Research Article

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Foliar residue dynamics of azadirachtins following direct stem injection into white and green ash trees for control of emerald ash borer

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Abstract

BACKGROUND: Azadirachtins are natural insecticides derived from the neem tree. The emerald ash borer (EAB) is an exotic invasive insect pest that infests various ash tree species and has the potential for significant economic, aesthetic and ecological impacts throughout North America. The initial translocation and foliar residue dynamics of azadirachtins were examined following direct injection into white and green ash trees growing in urban scenarios as a potential control for EAB.

RESULTS: Substantial concentrations of azadirachtins A and B [mean maxima >0.98 mg kg^{-1} fresh weight (f.w.)] were observed within 2 days of injecting a specifically designed formulation of azadirachtins. Foliar residues declined exponentially through time, with half-life estimates ranging from 5.1 to 12.3 days. At the time of leaf senescence, foliar residue levels approximated 0.01 mg kg^{-1} f.w., strongly mitigating the potential effects of non-target biota in soil or aquatic compartments.

CONCLUSION: The magnitude and duration of exposures observed in this field study were considered to be above the thresholds required for biological effectiveness against both larval and adult life stages of EAB. Results support the use of azadirachtins as an environmentally acceptable systemic insecticide for control of EAB and protection of high-value ash trees in urban environments.

Keywords: azadirachtin; uptake; dissipation; systemic injection; emerald ash borer

1 INTRODUCTION

A recent review[1] estimated 360 cases of alien insect species introductions into North American forests, with attendant economic losses approximating $2.1 billion. Meyerson and Mooney[2] noted that invasive pest introductions are very likely to increase in concert with the process of commercial globalization and increasing international trade. The Canadian Government Strategy on Invasive Species[3] also emphasized the alarming increase in the rate of introductions of invasive alien species and noted that they are now considered the second greatest threat to biodiversity after habitat loss. Langor *et al.*[4] documented more than 419 exotic insects and mites occurring on woody plants in Canada. As noted by Haack,[5] many exotic insects feed on trees and shrubs, with several recent arrivals threatening to spread across continental scales. Among the latter group of invasives, the emerald ash borer (*Agrilus planipennis*) stands out as a key problem species.

The emerald ash borer (EAB) was first discovered in North America near Detroit, Michigan, in 2002, and has since become established in 15 different US states (http://www.aphis.usda.gov/plant_health/plant_pest_info/emerald_ash_b/downloads/multistateeab.pdf), as well as throughout southern Ontario and into the province of Quebec in Canada (http://www.inspection.gc.ca/english/plaveg/pestrava/agrpla/regrestrice.shtml). While research has demonstrated that EAB infestations are not likely to develop on tree species other than ash,[6] surveys have indicated that all native ash species are susceptible.[7] Green ash trees are reportedly colonized earlier and succumb more rapidly than white ash when infested by EAB on the same site.[7] In the environs of the original infestations in the Great Lakes watershed, several species of ash, particularly green and white ash (*Fraxinus pennsylvanica*) and (*Fraxinus americana*), were extensively planted as shade trees in parks and street boulevards, as well as to replace elm (*Ulmus* spp.) which had been previously devastated by Dutch elm disease.[8] Ash was also planted in numerous marginal agricultural land reclamation projects in southern Ontario. In some cases, these

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and other species of ash also represent significant components of remnant natural forests, woodlots and riparian zones in this area.[9]

As noted by previous authors,[10] EAB adults feed on ash foliage particularly during the early summer to acquire energy for maturation and breeding; however, adults cause relatively little damage to the tree per se. Typically, leaf feeding occurs for a period of approximately 2 weeks before mating and oviposition begin. Eggs are laid in bark crevices of the main stem and branches of the tree from late June to early August and hatch within roughly 2 weeks. Larvae chew through the bark and feed on phloem until late autumn, thereby weakening the tree. Successive years of larval feeding activity effectively result in girdling of the tree, preventing sufficient uptake and translocation of water and nutrients and ultimately tree mortality.

