

Azadirachtin: An Effective Systemic Insecticide for Control of *Agrilus planipennis* (Coleoptera: Buprestidae)

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ABSTRACT The emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), an invasive pest discovered in North America in 2002, is now well established and threatens ash (*Fraxinus* spp.) trees throughout the continent. Experiments were conducted to 1) examine the efficacy of an alternative natural pesticide, azadirachtin, to control emerald ash borer, and 2) determine foliar uptake and dissipation patterns after systemic injections of azadirachtin into trunks of small (2.2 cm diameter at breast height [dbh]), uninfested green ash trees. We found no evidence of mortality of adult beetles. In contrast, fewer larvae completed their development at dose levels ≥ 1.7 mg (AI)/cm dbh and development ceased beyond the second instar at dose levels ≥ 13.6 mg (AI)/cm dbh. Substantial concentrations (11.2 $\mu\text{g/g}$ dry mass [SD = 7.55]) of azadirachtin were present in leaves within 7 d of treatment. After rapid initial uptake, concentrations in leaves declined logarithmically during the 55 d after injection. A similar pattern was observed in a separate experiment that examined the uptake and translocation of azadirachtin in larger green ash trees (22 cm dbh) treated with 250 mg (AI)/cm dbh with the EcoJect injection system. In another experiment, recently infested plantation green ash trees treated with doses ≥ 40 mg (AI)/cm dbh had significant reductions in adult emergence ≈ 1 yr postinjection. Given the inhibition of larval development, reduction of adult emergence, and the occurrence of foliar residues at biologically active concentrations, we conclude that azadirachtin is effective in protecting ash trees from emerald ash borer.

KEY WORDS emerald ash borer, azadirachtin, systemic injection, foliar uptake, larval mortality

Millions of ash (*Fraxinus* spp.) trees have died since summer 2002 when the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) was discovered near Detroit, MI (Cappaert et al. 2005), and Windsor, ON, Canada (Marchant 2005). An invasive wood-boring beetle from northeastern Asia, emerald ash borer attacks all species of ash native to the Great Lakes region. Rebek et al. (2008) suggested that most North American species of ash lack the natural defenses that Asian ash trees possess as a result of coevolving alongside the emerald ash borer. The increased susceptibility of most North American ash species to emerald ash borer attacks combined with the paucity of natural predators, parasites, and pathogens is a threat to billions of ash trees throughout North America (Haack et al. 2002).

Ash trees have been extensively planted in urban areas, often to replace elm (*Ulmus* spp.) trees that were killed by Dutch elm disease (Poland and McCullough 2006). Ashes were selected as replacements

due to their hardiness and ability to grow well in a variety of soil types. In addition, several ash species are common in eastern North America (Grimm 1983) and are a significant component of the remaining forests, woodlots, and riparian areas. Given the abundance of ash trees in these habitats and their susceptibility to this exotic pest, there is high potential for widespread economic, esthetic, and environmental damage (Cappaert et al. 2005).

Management of this insect pest in Canada and the United States has largely focused on slowing the spread of the emerald ash borer through establishment of quarantined and regulated areas; restrictions on movement of live ash trees and all types of firewood; detection surveys in high-risk areas such as campgrounds, parks, and nurseries; and communication strategies to educate the public about the pest (Marchant 2006, McCullough and Siegert 2006). Regulatory agencies in both North American countries initially attempted to remove and destroy outlier infestations but quickly determined that tree removal on a grand scale was not an economical or necessarily effective solution (Marchant 2006, McCullough and Siegert 2006). Infested trees that are now left in place are a significant source of adult beetles.

Control of invasive tree-boring insect pests such as the emerald ash borer, particularly when they occur in

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environmentally sensitive or urban areas, represents a unique challenge because conventional methods of insecticide application via broadcast foliar/bark sprays or soil injection may be publicly unacceptable or environmentally inappropriate (Capraert et al. 2005). Several parasitoid species that attack eggs and larvae have been identified (Liu et al., 2003, Yang et al. 2005, Zhang et al. 2005, Fuester and Schaefer 2006, Lyons 2008). These biocontrol agents may eventually provide long-term emerald ash borer control; however, they are still in the early stages of development as biocontrol agents and may never prove to be effective. There is an opportunity for development of a low-risk, fast-acting, and efficacious means of treating high-value trees such as those found in environmentally fragile or urban landscapes.

Currently, there are several products registered for the control of emerald ash borer in the United States. Most relevant to this study are trunk-injection products, many of which contain the active ingredient imidacloprid. In two recent articles by Kreuzweiser et al. (2008a,b), imidacloprid has been implicated for negative impacts on beneficial soil-dwelling arthropods. The relative safety associated with all neonicotinoids, including imidacloprid, has been questioned in Germany where its use has been suspended due to perceived negative impacts on honey bees (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit 2009). Products currently registered in the United States provide adequate control of emerald ash borer larvae; however, based on their residual effects, toxicity ratings and fate properties, their use in environmentally sensitive and urban areas may not be warranted. Currently, there are no pest control products with a full registration available for the control of emerald ash borer in Canada.

