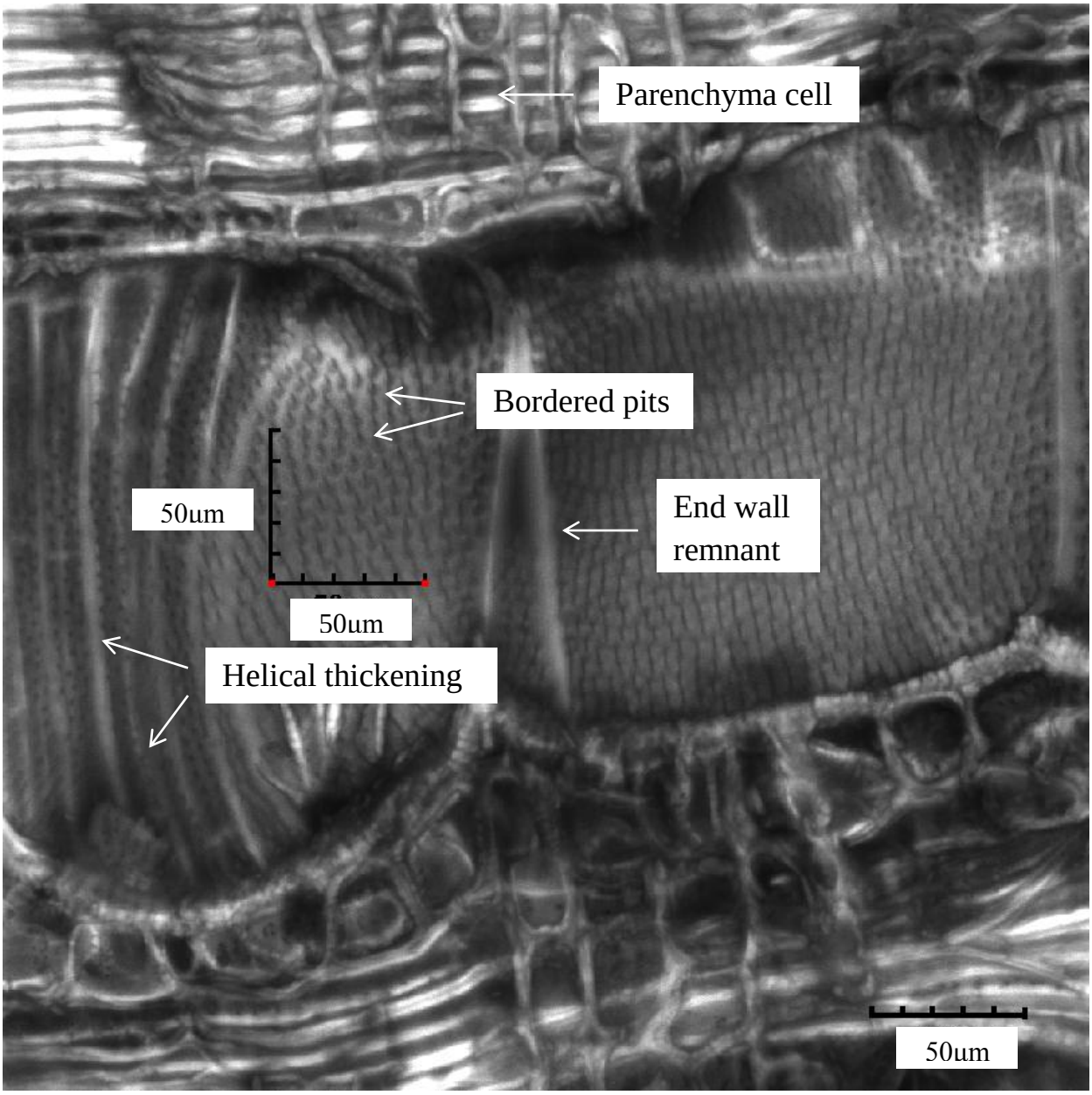


Figure 4.4: Radial cross section of a *Fraxinus quadrangulata* xylem element and surrounding cells from a tree injected with 15 ml of safranin orange. The image was obtained using a confocal laser scanning microscope equipped with helium-neon (λ=543 nm) and argon (λ=488 nm) lasers, a 20× dry objective and 1.8 zoom were used for magnification. The three-dimensional image consists of 87 slices obtained 1.0 µm apart.

Figure 4.5: Radial cross section of a *Fraxinus pennsylvanica* xylem element and surrounding cells of a tree injected with 15ml of safranin orange. The image was obtained using a confocal laser scanning microscope equipped with helium-neon (λ=543 nm) and argon (λ=488 nm) lasers, a 20× dry objective and 1.8 zoom were used for magnification. The three-dimensional image consists of 48 slices obtained 1.0 µm apart.