Given the abundance of ash trees in both urban and remnant natural forests throughout northeastern North America, their generic susceptibility to EAB attack and the invasive character of EAB, there is an exceedingly high risk of economic, ecological and aesthetic impacts.[10] An effective integrated pest management approach is therefore required to mitigate those risks. While several parasitoid insect species and microbial agents are currently being investigated as potential biocontrols,[7,11,12] none has been shown to provide control over field populations to date. Meanwhile, EAB-infested areas increase dramatically with each passing year, and thus there is an immediate need for non-destructive control options with the ability to protect ash trees of high economic, aesthetic or ecological value. In the United States, several chemical control techniques employing various application methods and systemic insecticides have been developed and are being used. Principal among these is the application of systemic insecticides such as imidacloprid or emamectin benzoate using either stem injection, soil injection or soil drench techniques.[13] Although some of these options have been shown to result in high levels of adult mortality and significant reductions in larval density, there are substantial environmental concerns associated with some of these compounds and application techniques. In particular, soil injection or soil drench techniques may result in unnecessary exposure and risk to non-target organisms in both the soil and potentially adjacent aquatic compartments.[14,15] In general, environmental concerns are exacerbated when the compounds involved are relatively persistent, and such issues may significantly influence registration or operational use in some jurisdictions.

In Canada, where major EAB infestations often occur in association with urban environments, legislative bans, as well as general public opposition to the use of conventional insecticides, significantly constrain potential chemical control options. Given that the treatment objectives in urban scenarios revolve around protection of relatively small numbers of high-value ash trees, single-tree application techniques are viable options. Among the variety of potential techniques available, direct stem injections using insecticides with a favorable environmental profile are considered most likely to receive registration and be accepted by the general public.

Azadirachtins are a family of natural tetranortriterpenoid compounds of botanical origin. They are particularly prevalent in seeds of the neem tree (*Azadirachta indica* Juss.) and exhibit a variety of exploitable biological activities, including significant antifeedant, antifertility and growth-regulating properties in insects.[16] Neem seed extracts are particularly rich in two closely related compounds, referred to as azadirachtins A and B (Aza-A and Aza-B), that are considered to be the putative active ingredients for observed effects on insects. Formulations prepared from these extracts have activity on a variety of wood-boring and foliar pests and varying levels of systemic activity.[17–20] Azadiracthins also exhibit relatively low toxicity to mammals, birds and non-target invertebrates.[21,22] A recent review on the environmental fate and effects of azadirachtins in relation to potential uses in Canadian forestry[23] demonstrated that formulations containing azadirachtins have low to moderate persistence in water, soil and foliage and generally do not present a significant risk to non-target species, with the exception of some freshwater zooplankton species. Given their general activity against wood-boring insect pests, systemic properties, low mammalian toxicity and positive environmental profile, azadirachtins are particularly well suited for development and use as a potential control of invasive wood-boring insect pests such as EAB.

In a recently published paper by the present authors,[24] proof of concept was provided through the demonstration of dramatic inhibition of larval growth and development, reductions in feeding galleries and ultimate mortality of EAB larvae at relatively low dose levels in small and large green ash following stem injections with the formulated product TreeAzin™. In the present paper, this potential is examined further by comparatively assessing foliar residue dynamics in both green and white ash trees growing under typical urban parkland and street boulevard scenarios typical of anticipated primary use patterns.