Azadirachtin is a natural product that has insecticidal properties and the potential for systemic control of wood-boring and foliar pests (Marion et al. 1990, Naumann et al. 1994, Lyons et al. 1996, Wanner et al. 1997, Duthie-Holt et al. 1999, Naumann and Rankin 1999, Helson et al. 2001, Poland et al. 2006). Azadirachtin is a term generally used to refer to a family of related natural tetranortriterpenoid compounds found in extracts of the seed kernels of the neem, *Azadirachta indica* Juss. tree. Azadirachtin A and B (AZA-A and AZA-B), the putative active ingredients in neem extracts, have powerful antifeedant, antifertility, and growth-regulating properties in insects (Koul et al. 1990, Schmutterer 1990, Naumann et al. 1994). They exhibit very low toxicity to mammals and birds (Schmutterer 1995). Thompson and Kreuzweiser (2007) recently reviewed the environmental fate and effects of azadirachtin in relation to the Canadian forest industry and demonstrated that formulations containing azadirachtin have low to moderate persistence in water, soil, and foliage and do not present a significant risk to nontarget species studied to date. Therefore, the low-risk toxicological characteristics of azadirachtin make neem products suitable for use in environmentally sensitive areas. The botanical origin of azadirachtin will make it more acceptable

for use in urban areas where public perception of chemical pesticides is often negative.

In this study, we examined the uptake and dissipation of azadirachtin after systemic injections into the trunks of ash trees and its efficacy for control of larval and adult emerald ash borers.

Materials and Methods

Azadirachtin Treatment of Nursery Green Ash Trees. We obtained 66 uninfested nursery (mean diameter at breast height [dbh] = 2.2 cm, SD = 0.31) green ash, *Fraxinus pennsylvanica* variety *lanceolata* Marshall, trees from a nursery outside of the emerald ash borer-quarantined area. Individual trees in black plastic pots (30 cm in diameter) were placed into the ground on 21 May 2003 in Windsor, ON, Canada. The potted trees were placed 1 m apart in four rows also spaced 1 m apart. Adult emerald ash borer beetles were common at the site because the city of Windsor used it for disposal of hundreds of infested trees. The site was fenced off within an industrial area and across the street from the Windsor Raceway, a horse race track. No living ash trees were found within a 100-m radius of the site. Whole trees, logs, branches, and some ash wood chips were disposed of in the yard that was under 24-h security monitoring for 2003 and were monitored during normal business hours throughout 2004. Experimental trees were watered every other day by hand until runoff occurred and the pots were thoroughly soaked. However, despite routine watering, extremely dry conditions in late June and July 2003 led to occasional drought stress in many of the experimental trees.

On 13 June 2003, individual trees were assigned to treatments according to a completely randomized design. For the purposes of this experiment, the technical grade active ingredient NeemAzal (42.3% total azadirachtin A+B, E.i.d. Parry, Bangalore, India) was used to prepare a proprietary formulation referred to as TreeAzin 2 (5% total azadirachtin A+B wt:vol). Treatments consisted of several dose levels of TreeAzin 2 (0, 1.7, 3.4, 6.8, 13.6, 27.3, and 54.5 mg azadirachtin A+B per centimeter dbh; mg [AI]/cm dbh). All treatments were replicated five times with the exception of the 1.7 mg (AI)/cm dbh dose level ($n = 3$). Successively higher dose levels of the active ingredient were achieved by increasing injection volumes by two-fold. The "0" dose level or solvent control involved injection of the blank formulation at maximal volume of formulants minus the active NeemAzal components. Injections were made using a digital pipette to deliver prescribed volumes (75–2400 μ l) into each of three holes (0.79 cm in diameter), spaced 2–3 cm apart and drilled into the base of each tree at a downward angle to a depth of \approx 4 cm. Holes were drilled \approx 10–15 cm above the root flare with a battery operated drill and a 0.79-cm (0.3-in.) drill bit.

Sampling of Leaves from Experimental Trees. Leaf samples from trees receiving the highest azadirachtin dose were collected 7, 14, 20, 28, 40, and 55 d after treatment (DAT) to establish the temporal pattern of

foliar residues. On each sampling date, 10 leaflets (five opposing leaflet pairs) were collected from positions stratified throughout the top, middle, and bottom portions of the tree crown. Five leaflets from each sample were used later on the day of sampling in the adult bioassay experiments. The remaining five leaflets were placed in appropriately labeled plastic sealable bags and transferred on ice to a dark freezer (less than -10°C) within 8 h of collection; they remained frozen until analysis of residues was performed.

Foliar Residue Analysis. Foliar samples were air-dried at ambient temperature (≈ 2 h); macerated at 4,500 rpm for 15 s by using a Knife Mill (Grindomix GM 200, Retsch GmbH, Haan, Germany); and thoroughly mixed by hand-tumbling the macerated sample in the original plastic sealable bag to ensure homogeneity. The Knife Mill and handling implements were washed, rinsed with acetone, and dried between each sample to eliminate potential cross-contamination.