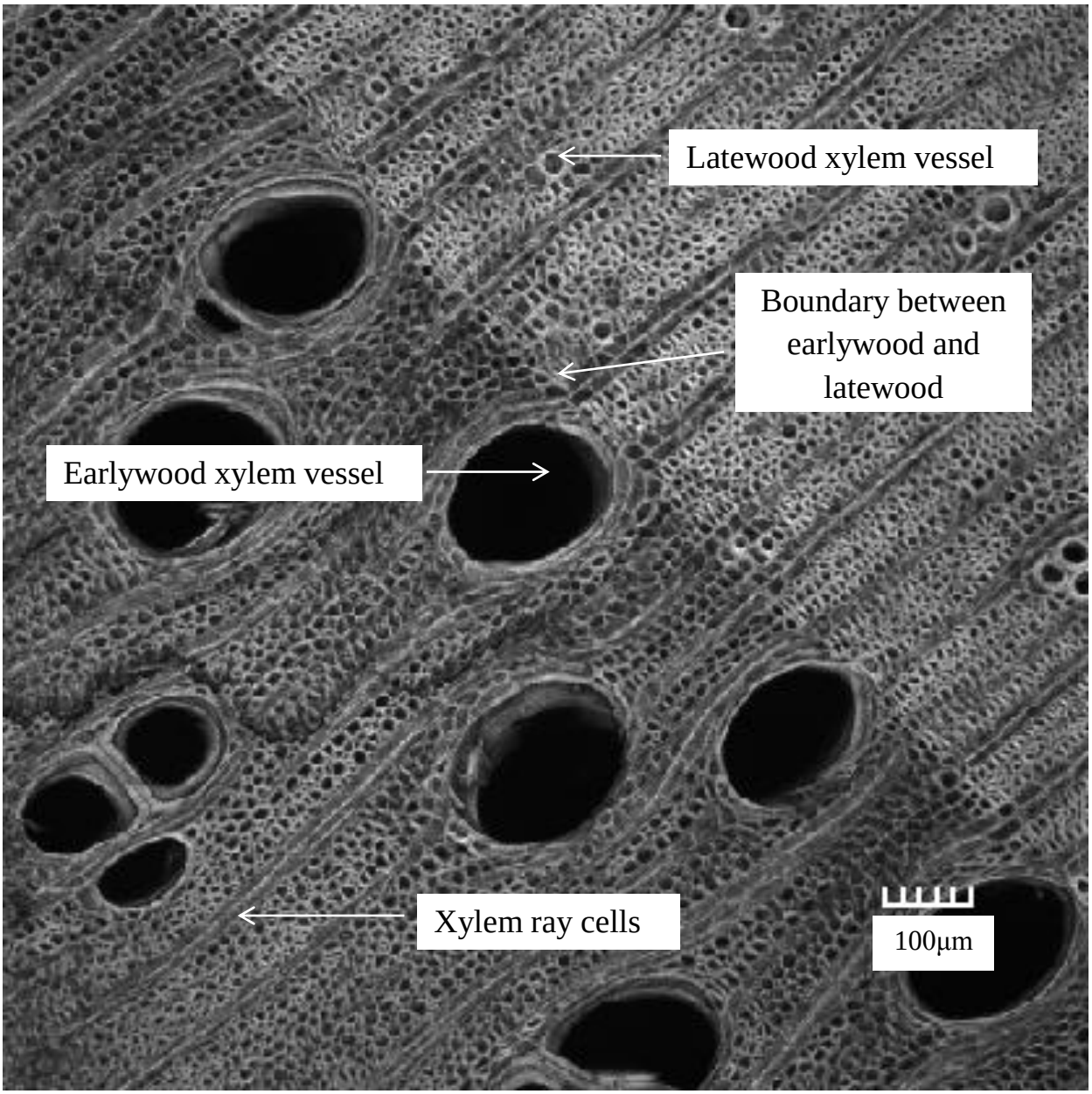


Figure 4.6: Transverse cross section of *Fraxinus pennsylvanica* discolored by trunk injected emamectin benzoate. The image was obtained using a confocal laser scanning microscope equipped with helium-neon (λ=543 nm) and argon (λ=488 nm) lasers. The three-dimensional image consists of eight slices obtained 14.5 µm apart. A 10X dry objective was used for magnification.

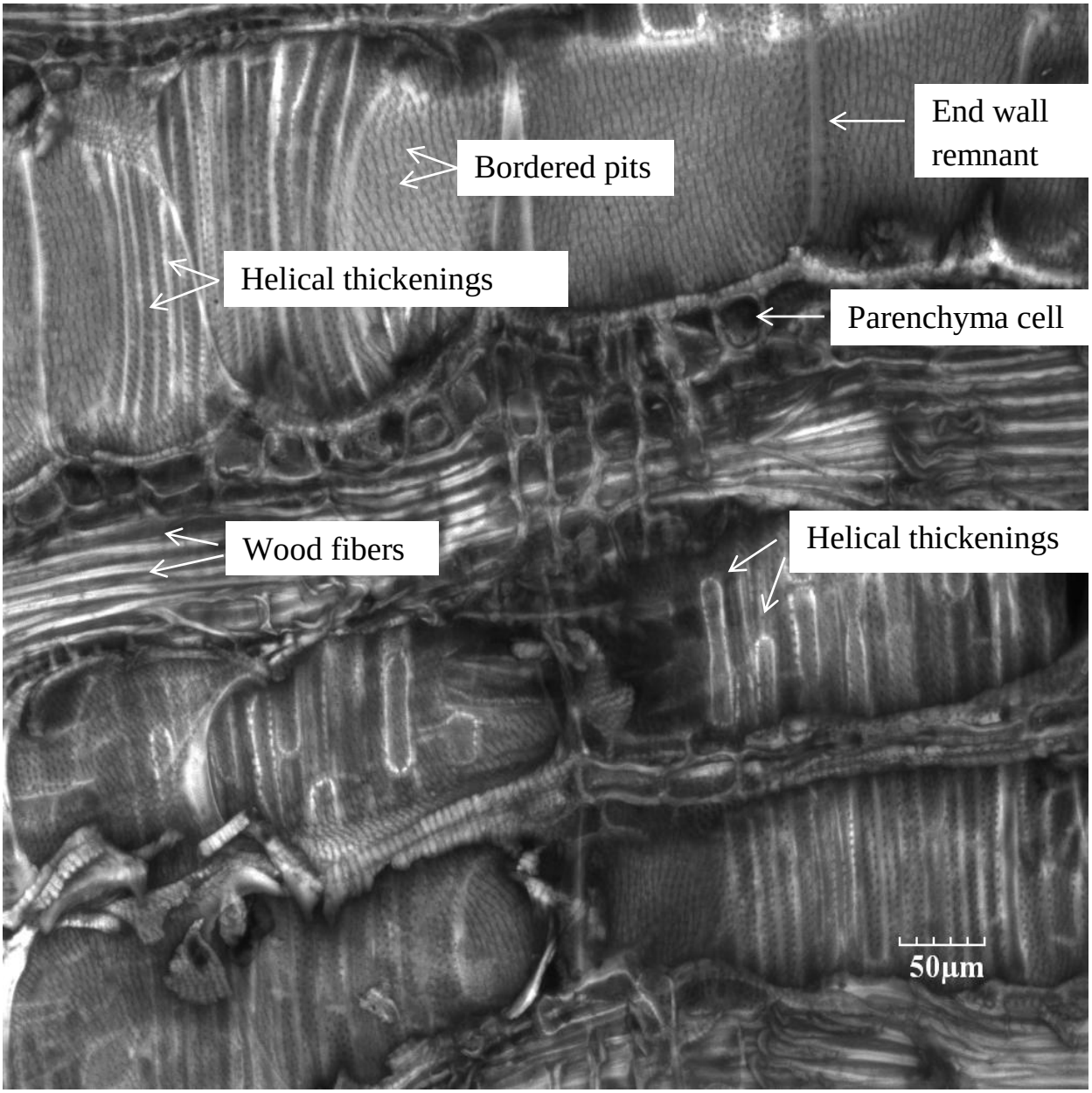


Figure 4.7: Radial cross section of a *Fraxinus quadrangulata* xylem element and surrounding cells from a tree injected with 15ml of safranin orange. The image was obtained using a confocal laser scanning microscope equipped with helium-neon (λ=543 nm) and argon (λ=488 nm) lasers. The three-dimensional image consists of 54 slices obtained 2.0 µm apart. A 20X dry objective was used for magnification.