2 EXPERIMENTAL METHODS

2.1 Experimental design, site and tree characterization

The experimental site chosen for this study was located in the vicinity of Carriage Hill Park, London, Ontario. The approximate site coordinates are (NAD 83 UTM 17T 477 681 4 763 209). The general site location, as well as the specific coordinates for each experimental tree, was obtained using a handheld Garmin GPSmap76CXx GPS unit (Garmin International Inc., Olathe, KS). At the time of study initiation in the summer of 2007, the general area was a known site of EAB infestation and being managed under quarantine restrictions. A few ash trees in the immediate area showed symptoms of latter-stage EAB infestation, including bark cracking, characteristic D-shaped exit holes and crown dieback. Within the park area and along the boulevard of nearby Masonville Crescent, a total of 13 ash trees of similar total height, main stem diameter and dimension of live crown canopy (Table 1) were selected and considered as replicate experimental units. Experimental trees were specifically selected for uniformity, to be free of physical damage on the main stem and as having healthy canopies with no evidence of crown dieback or chlorotic foliage. Stem diameter at breast height (dbh) was measured with a standard dbh tape. Measurements of tree height, as well as height and diameter of live crown, were made using a laser measuring device (LaserAce 300; MDL, Houston, TX).

The study was designed to compare white and green ash trees growing in the park setting ($n = 4$ for each species), with an additional separate comparison of residue dynamics in green ash trees ($n = 5$) growing in park or street boulevard conditions. As there were no white ash trees growing in nearby street boulevards, a similar comparison among white ash in the two different growing environments was not possible. Hence, the experiment may be considered as having three groups or classes (i.e. green ash – park setting, green ash – street boulevard setting, white ash – park setting). Mean tree measurements by species and growing condition class are given in Table 1. Comparative climatic data describing growing conditions (temperature and rainfall) in the year of study

Table 1. Characterization data for white (*Fraxinus Americana*) and green (*Fraxinus pennsylvanica*) ash trees selected for this experiment[a]

		Tree dbh (cm)		Tree height (m)		Canopy height (m)		Canopy diameter (m)	
Species	Class	Mean	CV[b] (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)
Green ash	Park	19	6	9	11	7	11	6	21
White ash	Park	19	15	9	8	7	25	6	29
Green ash	Boulevard	21	10	10	3	8	11	7	7

[a] ANOVA indicated no significant differences among means for tree diameter at breast height (dbh), tree height, height of live canopy or diameter of live canopy ($P > 0.10$).
[b] CV: coefficient of variation.

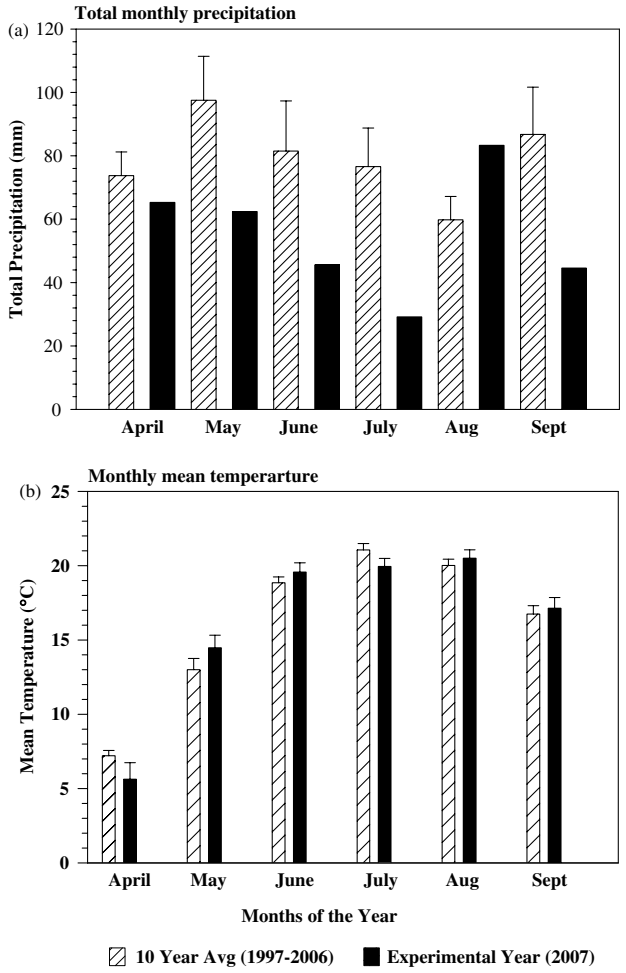

Figure 1. Monthly total precipitation (a) and mean air temperatures (b) for the experimental year (2007), as compared with the 10 year average for the period (1999–2006).