Subsamples (≈ 1 g) of macerated foliage were weighed to the nearest milligram, and then repetitively (five times) extracted in a mixture of water:acetonitrile (7:3), under moderate temperature (40°C) and pressure (13,790 kPa) by using an accelerated solvent extractor (Dionex ASE 200, Dionex Corporation, Oakville, ON, Canada). A similar subsample was taken for determination of percent moisture content, allowing final concentration data to be calculated and reported on a dry mass basis. Aqueous extracts were cleaned by liquid-liquid partition into hexane and ethyl acetate followed by further purification of solvent concentrates on NH_2 solid-phase extraction cartridges (catalog no. WAT020535, Waters, Mississauga, ON, Canada) eluted with methylene chloride. Eluates were evaporated under nitrogen using a Meyer N-Evap model 112 system (Organomation, Berlin, MA), and concentrates were redissolved in methanol. Methanol concentrates were filtered through an Acrodisc CR PTFE $0.45\text{-}\mu\text{m}$ filter and brought to final volume before quantification of azadirachtin A+B by high-performance liquid chromatography-mass spectrometry (LC-MS).

Liquid chromatography-mass spectrometry analyses were conducted using an Alliance 2690 system coupled to a ZMD mass selective detector (Waters, Milford, MA). The LC-MS technique involved isolation of the analytes from potential coextractive interferences by isocratic, reverse-phase chromatography on a Luna ODS column ($5\ \mu\text{m}$, 250 by 4.6 mm, 100 \AA ; Phenomenex, Torrance, CA) with methanol:water (65:35) as the mobile phase. The column was maintained at 33.0°C and eluted at a flow rate of 1.0 ml/min. Azadirachtin A and B showed retention times of ≈ 5.8 and 6.5 min, respectively, and were detected using the mass spectrometer operating in Atmospheric Pressure Chemical Ionization positive (APCI+) mode. Under these conditions, the detector was set to monitor masses of 743 and 726 atomic mass units for azadirachtin A as well as 685 and 667 atomic mass units for azadirachtin B. The analytical method was validated by analysis of foliage matrix blanks fortified with known amounts of azadirachtin A and azadirachtin B

to determine recovery efficiency, precision, and sensitivity. Method validation data demonstrated a high level of mean recovery efficiency ($>88\%$) for both azadirachtin A and azadirachtin B, good precision (coefficient of variance [CV] $<12\%$) for both analytes and good sensitivity with a conservatively estimated limit of detection (LOD) of $0.02\ \mu\text{g/g}$ dry mass (d.m.). All residue data were corrected for analytical recovery losses by multiplying by the reciprocal of the mean recovery efficiency and reported as total azadirachtin (A+B) residues. Residue data were statistically analyzed using SigmaPlot version 10 (Systat Software, Inc., San Jose, CA). Because raw residue data did not conform to the assumption of equal variance, temporal trends were examined by transforming total azadirachtin (A+B) concentrations to their natural logarithms which were then subjected to linear regression versus time (DAT).

Adult Feeding Bioassays. Large numbers of adult beetles were needed for feeding trials. Approximately 400 infested ash logs ≈ 1.5 m in length were placed in a 15.8-m-long truck trailer that served as a large emergence cage. After emergence, adult emerald ash borer beetles oriented toward the light and landed on or near the screened doorway. They were collected daily, placed in 600-ml plastic containers and used immediately in adult feeding bioassays. Leaflets from trees receiving the highest dose ($54.5\ \text{mg [AI]/cm dbh}$) and the solvent control ($0\ \text{mg [AI]/cm dbh}$) were collected 10, 20, and 28 DAT. Leaflets from trees receiving the highest dose were used to determine whether TreeAzin 2 affected adult survival. Digital photos were taken to quantify the leaflet areas at the start of the experiment. The leaflets were photographed with a Camedia D-Series camera (Olympus, Tokyo, Japan) under a 30- by 30-cm sheet of glass alongside a clear 15-cm ruler that was used to calibrate leaflet size. Leaf areas were determined with the aid of Scion Image analysis software (Scion Corporation, Frederick, MD). Five individual leaflets, one from each pair of leaflets sampled, were then placed in a 20 ml water-filled scintillation vial. The vials were covered with a 10- by 10-cm sheet of Parafilm to prevent beetles from falling into the water. We placed leaflets from each experimental tree along with six beetles (three male, three female) into a 600-ml clear plastic bioassay chamber. These chambers were created by taping two 300-ml plastic drinking cups top to top; small ventilation holes (≈ 1 mm in diameter) were made in the upper cup. Bioassay chambers were kept in a controlled environment chamber at $21 \pm 1^{\circ}\text{C}$ and a photoperiod of 14:10 (L:D) h at the University of Windsor's Department of Biology.

Beetles were exposed to leaflets from injected trees for 3 d. Subsequently, the leaflets were removed, photographed again, and replaced with fresh leaflets collected from untreated green ash trees. To determine leaf area consumed, the area of the leaflets at the end of the 3-d exposure period was subtracted from the initial leaflet area (Scion Corporation). All consumption values were based on three male and three female beetles exposed to five treated leaflets over 3 d. A

Student's *t*-test was used to compare the mean differences in consumption of leaf matter from trees injected with the highest dose (54.5 mg [AI]/cm dbh) and the solvent control (0 mg [AI]/cm dbh).