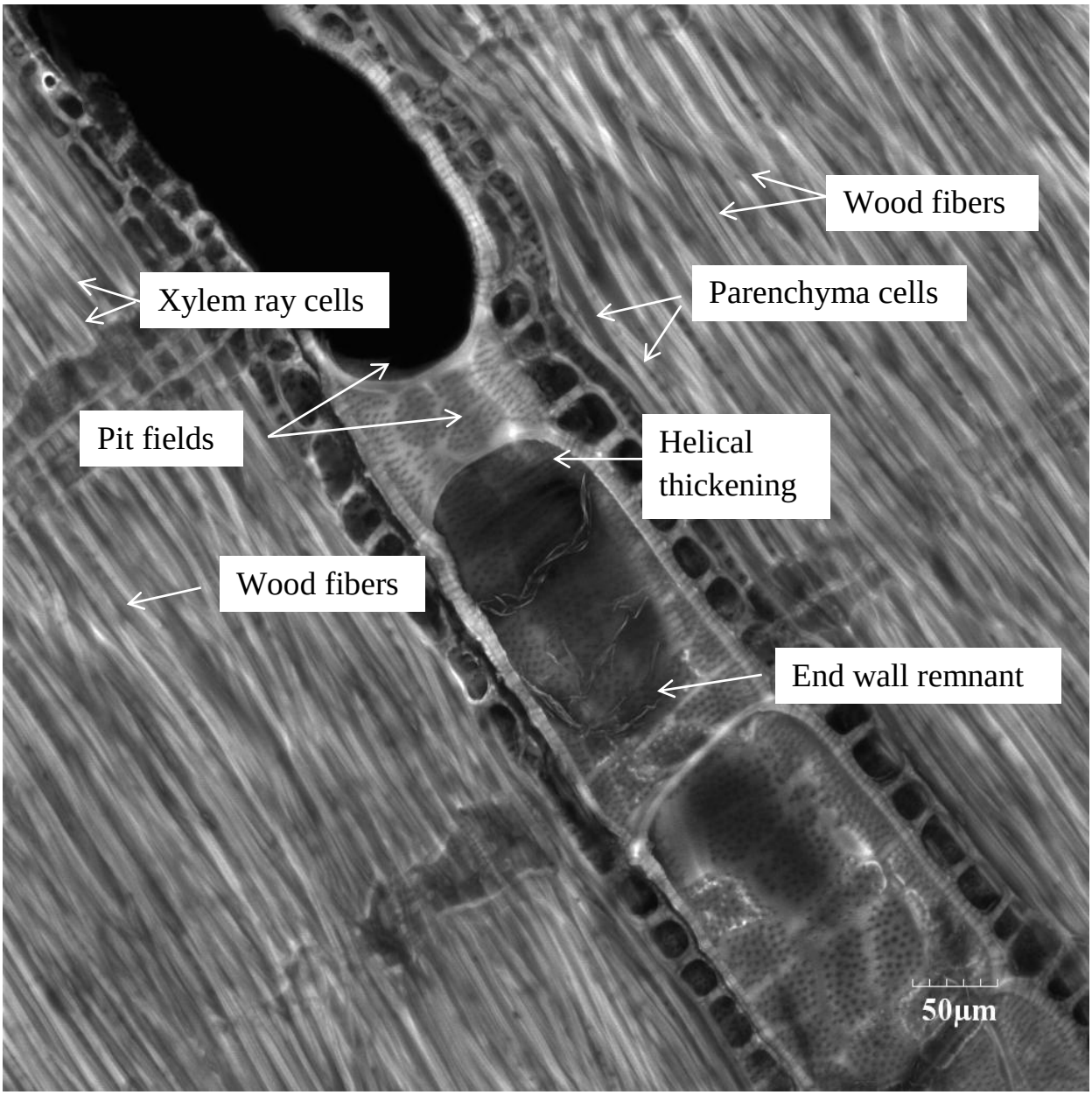


Figure 4.8: Radial cross section of a *Fraxinus pennsylvanica* xylem element and surrounding cells from a tree injected with 15ml of safranin orange. The image was obtained using a confocal laser scanning microscope equipped with helium-neon (λ=543 nm) and argon (λ=488 nm) lasers. The three-dimensional image consists of 39 slices obtained 1.0 µm apart. A 20X dry objective was used for magnification.

CHAPTER FIVE

Examination of the phloem of three ash species using fluorescent confocal laser scanning microscopy

ABSTRACT

Variability in ash (*Fraxinus* spp.) tree resistance to emerald ash borer (EAB, *Agrilus planipennis* Fairmaire), an exotic pest introduced from Asia, has sparked interest in ash physiology and defensive chemistry. Blue ash (*Fraxinus quadrangulata* Michx.), which is native to North America, does not share an evolutionary history with EAB, yet it has resistance equal to that of Manchurian ash (*Fraxinus mandshurica* Rupr.), a species that co-evolved with EAB in Asia. In contrast, green ash (*Fraxinus pennsylvanica* Marsh.) is highly preferred by EAB. Phenolic compounds and reactive oxygen species (ROS) are often produced by plants when they are injured. These compounds may serve as defensive compounds (e.g., anti-feedants), signal molecules or defensive compound precursors. Larval stages of EAB develop under the bark of ash trees, therefore interspecific differences in phloem anatomy and defensive chemical production could play an important role in tree resistance. I used confocal laser scanning microscopy (CLSM) to detect phenolic compounds, assess ROS production and examine the architecture of blue ash, green ash and Manchurian ash phloem. Autofluorescence indicates that phenolic compounds were present in blue ash and Manchurian ash phloem but there were no detectable levels of phenolic compound autofluorescence in green ash phloem. All three ash species produced ROS, but Manchurian ash trees produced at least three times more than blue ash and green ash trees. In addition to chemical defenses, blue ash and Manchurian ash phloem had a continuous layer of sclerenchymatous cells that could affect EAB larvae. The layer of sclerenchymatous cells was also present in green ash but it was not continuous.

INTRODUCTION

Emerald ash borer (EAB, *Agrilus planipennis* Fairmaire) (Coleoptera: Buprestidae) was first identified in North America near Detroit, Michigan in 2002 (Cappaert et al. 2005). A native of Asia, EAB has killed tens of millions of ash (*Fraxinus* spp.) trees in 19 states and two Canadian provinces (EAB.info 2013). In Michigan, EAB adults generally emerge in mid-May. Females feed for approximately three weeks before they begin laying eggs in the cracks and crevices of ash bark in mid-June (Poland and McCullough 2006). Ash tree canopies are fully flushed during this time (Barnes and Wagner 1981) and phloem is actively transporting nutrients from the canopy to the roots (Dickison 2000). First instar EAB larvae tunnel through the bark into the phloem and cambium where they feed on the nutrient rich tissues and develop through a total of four instars (Poland and McCullough 2006). Tree death occurs when galleries made by tunneling larvae cut off water and nutrient conducting pathways (Cappaert et al. 2005).