versus averages for the preceding 10 years for this area (Fig. 1) were extracted from the National Climate and Information Data Archive (http://www.climate.weatheroffice.gc.ca/) for the London International Airport and London CS stations.

2.2 Stem injection methods

TreeAzin™ systemic insecticide is a proprietary formulation of azadirachtins owned by the Canadian Forest Service and licensed worldwide for distribution by BioForest Technologies Inc. The product is exempt from the current Cosmetic Pesticide Ban legislation which is now in place in the province of Ontario, where it is scheduled as a class 4 or 'low-risk' pesticide, and is also listed by the Organic Materials Review Institute for use in organic crops. TreeAzin™ contains 50 mg mL^{-1} or 5% total azadirachtins A + B as the active ingredients. The formulation was prepared on 19 June 2007, using technical, active powder material provided by EID Parry (Bangalore, India). The certified total azadirachtins (A + B) content of lot 138 used to prepare the formulation tested in this study was 42%, a value verified as accurate on the basis of an independent analysis via a liquid chromatography diode array detector (LC-DAD). Following preparation of the TreeAzin™ formulation (lot 2007-1), analysis of a subsample showed a concentration of 4.6 µg mL^{-1} or 92% of the expected total azadirachtin (A + B) content. The ratio of active ingredients (Aza-A : Aza-B) in the final formulation was approximately 75 : 25%.

In this study, all experimental trees were injected with TreeAzin™ on 26 June 2007 at a rate of 0.2 g AI cm^{-1} dbh (equivalent to 4 mL of TreeAzin™ per cm dbh) under an experimental use permit (48-RP-07) granted by the Pest Management Regulatory Agency of Health Canada. Stem injections were made with the EcoJect® system for direct stem injection of systemic pesticides, which was recently introduced by BioForest Technologies Inc, Sault Ste. Marie, ON. Stem injections were made following general instructions as documented in the operating manual[23] but employing four injection ports per tree as a standard protocol. The creation of injection ports involved drilling four 0.58 cm (15/64 inch) holes at equal spacings about the circumference of the main stem at approximately 15–30 cm above the ground. Each hole was drilled to a depth of 1.3–1.9 cm (0.5–0.75 inches) into the xylem tissue at a downward angle of 20–45°. A plastic EcoJect® nozzle was then inserted snugly into each port and mated to an 8 mL canister that had been prefilled with the TreeAzin™ formulation using the EcoJect® pump system. Once attached to the nozzle, the spring-loaded system automatically injected the formulation into the tree. Details pertaining to the stem injections for each tree are provided in Table 2, and an example of the stem injection set-up as employed in this study is shown in Fig. 2. Given the injection volumes required, a total of eight full canisters were routinely applied to each tree (two canisters deployed in sequence at each port). Residual volumes required to attain the total volume to be injected for any individual tree were subsequently applied using measured volumes in partially filled canisters.

2.3 Foliar sampling

Composite samples of canopy foliage were obtained from each experimental tree at several discrete time periods. Sampling was initiated on 28 June 2007, 2 days after treatment (DAT), and thereafter at 7, 14, 29, 43, 56, 70 and 83 DAT as well as at 365

Table 2. Calculated volumes and masses of total azadirachtins (A + B) injected into each experimental tree[a]