Data on percentage of mortality determined at each monitoring interval were statistically analyzed using analysis of variance (ANOVA) with data transformed when necessary to meet standard assumptions of normality and homogeneity of variance. For some of the interval data sets, removing outliers and applying transformations did not yield normally distributed data. In these cases, the nonparametric Kruskal-Wallis test was used. For all ANOVA and Kruskal-Wallis tests, the independent variable was the insecticide dose in mg (AI)/cm dbh and the dependent variable was the percentage of mortality of emerald ash borer adults. All statistical analyses were performed with a type I error rate of $\alpha = 0.05$, using SAS/STAT software version 8.02 for Windows (SAS Institute 2001). Mortalities in treated groups were adjusted for natural mortality where appropriate (Abbott 1925).

Larval Mortality. The research site had high populations of emerald ash borer adults during summer 2003. Beetles were frequently seen on the experimental trees and had the opportunity to oviposit on them. To assess the potential effects of azadirachtin treatments on developing emerald ash borer larvae, the experimental trees were left on site for ≈ 11 mo post-treatment, after which larval infestations were quantified. The trees were watered by hand until runoff and the pots were thoroughly soaked every other day during summer and once per week in the fall until the first frost occurred. Between 21 May and 9 June 2004, the trees were cut down, branches were removed, and the tree boles were debarked with a draw knife. Emerald ash borer larval galleries were categorized as complete or incomplete. Complete galleries ended with a D-shaped emergence hole or contained a pupal chamber, prepupa, or live third- or fourth-instar larva. Incomplete galleries were characterized by shorter lengths, much smaller gallery diameters, absence of larvae, and no exit holes.

We analyzed the results of the emerald ash borer larval response experiment by using parametric one-way analyses of variance or nonparametric Wilcoxon-Mann-Whitney tests. Where significant differences were detected by ANOVA, differences among treatment means were determined using Tukey's pairwise comparison of means. Separate analyses were conducted to determine differences in mean number of complete, incomplete and total galleries (complete + incomplete) in relation to dose level.

Uptake and Translocation of Azadirachtin in Larger Ash Trees. In a separate experiment, the uptake and translocation of azadirachtin after systemic injections into larger ash trees was investigated. We selected ash trees (mean dbh = 22 cm, SE = 0.51; $n = 5$) representative of high-value trees that landowners may want to protect, in the Olde Family Estate plantation (UTM 17 T 0373508 4685435), and ≈ 300 m from the southeast shore of Lake St. Clair in southwestern On-

tario. The plantation covered an area of ≈ 10.7 ha, and the experimental trees were located in the eastern portion of this land area. The trees were believed to be white ash, *Fraxinus americana* L., and ≈ 25 yr old. Each tree was injected with the proprietary TreeAzin 2 formulation at a dose of 250 mg (AI)/cm dbh. Injections were made on 2 July 2004, by using the newly developed EcoJect injection system (BioForest Technologies Inc., Sault Ste. Marie, ON, Canada). The higher treatment rate used in this trial was determined in accordance with calculations as presented in the EcoJect manual (BioForest Technologies Inc. 2009) and reflects the relatively larger size of the experimental trees at this particular study site. Injections were made using one injection port evenly spaced approximately every 5 cm around the main bole at a height of ≈ 15 cm above ground level to match anticipated application methods. Foliar samples were collected from each treated tree at 11, 15, 22, and 28 DAT by using a pole pruner. Sampling positions were standardized at the four cardinal directions (north, south, east, and west) from the outer branches of both the lower and upper half of the tree crown. Two leaflets were removed from each sampling position for a total of 16 subsamples. From each of the 16 leaf subsamples, two opposing leaflets were excised and pooled to form a single aggregate sample for each individual tree and placed in an appropriately labeled plastic sealable bag. Pooled samples were stored frozen before maceration and homogenization as described above. Subsamples (≈ 1.3 g) of macerated foliar material were extracted and analyzed for azadirachtin residues with a linear regression as previously described under foliar residue analysis of nursery trees.

Azadirachtin Treatment of Plantation Green Ash Trees. In 2005, experiments were conducted in a green ash plantation in Essex County, ON (UTM 17T 348801 4670892) that was ≈ 10 ha, with rows spaced 3 m apart, and individual trees spaced 2 m apart. Agricultural fields surrounded this plantation. Experiments were conducted to determine the effects of azadirachtin treatments on adult emergence ≈ 1 yr postinjection. Four injection dates were selected to determine the effect of injection timing on the development of larvae: one in May to coincide with the start of adult beetle emergence, one in June during the peak of oviposition, and one each in July and August. The carrier compounds of TreeAzin 4 were slightly modified from TreeAzin 2 to improve uptake and to increase ease of injection; no alterations were made to the active ingredients between the two formulations. The nursery had been infested with emerald ash borers for several years and by the end of 2006, the trees that were left untreated had become severely damaged or had died.