Ash species have varying levels of inherent resistance to EAB (Anulewicz et al. 2007; Rebek et al. 2008; Duan et al. 2012; Tanis and McCullough 2012). Manchurian ash (*Fraxinus mandshurica* Rupr.), an Asian species and blue ash (*Fraxinus quadrangulata*, Michx.), a North American species, are relatively resistant to EAB (Liu et al. 2003, Duan et al. 2012, Tanis and McCullough 2012; Tanis, Chapter three). In southeast Michigan woodlots, blue ash survival was > 60% in 2011, several years after EAB populations peaked in the area (Tanis and McCullough 2012). In contrast, green ash (*Fraxinus pennsylvanica* Marsh.), another North American species, is heavily attacked by EAB and killed. In forested parklands in southeast Michigan and northwest Ohio, green ash mortality exceeded 99% in 2009 (Herms et al. 2009; Gandhi and Herms 2010b; Knight et al. 2010).

Blue ash, green ash and Manchurian ash are evolutionarily diverse. On a phylogenetic tree based on floral morphology, blue ash, a monoecius species, is placed in the Dipetalae section along with *Fraxinus dipetala* Hook. & Arn. and *Fraxinus anomala* Torr. ex S.Watson, both of which are native to the southwestern United States (Wallander 2008). Green ash, a dioecious species, is placed in the Melloides section with eight other North American species. Manchurian ash, also a dioecious species, is placed in the Fraxinus section along with black ash (*Fraxinus nigra* Marsh.) (Wallander 2008), a species that is highly susceptible to EAB (McCullough 2013). Variability in ash tree resistance has sparked interest in ash physiology and defensive chemistry.

Ash tree resistance to EAB likely involves a suite of defensive mechanisms (Cipollini et al. 2011), including the production of chemical defenses in the phloem, where EAB larvae feed and develop (Eyles et al. 2007, Cipollini et al. 2011, Whitehill et al. 2012). Blue ash, green ash and Manchurian ash have distinct phloem chemistry profiles. For example, several lignin compounds that could function as larval antifeedants or growth retardants are unique to Manchurian ash (Cipollini et al. 2011, Whitehill et al. 2012).

Confocal laser scanning microscopy (CLSM) can be used to detect the autofluorescence of defensive compounds in living cells (Roshchina 2012). Autofluorescence is a natural phenomenon that occurs when organic compounds emit a specific wavelength of light after absorbing light from a different wavelength. For example, chloroplasts emit green light when they have absorbed red light. Autofluorescence that is detected by CLSM can serve as a diagnostic indicator (Roshchina 2012) since many phenolic compounds (e.g., hydroxycinnamic acids, coumarins, stilbenes and styrlypyrones) autofluoresce when illuminated with blue lasers (Hutzler et al. 1998; Pfündel et al. 2006; Roshchina 2012). In Norway spruce (*Picea abies* L.), CLSM autofluorescence techniques were used to determine the location of phenolic compounds

in needles. As expected, autofluorescence was recorded in phloem near resin ducts, but high concentrations of phenolic compounds were also present in the cell walls of the xylem (Hutzler et al. 1998). While ash phloem chemistry has been the focus of considerable research (Eyles et al. 2007, Cipollini et al. 2011, Whitehill et al. 2011, 2012, Hill et al. 2012), the anatomical features that may affect larvae directly or indirectly through chemical defense production remain unexplored. My first objective was to assess phenolic autofluorescence in the phloem of resistant blue ash and Manchurian ash and highly susceptible green ash using CLSM.

In addition to defensive phenolic compounds, ash trees also likely produce a variety of reactive oxygen species (ROS) when injured (Rajarapu et al. 2011). Reactive oxygen species are biologically active compounds that contain oxygen. Peroxide, a common ROS produced by plants, can serve as a defensive compound, defensive compound precursor or a signal for injury (Maffei et al. 2007). When insects feed on plant tissues, high concentrations of ROS typically flood the injured area in a phenomenon called an "oxidative burst" (Maffei et al. 2007). When insects ingest these injured tissues, high concentrations of ROS can cause cellular oxidative stress (lipid, DNA and/or protein degradation), which could potentially kill the insect. In order to counteract cellular oxidation, insects must produce antioxidant enzymes to detoxify the ROS compounds before their tissues are damaged (Rajarapu et al. 2011). Rajarapu et al. (2011) suggest that the ability of EAB to produce antioxidant enzymes is at least partially responsible for their "rampant invasion of North American ash", but the extent to which different ash species produce ROS is unknown.

The production of ROS in living tissues can be detected with CLSM. Amplex® Red (Invitrogen, Molelcular Probes Inc., Eugene OR, USA), a fluorescent probe that can be absorbed by living cells (Maffei and Bossi 2006, Maffei et al. 2007, Ozawa et al. 2009, Bricchi et al.

2010). The reaction that occurs between Amplex Red and ROS produces a compound called resorufin in a 1: 1 peroxide: resorufin ratio. When resorufin is produced, it emits red light that can be detected with CLSM (Invitrogen.com, Accessed 14 April 2012). My second objective was to assess ROS production in blue ash, green ash and Manchurian ash using Amplex Red and CLSM.

Relatively few studies have examined how anatomical characteristics of deciduous trees might contribute to defense against phloem boring larvae (Dunn et al. 1990, Cipollini et al. 2011). However, there is evidence for anatomical resistance mechanisms (i.e., lignification of attacked tissue) (Heering, 1956, Lieutier, 2008, Dunn et al., 1986, Hanley et al., 2007). While debarking blue ash and Manchurian ash trees for a concurrent study, I observed a distinct layer of cells that could act as a barrier to tunneling larvae. The cell layer was visually apparent and so hard that drawknives glanced off the layer instead of penetrating into the cambium. Many studies incorporate CLSM to examine the internal architecture of wood because high resolution, three-dimensional images can be acquired with relatively little tissue preparation (Kitin et al. 2004, 2009). My third objective was to examine phloem architecture of blue ash, green ash and Manchurian ash with CLSM to more thoroughly evaluate variations in phloem structure that could potentially affect EAB larvae.