Tree number	Species	Diameter dbh (cm)	Treatment rate (g cm^{-1} dbh)	Total Aza (mg)	Total formulation (mL)	Residual volume (mL)
20	Green	19.0	0.2	3800	76.0	12.0
11	White	21.0	0.2	4200	84.0	10.5
13	Green	19.0	0.2	3800	76.0	9.5
17	White	15.7	0.2	3140	62.8	7.9
14	Green	17.0	0.2	3400	68.0	8.5
15	Green	19.8	0.2	3960	79.2	9.9
16	White	21.0	0.2	4200	84.0	10.5
18	White	16.7	0.2	3340	66.8	8.4
21	Green	19.5	0.2	3900	78.0	9.8
22	Green	20.0	0.2	4000	80.0	10.0
23	Green	20.0	0.2	4000	80.0	10.0
24	Green	24.0	0.2	4800	96.0	12.0
25	Green	20.0	0.2	4000	80.0	10.0

[a] All injections were completed using EcoJect® canisters containing 8 mL of TreeAzin™ formulation (50 mg mL^{-1} of total azadirachtins A + B).

Figure 2. Direct stem injection of TreeAzin™ into ash trees using EcoJect® canisters and the exemplary growing conditions for green ash growing in a street boulevard. Note: under the standard protocol used for stem injections in this study, a total of four equally spaced injection ports were used for each tree. The fourth injection port and canister on the opposite side of the tree are not visible in this photograph.

DAT. Sampling at 70 and 83 DAT was undertaken to coincide with the general onset of leaf senescence and leaf fall for ash trees in this area. With the aid of a pole pruner, randomly selected twigs were excised from each of eight positions, one in each of the four cardinal directions in both the upper and lower half of the canopy. From the excised twig, a representative compound leaf was removed, and, from each of these, two opposite leaflets were taken and placed into a Ziploc™ bag. Each bag was uniquely labeled to identify the tree species, individual tree and date of sampling. As such, composite samples comprised 16 individual leaflets representative of those throughout the canopy at any given sample date and integrated, to some degree, the expected variation in residues within the tree.

Immediately following collection, composite foliar samples were placed in an insulated cooler together with frozen ice packs and stored under cool, dark conditions. Following completion of sampling for all 13 experimental trees, foliar samples were transferred into frozen storage (below −10 °C) at a nearby storage facility and maintained under frozen condition pending extraction and analysis.

2.4 Sample preparation and analysis

Composite foliar samples were thawed and allowed to air dry under dark, ambient conditions overnight prior to maceration. Maceration of semi-dried samples was achieved in clean glass mason jars (250 mL) inverted and fitted to a conventional kitchen blender (Oster, Niles, IN). Subsamples (1 g) of the macerated foliage were subjected to multiple extractions (five cycles) with 100% acetonitrile (CH_3CN) using an accelerated solvent extractor (ASE) (Dionex ASE 200; Dionex Inc., Sunnyvale, CA) operated at ambient temperature and high pressure (1.38 10^7 Pa). Prior to extraction, macerated foliar subsamples were mixed homogeneously with C_{18} silica (1 g) and diatomaceous earth (0.75 g) to enhance solvent–matrix interaction. The mixture was placed in the extraction cell together with 0.5 g of primary/secondary amine bonded silica (PSA; Supleco, Bellafonte, PA) to effect an automated clean-up. Raw extracts obtained from ASE were adjusted to a constant volume (25 mL) with CH_3CN. An aliquot (5 mL) was further treated by shaking with PSA (0.1 g) for 1 min. Solvent extracts were evaporated to near dryness, reconstituted in acetonitrile/water (50 : 50, v/v) and filtered using a nylon 0.2 µm Acrodisc® prior to LC-MS analysis.