Thirty trees (mean dbh = 5 cm) per treatment were randomly selected and treated on four different injection dates and with one to three different doses. Another 30 trees were designated as untreated controls. A stock solution of TreeAzin 4 was prepared and administered in different volumes to achieve different doses. TreeAzin 4 was applied with a pipette into four

evenly spaced holes drilled into the base of each tree. Operational drilling methods were the same as used in the 2003 trials on potted green ash trees. On 16 May 2005, 21 June 2005, 19 July 2005, and 16 August 2005, 30 trees were treated with 40 mg (AI)/cm dbh. On 21 June 2005, two additional doses (20 and 80 mg [AI]/cm dbh) were administered to 30 trees per dose. On 25 April 2006, the number of old exit holes (i.e., the holes produced by emerging adults in 2005) were counted on each tree to a height of 2 m. On 30 August 2006, the total number of exit holes on each tree was counted, and the number of old holes was subtracted from this count to determine the number of new exit holes (i.e., the holes produced by adults emerging in 2006). Beetles that produced the new exit holes had potentially been exposed to azadirachtin during some or most of their larval development. Effectiveness of the treatments was measured by comparing the number of new exit holes in the controls with the number of new exit holes in treated trees.

Data on the number of exit holes was not normally distributed. Consequently, trees were assigned ranks based on the number of exit holes, and a nonparametric ANOVA-on-ranks test was conducted. For the data from trees injected with the intermediate dose of 40 mg (AI)/cm dbh on four different dates, separate tests were conducted on the number of old exit holes and number of new exit holes to determine whether the timing of treatment affected larval mortality. If significant differences were detected with the initial test, the data were further partitioned by injection date and nonparametric Mann-Whitney tests were conducted to examine differences between the trees injected on each date compared with data from the untreated control trees. Two additional analyses of ranks were completed to determine whether the number of old exit holes differed before the experiment started and if there was a dose effect in the number of new exit holes. If significant differences were detected, the injection dose results were further partitioned and nonparametric Mann-Whitney tests were conducted to examine differences between the individual doses and the untreated control trees.

Results

Adult Emerald Ash Borer Response. Azadirachtin treatments had no observable effect on adult emerald ash borer beetles. No significant differences in mortality were observed in bioassays where adult emerald ash borers fed on foliage collected 10, 20, or 28 d after injection of azadirachtin ($P > 0.05$). Maximum mean mortality levels in treated groups observed in these bioassays ranged from 10 to 50% (0–12% after Abbott's adjustment for natural mortality). In all three bioassays, mean mortality increased with time.

The amount of leaf material consumed by emerald ash borer adults exposed to leaves from azadirachtin-treated trees also did not differ significantly between the two doses tested ($t = 1.20$, $P = 0.2643$). The mean amount of leaf material consumed in the solvent control treatment and the high dose (54.5 mg [AI]/cm

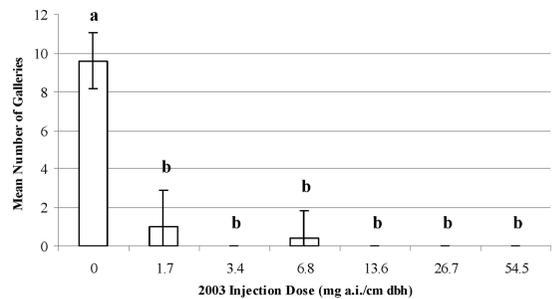


Fig. 1. Mean number of complete emerald ash borer larval galleries as a function of TreeAzin 2 dose ($n = 3$ for 1.7 mg [AI]/cm dbh, $n = 5$ for all other doses). Doses ≥ 1.7 mg (AI)/cm dbh reduced larval development, and no larvae survived in trees treated with ≥ 13.6 mg (AI)/cm dbh. The different letters denote significant differences between the doses tested; a nonparametric Wilcoxon-Mann-Whitney test was used to analyze these results ($\chi^2 = 27.01$, $P = 0.0003$).

dbh) treatment was 17.4 ± 2.15 and 13.7 ± 2.15 cm², respectively.

Larval Emerald Ash Borer Response. Azadirachtin treatments had a strong effect on larval emerald ash borer development. A significant treatment effect was seen in the number of complete galleries (Fig. 1; $\chi^2 = 27.01$, $P = 0.0003$). The nursery trees injected with azadirachtin doses ≥ 1.7 mg (AI)/cm dbh had significantly fewer complete galleries than the solvent control trees. No complete galleries were present in trees injected with doses ≥ 13.6 mg (AI)/cm dbh azadirachtin, and no larvae survived beyond the second-instar stage at these doses. In contrast, differences among treatment means for the number of incomplete galleries ($F_{7,27} = 1.54$; $P = 0.198$) and the total number of galleries ($F_{7,27} = 1.23$; $P = 0.321$) did not differ between treatments.

Uptake and Distribution of Azadirachtin in Systemically Injected Ash Trees. Seven days after treatment, the highest mean (\pm SD) amount of total azadirachtin A+B residues recovered from foliage was 11.2 ± 7.6 $\mu\text{g/g}$ d.m. These residues were the result of caliper-sized (2.2 cm dbh) trees being injected with 54.5 mg (AI)/cm dbh. The coefficient of variation was 67%, indicating variability in initial uptake and translocation among treated trees. Thereafter, mean residue levels followed a logarithmic decline with time, with the lowest mean foliar residue level of 0.81 $\mu\text{g/g}$ d.m. observed at 55 d after treatment. For ease of viewing, untransformed residue means are graphically represented in Fig. 2a. In all samples, azadirachtin A was the predominant compound, on average comprising 77% of the total residue. Linear regression of ln-transformed residue concentrations versus days after treatment accounted for 64% of the variance in the data and demonstrated a highly significant decline of residue with time ($\text{Ln } Y = 2.3588 - 0.0562 \times X$; $F_{1,27} = 47.17$; $P < 0.0001$). The estimated time to 50% dissipation (DT_{50}) was 21 d.