METHODS

Ash Plantation

On 26 April 2006, blue ash, green ash and Manchurian ash trees were planted (2.4 m apart) at the Michigan State University Tree Research Center in Okemos, Michigan (Ingham County) (Tanis, Chapter Four). Trees were fertilized annually and watered twice per week or as needed with drip irrigation. All trees used in our experiments were seven years old at the time of experiments.

Tissue Sampling

Phenolic Compound Autofluorescence

On 31 July 2012, four tissue samples consisting of phloem, cambium and sapwood (approximately 2.0 × 2.0 × 0.5 cm) were removed from breast height (1.4 m aboveground) with a drawknife from the cardinal directions from three blue ash, green ash and Manchurian ash trees (four samples per tree, 36 total samples). Samples were sealed in plastic bags, placed in an opaque box to reduce exposure to light and immediately transported to the Michigan State University Microscopy Center. Upon arrival, two thin cross sections approximately 200 µm thick were removed from the transverse plane of each phloem sample (eight sections per tree, 72 total samples) with a razor blade. Samples were immediately analyzed for blue auto-florescence with CLSM, they did not receive any dye treatments.

Reactive Oxygen Species (ROS) Production

Six to 10 additional cross sections were removed from each phloem sample as described above and incubated in Amplex Red reagent in the dark for 1 hr per Amplex Red hydrogen peroxide/peroxidase assay kit instructions (Bricchi et al. 2010, Invitrogen.com 2012). I incubated an excess of cross sections in order to ensure I had multiple high quality sections to

choose from. The two thinnest cross sections from each phloem sample (eight sections from each tree, 72 total samples) were analyzed using CLSM.

Phloem Anatomy

While evaluating phloem cross sections for autofluorescence, I also assessed potential differences in anatomy. The unique cells observed while debarking blue ash and Manchurian ash trees during a concurrent study were clearly visible through the oculars, but I was unable to obtain a high resolution image because samples were living and the cytoplasm had not been cleared. Therefore, I dyed, dehydrated and cleared the phloem cross sections that were used to assess autofluorescence. Immediately after autofluorescence observations were complete, cross sections were placed into fixing solution (50% ethanol, glycerol, formaldehyde, 18:1:1) for at least 72 hours. After fixation, cross sections were placed in a solution of safranin orange dye (1% safranin, Carolina Biological Supply Company, Burlington, NC, USA) for 15 minutes, then dehydrated through 30, 50, 75, 90 and 100% ethanol (Decon Labs, Inc., King of Prussia, PA, USA), then rehydrated backward through the same solutions. Cross sections remained in each alcohol solution for 10 minutes. Solutions were changed multiple times during each cycle to remove excess dye. After rehydrating, cross sections were placed in de-ionized water for 15 minutes then processed through 25, 50 and 75% glycerol solutions (1 hour each with frequent decanting) and finally left overnight in 100% glycerol. Images were obtained with a confocal microscope the next day.

Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy images are obtained by raster scanning, a process that uses lasers to scan point by point through a series of slices from the highest to the lowest focused portions of the sample (Wymer et al. 1999). The number of slices (defined by the user)

depends on the thickness of the sample and the resolution necessary to produce an image of the required quality (Hutzler et al. 1998). To obtain a three dimensional image, multiple slices (called a z-scan) are compiled by software programs that detect and amass only the focused portions of each slice (Wymer et al. 1999). After compiling an image, vertical and horizontal fluorescent profiles can be overlain to show the frequency and intensity of fluorescence. I used an Olympus Fluoview 1000® (Olympus Corporation, Tokyo, Japan) confocal microscope equipped with a 10 × dry objective for magnification throughout all experiments. The total area of each image was 1.59 mm^2.

Phenolic Compound Autofluorescence

Phloem cross sections that were assessed for autofluorescence were illuminated with a blue Diode (λ=405) laser to detect autofluorescence of phenolic compounds and a helium-neon (λ=543) laser to show the basic structure of each sample. Three-dimensional images were produced for each phloem cross section using a double channel z-scan. The double channel z-scanning option enables the user to assign colors to images that have been produced by different lasers. I chose blue to label phenolic autofluorescence (detected by the blue Diode laser) and orange to label the structural architecture of the phloem (detected by the helium-neon laser) (Figure 5.1). Colors are completely arbitrary and are used to clarify presentation. The number of slices ranged from 24 to 81 per sample while slice depth was 11.5 µm for all samples.

Reactive Oxygen Species (ROS) Production

Phloem cross sections incubated with Amplex Red were illuminated with argon (λ=488) and helium-neon (λ=543) lasers, a laser combination that detects the red fluorescence produced when Amplex Red reacts with peroxide (Ozawa et al. 2009). Three-dimensional images were produced for each phloem cross section and red was used to label areas where fluorescence was

present (Fig. 5.2). Slice depth was 5.0 µm for all images and the number of slices ranged from 10 to 31 per sample. Vertical and horizontal fluorescence frequency profiles were added to each image to provide a reference for the intensity and distribution of fluorescence (Fig. 5.2).

Phloem Anatomy

Cross sections used to assess differences in phloem anatomy were illuminated with argon and helium-neon lasers, a laser combination that detects the red fluorescence produced by safranin dye. A three dimensional image was produced for each phloem cross section. Images consisted of 34 to 79 slices per sample, taken 5.0 µm apart.

RESULTS

Phenolic Compound Autofluorescence

Phenolic autofluorescence was detected in blue ash and Manchurian ash phloem samples but not in green ash phloem samples (Fig. 5.1). Phenolic autofluorescence was detected in 40 to 50% of the phloem area of every blue ash phloem sample. Autofluorescence located 290 to 370 µm (mean depth (±SE) = 323.3 ± 12.02 µm) beneath the outside edge of the bark but never occurred within the bark (Fig. 1B1 and 1B2). Phenolic compound autofluorescence was never detected in green ash phloem samples; all images were completely black (Fig. 1G2). In all Manchurian ash samples, at least 70% of the phloem area contained autofluorescing phenolic compounds (Fig 1.M2). These phenolic compounds were distributed throughout all samples and were always present in the bark.

Reactive Oxygen Species (ROS) Production

Production of ROS was recorded in all phloem samples regardless of ash species. Distribution and concentration of ROS, however, varied considerably among species. Where ROS were produced within the phloem samples, the tissues appear bright red (Fig. 5.2). Anomalies in fluorescent frequency profiles correspond to areas within each phloem sample where ROS fluorescence was detected. These fluorescent profiles indicated by the horizontal and vertical traces (Fig. 5.2), suggest the production of ROS occurred in localized areas within the blue ash and green ash phloem samples (Figs. 5.2A and 5.2B). In contrast, ROS production was more or less continuous in the Manchurian ash phloem (Fig. 5.2C). The images confirm the patterns indicated by the frequency traces. Blue ash and green ash ROS fluorescence appeared much more mottled, whereas ROS fluorescence appeared in two bands in the Manchurian ash phloem (Fig. 5.2).