The analytical instrumentation comprised a Waters Alliance 2690 LC (Waters, Milford, MA) coupled to a ZMD single quadrupole mass spectrometer through an orthogonal Z-spray interface. Chromatographic separation was achieved by injecting 25 µL of the filtered final sample into a C_{18} column (Kinetex, 50 × 2.1 mm i.d., 2.6 µm; Phenomenex, Torrance, CA), using a mobile phase of 10 µM sodium acetate in methanol/10 µM sodium acetate in water at a flow rate of 200 µL min^{-1}. In the mobile phase gradient, the percentage of methanol changed with time as follows: 0.0 min 30%; 1.0 min 30%; 3.0 min 60%; 4.0 min 90%; 9.8 min 90%; 9.9 min 30%; 15.0 min 30%. Analyses were conducted under positive ESI mode with selected ion monitoring using ions 743.2 and 685.2 for quantitation of azadirachtins A and B respectively. Confirmation of the analytes was made using additional ions 725.2 and 665.2 for Aza-A, as well as 667.2 plus 567.2 for confirmation of Aza-B.

The analytical method was validated in accordance with standard guidelines[25] using green ash foliage matrix blanks fortified at concentrations of 0.01, 0.1 or 1.0 mg kg^{-1} fresh weight (f.w.) of Aza-A and Aza-B. During the application of the method, similarly fortified samples were run as concurrent quality controls. Following testing for equality of recovery efficiency among the three different fortification levels, correction factors were calculated for each analyte. Correction factors were calculated on the basis of the reciprocal of overall mean recovery efficiencies. Multiplying raw data values by these factors corrected for the small analytical recovery losses observed. The resultant foliar concentration estimates were reported as the sum total of azadirachtin (A + B) residues, typically in units of mg kg^{-1} f.w. Where concentrations on a dry mass (d.m.) basis (mg kg^{-1} d.m.) were of interest, these were calculated on the basis of the average moisture content of 57% [15% coefficients of variation (CV)] for samples (n = 14) selected to represent the average moisture content observed for representative field samples.

2.5 Statistical analyses

Fundamental summary statistics, including means, standard deviation and CV, were calculated using Microsoft Excel 2003 (Microsoft Corporation). Analysis of variance (ANOVA) and non-linear regression analyses were conducted using SigmaPlot v.11 (Systat Software Inc., 2008). All data were tested for normality using the Kolmogorov–Smirnov test (with Lilliefors correction) and for homogeneity of variance using the Spearman rank correlation between the absolute values of the residuals and the observed value of the dependent variable. These tests are standard default procedures in the SigmaPlot program. Where data did not conform to fundamental assumptions, data were subjected to Kruskal–Wallis ANOVA on ranked data also using SigmaPlot software.

Scatter plots of foliar residues relative to time (days after treatment) showed consistent curvilinear dissipation patterns. Therefore, non-linear regression techniques were used to model the dissipation curves for each of the three test cases. Data for each combination of ash species and growing conditions were examined separately. Best-fit non-linear models were chosen as those meeting standard regression assumptions and exhibiting the highest coefficient of determination (r^2) values and the lowest standard error of the estimates (SEE) and where all parameters were significant ($P < 0.05$). In all three test cases, exponential decline models of the form $y = a^* \exp(-b^* x)$ were determined as best fits to the data. As variance was clearly non-homogeneous, and correlated directly with the value of y, weighted regression using the reciprocal of $1/Y$ was employed in all cases.

3 RESULTS AND DISCUSSION

3.1 Analytical-quality control results

Statistical analysis of analytical-quality control samples fortified at levels of 0.01 ($n = 14$), 0.1 ($n = 17$) and 1.0 ($n = 16$) mg kg^{-1} f.w. showed no significant differences ($P = 0.0726$) in mean recovery efficiencies. As such, overall average values were calculated together with estimates of associated variability. The overall mean and CV for azadiracthins A and B were 92.6% (26%) and 89.9% (25%) respectively. These results demonstrated excellent recovery efficiency and reasonable levels of precision for quantitation for analysis of azadirachtins A and B in ash foliage. The analytical method demonstrated sufficient specificity and robustness for the purposes of this experiment and showed LOQ for both analytes of 0.01 mg kg^{-1} of ash foliage on a fresh weight basis.