We observed a similar pattern of rapid initial uptake and subsequent logarithmic decline of foliar residues with time in larger (22 cm dbh) ash trees systemically

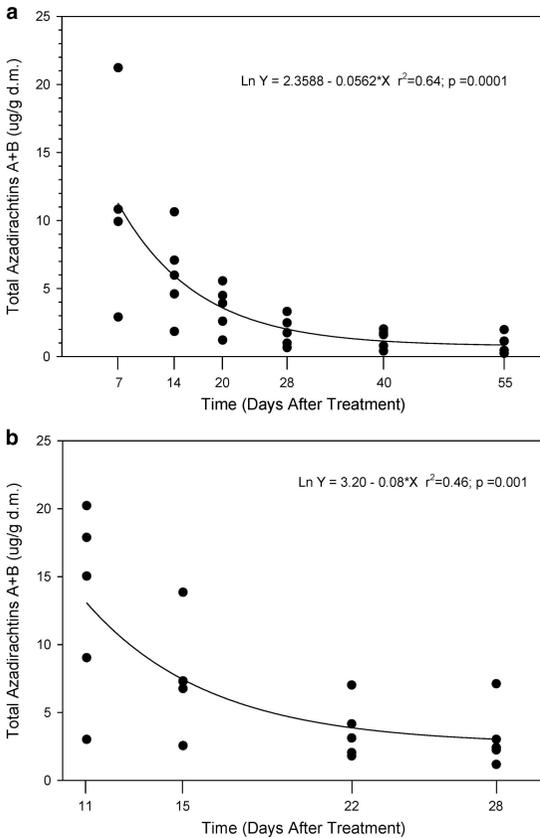


Fig. 2. Uptake and dissipation of total azadirachtin in foliage of relatively small (2.2 cm dbh, $n = 5$; a) and large (22 cm dbh, $n = 5$; b) ash trees after systemic injections with TreeAzin 2. The small tree data are from ash trees injected with azadirachtin at a dose of 54.5 mg (AI)/cm dbh in the 2003 azadirachtin treatment of nursery green ash tree experiment and the large tree data are from trees injected with azadirachtin at a dose of 250 mg (AI)/cm dbh in 2004 for the uptake and translocation of azadirachtin in larger ash trees experiment.

injected with TreeAzin 4 using the Ecoject system. For ease of viewing, untransformed residue means are graphically represented in Fig. 2b. Mean (\pm SD) foliar residues were greatest ($13.04 \pm 6.99 \mu\text{g/g d.m.}$) on the first sampling event 11 d after treatment. The coefficient of variation value of 54% indicates variability in the uptake, translocation, and distribution of azadirachtin residues among the five replicate trees. Again, azadirachtin A consistently accounted for the majority (54%) of total azadirachtin residues observed in the foliage. Linear regression of ln-transformed residue concentrations versus DAT accounted for 46% of the variance in the data and demonstrated a significant decline of residue with time ($\text{Ln } Y = 3.20 - 0.08 \times X$; $F_{1,18} = 15.1$; $P = 0.001$). The estimated DT_{50} of 20 d determined for azadirachtin in foliage of the larger trees was similar to the value of 21 d estimated for the plantation trees in the previous experiment.

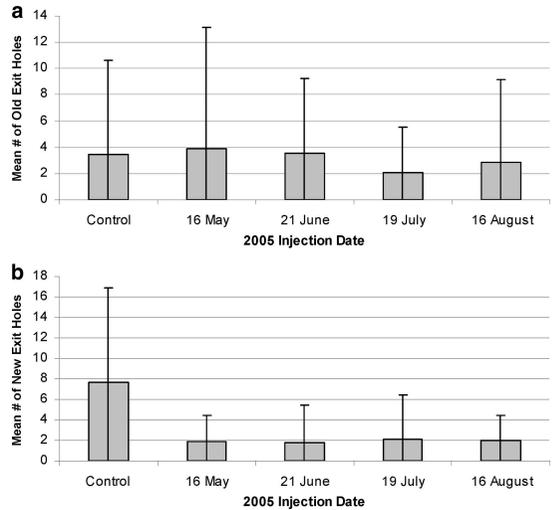


Fig. 3. Mean number of old (a; $P = 0.994$) and new (b; $P = 0.033$) emerald ash borer emergence holes as a function of four different injection dates with TreeAzin four at 40 mg (AI)/cm dbh ($n = 30$ for each injection date). Overall, a significant injection timing effect was seen in the number of new emergence holes counted.

Effects on Emerald Ash Borer Emergence in Plantation Trees. The nursery trees treated in 2005 did not differ in the mean number of old exit holes (representing beetles that developed before treatment) among the four different injection dates (ANOVA-on-ranks; $\text{df} = 4$, $H = 0.222$, $P = 0.994$). However, there were significant differences in the mean number of new exit holes among the four different injection dates (ANOVA-on-ranks; $\text{df} = 4$, $H = 10.462$, $P = 0.033$; Fig. 3). Further partitioning of the injection date results indicates there were significantly fewer new exit holes in trees treated on 16 May, 21 June, and 19 July when compared with untreated controls (Table 1). The 16 August treatment had numerically less but not significantly fewer number of new exit holes ($P = 0.052$). These results suggest that treatments of trees made late in the summer are less effective in reducing adult emergence the following summer, perhaps because larger and more mature larvae are less sensitive to azadirachtin.