The area (μm^2) where ROS were produced comprised 13.3 ± 1.2 % and 16.1 ± 2.6% of the total blue ash and green ash phloem area, respectively. In contrast, the area where ROS were produced in Manchurian ash phloem samples comprised 72.2 ± 3.2 %, which was 82 and 78% higher than blue ash or green ash, respectively.

Phloem Anatomy

The layer of cells I observed when peeling blue ash and Manchurian ash trees was visible in phloem tissues when I assessed phenolic autofluorescence. I determined that the layer of cells was a sclerenchymatous cylinder as described by Wilson et al. (1983). Sclerenchymatous cylinders can be either continuous or semi-continuous. Continuous cylinders are comprised of interconnecting cells that occur in a ring. Semi-continuous cylinders are frequently interrupted by parenchyma cells and groups of sclerenchyma cells look more like bundles rather than a cylinder.

The blue ash sclerenchymatous cylinder was continuous (Fig. 5.3A). It was located 390 to 560 µm (mean (±SE) depth = 461.7 ± 18.3 µm) from the outside edge of the bark and was 40 to 75 µm thick (mean thickness = 52.0 ± 5.8 µm). In contrast, the green ash sclerenchymatous cylinder was semi-continuous (Fig. 5.3B). The bundles of sclerenchyma occurred 290 to 490 µm (mean depth = 330.0 ± 10.4 µm) from the outside edge of the bark and were between 60 and 95 µm thick (mean thickness = 82.0 ± 6.6 µm). As expected from our previous observations, the Manchurian ash sclerenchymatous cylinder was also continuous in nature (Fig. 5.3C). It was located 420 to 570 µm (mean depth = 495.0 ± 11.6 µm) beneath the outside edge of the bark and was between 80 and 140 µm thick (mean thickness = 103 ± 5.4 µm).

DISCUSSION

In his classic review of tree resistance, Hanover (1975) suggests multiple ways trees can defend themselves against insect pests. Resistant trees might produce defensive compounds to attract insect predators or repel pests, they may have anatomical structures to deter insects, and/or their nutritional content might be such that they are less preferred by or unsuitable for insect pests. Any one of these mechanisms, or a combination thereof, could influence adult and larval insect stages by altering feeding behavior, survivability and oviposition preference (Hanover 1975). I found blue ash and Manchurian ash trees produce defensive chemicals and have anatomical features in the phloem that could affect their resistance to EAB.

Detecting the autofluorescence of phenolic compounds in plant tissues with CLSM is a new technique that is gaining popularity (Hutzler et al. 1998). Many naturally occurring compounds produce autofluorescence of a known wavelength. Illumination of tissues with a blue light source has been used successfully to identify the autofluorescence of coumarin and polyresinol compounds in Norway spruce (Hutzler et al. 1998). Studies that have assessed the phloem chemistry of ash species indicate these two classes of compounds are unique to resistant ash species and could be harmful to EAB larvae (Whitehill et al. 2012). I chose to use blue autofluorescence techniques to assess the distribution of phenolic compounds in ash phloem because autofluorescence can detect an array of defensive compounds. This is important because the compounds in ash that confer resistance to EAB remain unknown (Eyles et al. 2007, Cipollini et al. 2011, Whitehill et al. 2012). Phenolic autofluorescence was intense in Manchurian ash phloem and bark. It was present to a lesser degree in blue ash phloem, but was completely absent in the blue ash bark. The complete lack of phenolic compound autofluorescence in the green ash phloem was unexpected. Multiple studies have identified

phenolic compounds in green ash phloem (Eyles et al. 2007; Cipollini et al. 2011; Whitehill et al. 2012). Intraspecific differences in phenolic chemistries could be highly variable depending on tree age, phenology and genotype (Cipollini et al. 2011; Whitehill et al. 2012). My study provides visual evidence of the presence and distribution of phenolic compounds in Manchurian ash and blue ash phloem and bark, but the exact identity of those compounds is unknown. The CLSM technique apparently did not detect all phenolic compounds, as evidenced by the lack of autofluorescence in green ash phloem tissues. The autofluorescence detected by CLSM, however, indicates one or more phenolic compounds were present in blue ash and Manchurian ash phloem that were not present in green ash phloem. Blue ash and Manchurian ash trees are relatively resistant to EAB while green ash is highly susceptible, further research is necessary to determine the identity of the autofluorescing compounds.

Many plants produce reactive oxygen species in response to herbivory (Peltonen et al. 2005, Witzell and Martin 2008). These compounds can serve directly as defensive compounds but can also serve as signals that turn on stress-tolerance genes (Potters et al. 2007). Through coevolution, many insects have developed antioxidant enzymes to metabolize and detoxify ROS. Rajarapu et al (2011) suggest the ability to produce these enzymes is a trait often exhibited by invasive insects, which could partially explain how EAB has been able to attack and infest a variety of ash species native to North America.

Reactive oxygen species are one of the first compounds produced when plants are injured (Bi and Felton 1995). Fluorescent CLSM has been used to detect and visualize ROS production in leaves injured by mechanical means and feeding insects (Maffei and Bossi 2006, Maffei et al. 2007, Ozawa et al. 2009, Bricchi et al. 2010), but to our knowledge, CLSM has not been used to visualize ROS production in ash phloem. I induced ROS production by cutting into the phloem

with a drawknife. Although this does not exactly mimic the injuries created by tunneling EAB larvae, I feel the injury response was similar. Production of ROS occurred in all of the ash species I assessed. While blue ash and green ash trees produced detectable levels of ROS, it was highly localized in the phloem tissues and occurred in <20% of the phloem area. In contrast, ROS appeared in two large swathes of tissue in Manchurian ash and occurred in almost 80% of the phloem area.

In addition to phenolic and ROS chemical defenses, blue ash and Manchurian ash trees may also utilize anatomical defenses in the form of a sclerenchymatous layer of tissue in the phloem. Continuous or semi-continuous sclerenchyma cylinders have been described for a variety of woody species (Wilson et al. 1983). In *Populas tremula* L. (Norberg and Meier 1966) the sclerenchyma cylinders were described as crystalline and appeared to be highly organized. In healthy, mature lilac (*Syringa vulgaris* L.), the sclerenchyma cylinder regenerated itself when tissues were mechanically wounded but as trees became stressed they lost their regenerative ability (Wilson et al. 1983).

Many studies suggest anatomical defenses can serve as important mechanisms for tree resistance, but few studies actually investigate those mechanisms in hardwood trees (Dunn et al. 1986; Hanley et al. 2007; Mithöfer et al. 2009; Cippollini et al. 2011). In our concurrent host preference study, the density of larvae on blue ash and Manchurian ash was so low (1.9 ± 0.93 and 1.5 ± 0.66 galleries per m^2 of phloem, respectively) I was unable to determine how EAB might be affected by the cylinder. Further investigation using artificially placed EAB eggs on blue ash and Manchurian ash trees would be necessary to determine how larvae are affected, but my assessment suggests the sclerenchyma layer could act as an obstacle for tunneling EAB larvae.

Blue ash continues to be an anomaly among the North American ashes. I identified phenolic compounds and a continuous sclerenchymatous cylinder in blue ash phloem, despite the fact it has not coevolved with any North American EAB congeners (Drooz 1985). In contrast, I detected no phenolic compound autofluorescence in green ash phloem and the sclerenchyma cylinder was frequently interrupted with parenchyma cells. While ROS production was comparable between blue ash and green ash, it was extremely low in both species compared to Manchurian ash. Results indicate that upon hatching, neonate larvae likely encounter phenolic compounds almost immediately when they begin to chew into the outer bark of Manchurian ash. If larvae persist, they will continue to encounter defensive compounds and the sclerenchymatous cylinder in Manchurian ash phloem. Larvae have no way to avoid or escape these tissues and are subject to the defensive compounds they encounter therein. Although my results provide evidence that blue ash and Manchurian ash trees are unique among ash trees commonly planted in eastern North America, further research is necessary to determine how their phloem characteristics and chemistry specifically affect EAB larvae.

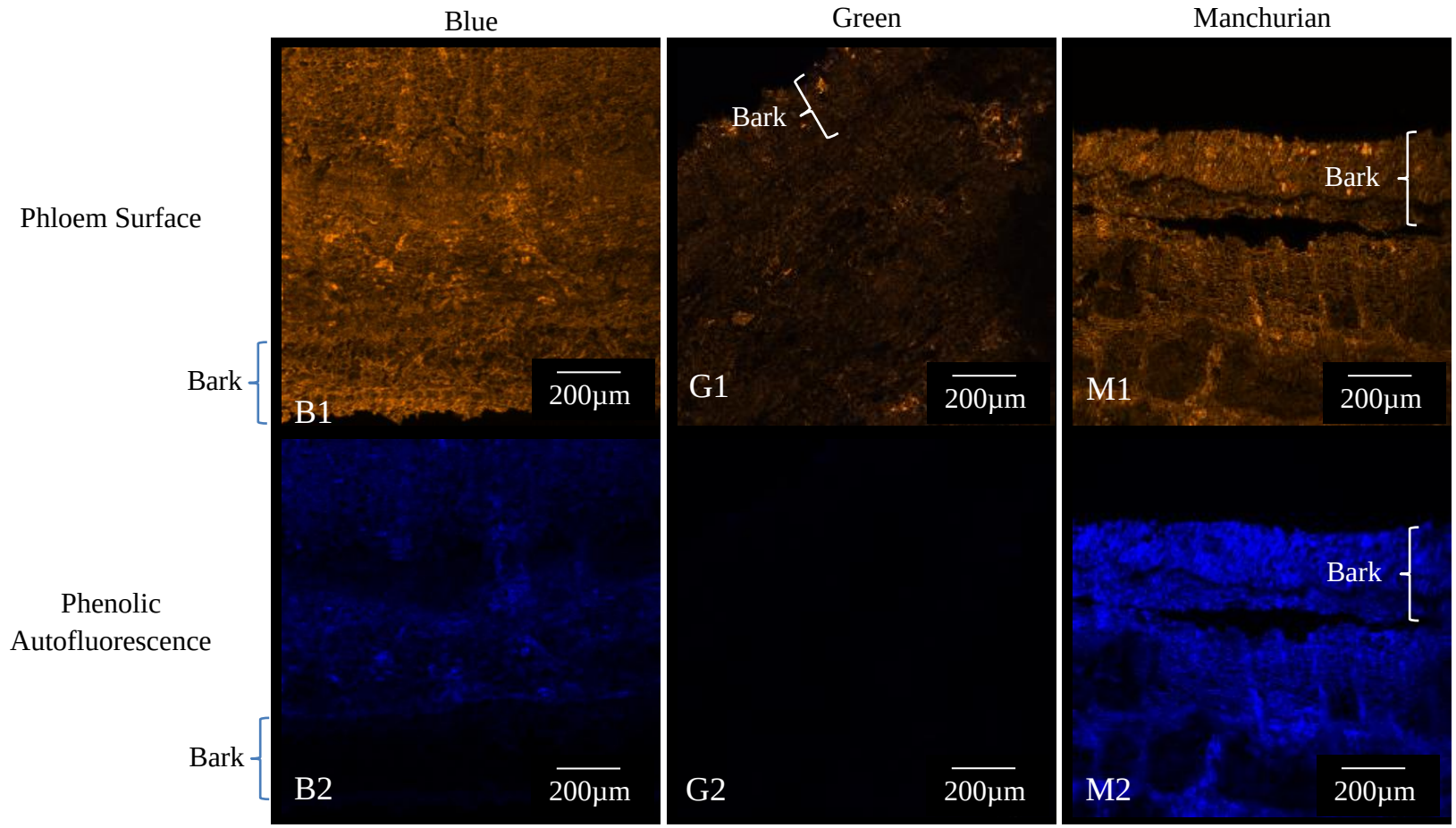


Figure 5.1: Transverse samples of blue ash (B1, B2), green ash (G1, G2) and Manchurian ash (M1, M2) phloem. Samples were illuminated with a helium-neon laser (λ= 543 nm) to show basic phloem structure (B1, G1, M1) or a blue Diode laser (λ=405) to detect phenolic compound autofluorescence (B2, G2, M2). Representative images were obtained with a confocal laser scanning microscope equipped with a 10× dry objective. Three dimensional images of blue ash, green ash or Manchurian ash consist of 79, 24 or 34 slices, respectively. Slices were obtained 11.5 µm apart. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