Table 1. Analysis-of-variance-on-ranks on the adult emergence response of emerald ash borer to TreeAzin 4 trunk injection treatments of 40 mg (AI)/cm dbh administered on different dates^a

Source	df	H	P
No. new exit holes			
Control vs. all injection dates	4	10.462	0.033
Control vs. 16 May 2005			0.029
Control vs. 21 June 2005			0.007
Control vs. 19 July 2005			0.025
Control vs. 16 Aug. 2005			0.052

^a Results were partitioned with nonparametric Mann-Whitney tests to examine the effects of each individual injection date when compared with the untreated control.

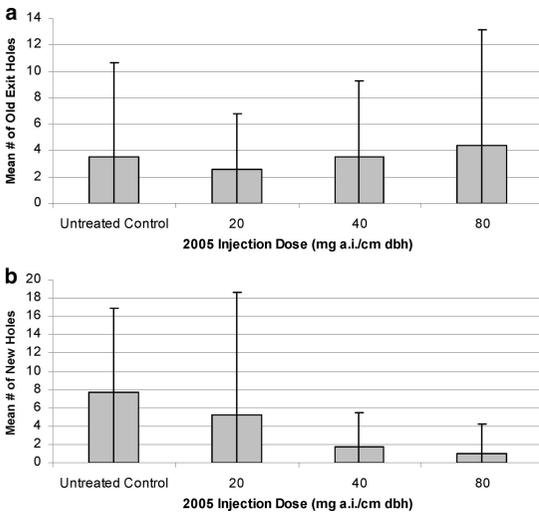


Fig. 4. Mean number of old (a; $P = 0.986$) and new (b; $P = 0.003$) emerald ash borer emergence holes as a function of different doses of TreeAzin 4 injected on 21 June 2005 ($n = 30$ for each treatment). Overall, a significant dosage effect was seen in the number of new emergence holes counted.

Among the different doses injected, no significant differences were seen in the number of old exit holes that reflected the background infestation level (ANOVA-on-ranks; $df = 3$, $H = 0.145$, $P = 0.986$). In contrast, there was a significant dose effect on the number of new exit holes (e.g., larvae developing after injection) in the treated plantation trees (ANOVA-on-ranks; $df = 3$, $H = 14.314$, $P = 0.003$). The mean number (\pm SE) of old and new exit holes counted for the treatments with different doses of azadirachtin are presented in Fig. 4. Further partitioning of these results indicates that only the trees treated with the lowest dose (20 mg [AI]/cm dbh) had numbers of new emergence holes similar to those in the untreated control trees (Table 2).

Discussion

Current pest management trends have indicated that the preferred method of treating insect pest problems in the urban landscape is with either soil or trunk injection of systemic insecticides (Mota-Sanchez et al. 2009); traditional broadcast spraying methods are no

Table 2. Analysis-of-variance-on-ranks on the adult emergence response of emerald ash borer to TreeAzin 4 trunk injection treatments of different doses administered on 21 June 2005. Results were partitioned with Mann-Whitney tests to examine the effects of individual doses when compared with the untreated control

Source	df	H	P
No. new exit holes			
Control vs. all treatment doses	3	14.314	0.003
Control vs. 20 mg (AI)/cm dbh			0.103
Control vs. 40 mg (AI)/cm dbh			0.007
Control vs. 80 mg (AI)/cm dbh			<0.001

longer acceptable by pesticide regulators, environmental advocates and most importantly, homeowners and the public at large. A wide variety of active ingredients has been formulated into trunk injection treatments and is available for use in the United States (Lawson and Dahlsten 2003, Poland et al. 2006, Grosman et al. 2009, Mota-Sanchez et al. 2009). The recent rise in popularity of this insecticide application method has led to a wealth of research on the within tree movement and persistence of trunk-injected products. A recent study by Mota-Sanchez et al. (2009) confirmed that injected imidacloprid accumulates in the leaves of treated trees and steadily increases over the growing season. However, 1 yr postinjection, foliar, trunk, and root imidacloprid levels sharply declined, indicating that after entering the transport system of a tree, the injected product most likely becomes xylem-mobile. For relatively newer active ingredients such as emamectin benzoate and fipronil, within tree movement and flow dynamics are not well or fully understood (Grosman et al. 2009). Most if not all of these trunk-injection treatments cause mortality if a lethal dose of treated foliage or stem tissue is consumed. Based on the low toxicity rating and public perception of azadirachtin as a pesticide, where TreeAzin fits best within this niche market is as a management option for environmentally sensitive urban landscape areas.

We observed no feeding inhibition of adult emerald ash borers, even though azadirachtin is known to possess potent antifeedant properties affecting both larvae and adults of some insect species, especially under exposure regimes and concentrations similar to those of this study (Schmutterer 1995). Adults that fed on leaflets from green ash trees injected with the highest dose of azadirachtin (54.5 mg/cm dbh) exhibited no significant mortality or antifeedant effects even though mean exposure concentrations were in excess of 3.6 $\mu\text{g/g}$ d.m. The lack of adult mortality observed in this experiment is consistent with results from other studies (Larew et al. 1987: birch leafminer, *Fenusa pusilla* [Lepeletier]; Marion et al. 1990: birch leafminer; Schmutterer 1990: planthopper *Nilaparvata lugens* Stål, and fall armyworm, *Spodoptera frugiperda* [J.E. Smith]; and Japanese beetle, *Popillia japonica* Newman; Ascher 1993: *Oncopeltus fasciatus* Dallas, and tobacco cutworm, *Spodoptera litura* F.). Azadirachtin is known to reduce the fecundity of some adult insects, including some beetles, without causing death (Koul et al. 1990, Schmutterer 1990, Naumann et al. 1994, Ji et al. 1998, Athanassiou et al. 2005). Therefore, azadirachtin may negatively affect emerald ash borer populations through indirect reproductive effects on adults, an aspect that should be studied in the future to fully evaluate the potential of azadirachtin for emerald ash borer management.

Azadirachtin treatments of nursery green ash trees reduced the number of completed larval galleries at dose levels as low as 1.7 mg (AI)/cm dbh. At dose levels ≥ 13.6 mg (AI)/cm dbh, no larvae developed beyond the second instar. This result is consistent with the primary mechanism of action for azadirachtin:

inhibition of larval feeding through both antifeedant and insect growth-regulating properties (Gill and Lewis 1971, Ascher 1993). Studies by Naumann et al. (1994) and Duthie-Holt et al. (1999) examined azadirachtin as a control option for two scolytid beetle species, the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, and the pine engraver, *Ips pini* (Say). Their results also indicated that azadirachtin decreased the number of larvae surviving to adulthood but did not halt gallery production completely. Studies that have tested azadirachtin for control of other tree pests, such as the spruce budworm, *Choristoneura fumiferana* (Clemens) (Thomas et al. 1992, Wanner et al. 1997, Helson et al. 2001) and the birch leafminer (Marion et al. 1990), also indicated that high levels of larval mortality can be achieved as a consequence of larvae consuming a lethal dose of azadirachtin through feeding. Thomas et al. (1992) noted that spruce budworm larvae were still actively feeding before death.

The effects of azadirachtin on emerald ash borer development also were examined in the plantation tree experiments. By treating trees at various dates in 2005 and allowing ≈ 1 yr to lapse before assessing the numbers of emergence holes, we recreated a typical operational trunk injection scenario: the treatment of trees known to be in the early stages of emerald ash borer infestation. The results of these experiments suggest that treating trees at an early stage of infestation with a dose of at least 40 mg (AI)/cm dbh can provide enough control to sustain the life of an ash tree for at least 1 yr posttreatment. Trials need to be conducted to determine whether higher doses might be required for larger trees. Furthermore, azadirachtin can provide control of emerald ash borer with injections during the months of expected egg-laying activity from approximately mid-May to at least mid-July.

Substantial concentrations of azadirachtin were expressed in the canopies of systemically injected trees within 7 d of treatment and persisted at detectable levels ($>0.006 \mu\text{g/g d.m.}$) up to 55 d after injection. These results, in combination with significant losses of larvae as evidenced by reduced numbers of completed larval galleries, suggest that azadirachtin translocates rapidly and easily into the canopy of ash trees after systemic injection with the TreeAzin 2 formulation. Foliar residues showed a slow logarithmic decline attributable to metabolic degradation, redistribution and/or dilution through growth. The similarity in maximal initial residues in the two experiments graphically represented in Fig. 2a and b is surprising given the approximately five-fold greater volume of TreeAzin 2 solution applied in the second study to the larger green ash trees. The greater treatment volume may have been offset by greater canopy volume through which the active ingredient was distributed. With a dissipation time (DT_{50}) of 20–21 d, our results suggest that toxicologically significant concentrations of azadirachtin are likely to occur throughout the majority of the larval feeding period in green ash trees of this size systemically treated in early summer.

Naumann et al. (1994) examined the translocation of azadirachtin in lodgepole pine as a chemical control

tool for mountain pine beetle control. Their study indicated that azadirachtin was detected within terminal twig samples but was not consistently detected in bark samples that included the underlying xylem tissue. However, the correlation between mountain pine beetle larval mortality and azadirachtin dose led these researchers to conclude that azadirachtin was present in the stem tissue post injection and provided effective control. Our studies demonstrate substantial residues of azadirachtin in ash trees sufficient to kill emerald ash borer larvae; however, additional studies are required to examine in detail the residue dynamics in the stem tissue of injected ash trees. Understanding these dynamics will allow accurate determination of the magnitude and duration of exposures required to control actively feeding emerald ash borer larvae and minimize feeding damage.

In this study, systemic injections of azadirachtin killed emerald ash borer larvae in situ. Given the dramatic effect on larval development and reductions in feeding galleries, even at relatively low dose levels, further research and development of systemic injection of azadirachtin for protection of ash trees is clearly warranted. This is particularly important given the inherent advantages of systemic injection techniques in sensitive and urban environments.

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