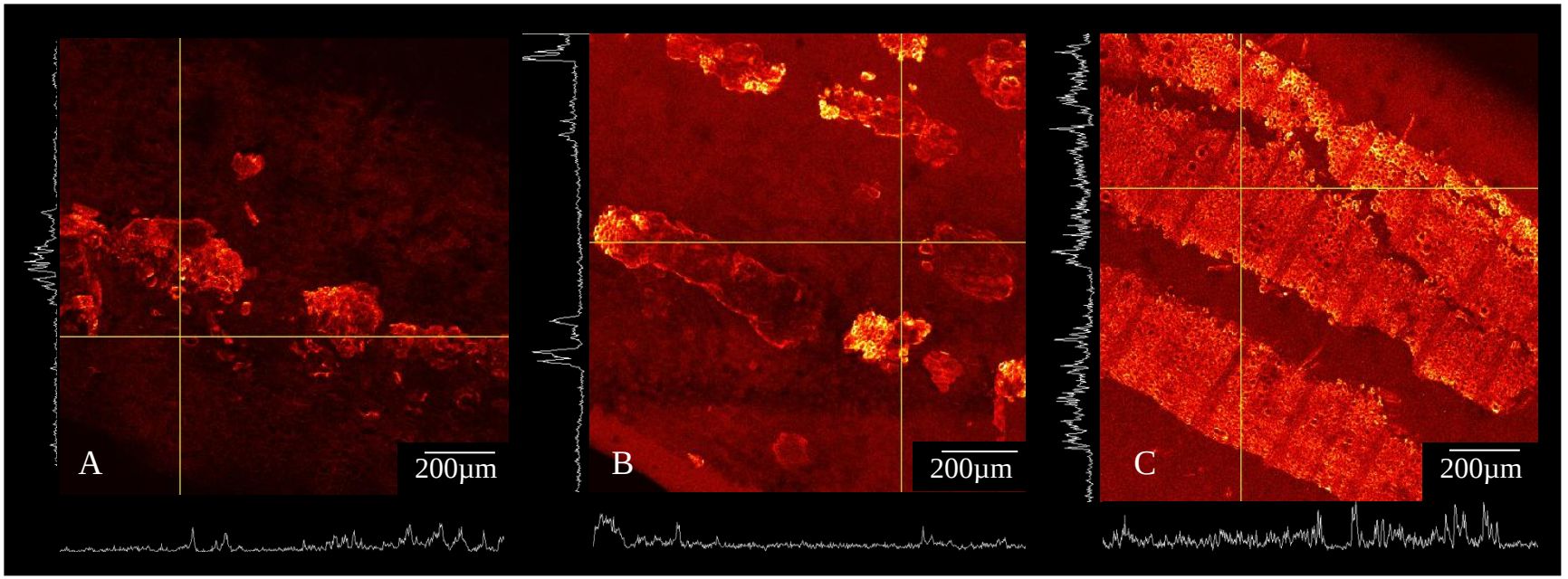


Figure 5.2: Transverse cross sections of (A) blue ash, (B) green ash and (C) Manchurian ash phloem tissues. Tissues were incubated with Amplex® Red peroxidase assay and illuminated with argon (λ=488) and helium neon (λ= 543 nm) lasers. Representative images were obtained with a confocal laser scanning microscope equipped with a 10× dry objective. The three dimensional images consist of 24, 11 or 12 slices, respectively, obtained 5.0 µm apart. Fluorescence profiles correspond to tissues located under corresponding yellow horizontal or vertical lines. Lines were placed in areas where fluorescence was strongest.

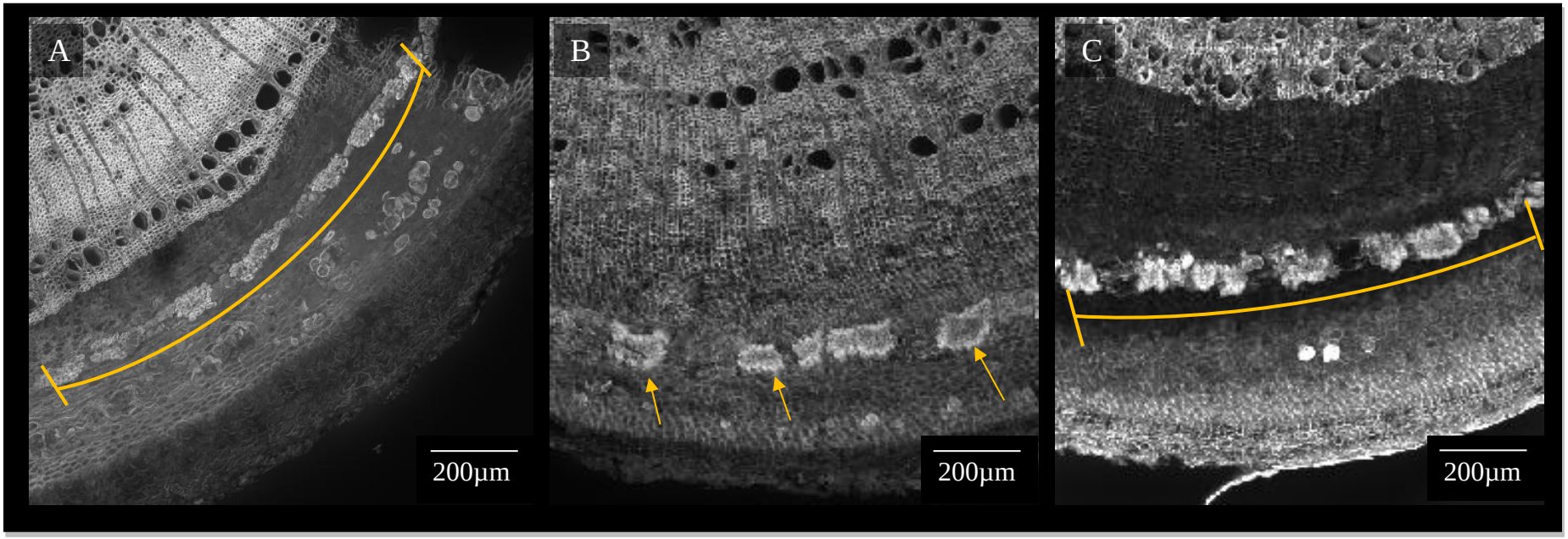


Figure 5.3: Transverse cross sections of (A) blue ash, (B) green ash and (C) Manchurian ash phloem tissues. Tissues were dyed with safranin orange (1%) and illuminated with argon (λ=488) and helium neon (λ= 543 nm) lasers. The three dimensional images consisted of 61, 47 and 56 slices, respectively, taken 5.0 µm apart. Representative images were obtained with a confocal laser scanning microscope equipped with a 10× dry objective for magnification. Tissues directly above the orange arc or the orange arrows indicate sclerenchymatous tissues.

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LITERATURE CITED

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