3.2 Tree measurements, environmental conditions and uptake rates

Uptake and translocation of pesticides in trees comprise a dynamic process influenced by a substantial number of variables, including those associated with tree dimensions, tree physiology and health, growing environment and local climatic conditions. As evidenced by the mean data presented in Table 1, experimental trees were very similar in dimensional attributes that might influence uptake, translocation and expression of foliar residues. Statistical analysis of these data showed no significant differences in mean tree height ($P = 0.125$), diameter at breast height ($P = 0.258$), live canopy height ($P = 0.225$) or live canopy diameter ($P = 0.874$).

Prior to initiation of this study, local climatic conditions were generally dry and hot (Fig. 1). Monthly mean air temperatures were slightly in excess of 10 year averages for each of the months of April, May and June. Total precipitation, however, was substantially lower than the long-term averages for each of these months. It is postulated that uptake and perhaps translocation may be somewhat impaired under such suboptimal conditions. This is particularly true for parkland trees, as the area was not routinely irrigated and soils were notably more compacted. In contrast, experimental green ash trees growing in street boulevards were being maintained by homeowners. Maintenance included the use of cedar mulches (Fig. 2) and probably routine watering and fertilization, as may be inferred by vigorously growing green grass about each tree, in contrast to the dry, yellow conditions of grass that were observed in the park prior to and throughout the experimental period.

3.3 Initial foliar concentrations

Initial foliar residue levels may be viewed as an integrative metric of translocation dynamics incorporating differentials in species, growing conditions and physiological factors. In addition, the magnitude and duration of azadirachtin residue concentrations are key determinants of dose and therefore potential effects on foliar-feeding EAB adults. In spite of the relatively slow uptake observed in this experiment, significant concentrations of azadirachtins A and B were observed in the first foliar samples collected only 2 days after treatment (Fig. 3; Table 3). This result demonstrates that, in spite of the suboptimal conditions noted above, rapid translocation of azadirachtins occurred in both green and white ash trees. The relative proportions of Aza-A and Aza-B observed in foliage at 2 DAT in both green ash (73 : 27%) and white ash (71 : 29%) were essentially the same as the proportion of these analytes in the injected formulation, suggesting equivalent initial uptake of both compounds. Substantially higher, but statistically non-significant, differences in initial concentrations were observed among green ash trees growing in boulevard conditions as opposed to relatively poor parkland conditions. In contrast, significantly ($P < 0.05$) higher residue levels were observed in white ash compared with green ash under parkland conditions, suggesting that white ash may have an innately greater capacity to translocate azadirachtins.

Mean initial foliar concentrations of azadirachtins A + B as observed in this study (<5.5 mg kg^{-1} d.w. on DAT 2) were substantially below the mean levels of 13.04 mg kg^{-1} d.w. reported for samples taken at 11 DAT from similarly sized ash trees injected using the EcoJect® system,[19] even after accounting for slight differences in the injection rates employed in these two studies (0.2 versus 0.25 g AI cm^{-1} dbh). These comparative results suggest that complete uptake of azadirachtins was impaired under the suboptimal conditions of this study.

In spite of the relatively low initial foliar concentrations, all experimental trees in the present study showed substantive initial concentrations (>1 mg kg^{-1} f.w.) of total azadirachtins in foliage, which remained above 0.1 mg kg^{-1} up to 29 days after treatment. As such, foliar residue data from this experiment indicate that both adults and larval EAB would have been exposed to substantial levels of azadirachtin residues throughout their respective maturation and developmental feeding periods. The initial foliar residue levels as observed in this study are above the threshold level of 1 mg kg^{-1}, which has been observed to significantly reduce fecundity of foliar-feeding EAB adults in laboratory studies (Thompson D *et al.*, unpublished). As the destructive sampling of trees was impossible in this urban scenario, it was not possible to directly estimate phloem residue levels or larval efficacy parameters in this experiment. However, based on

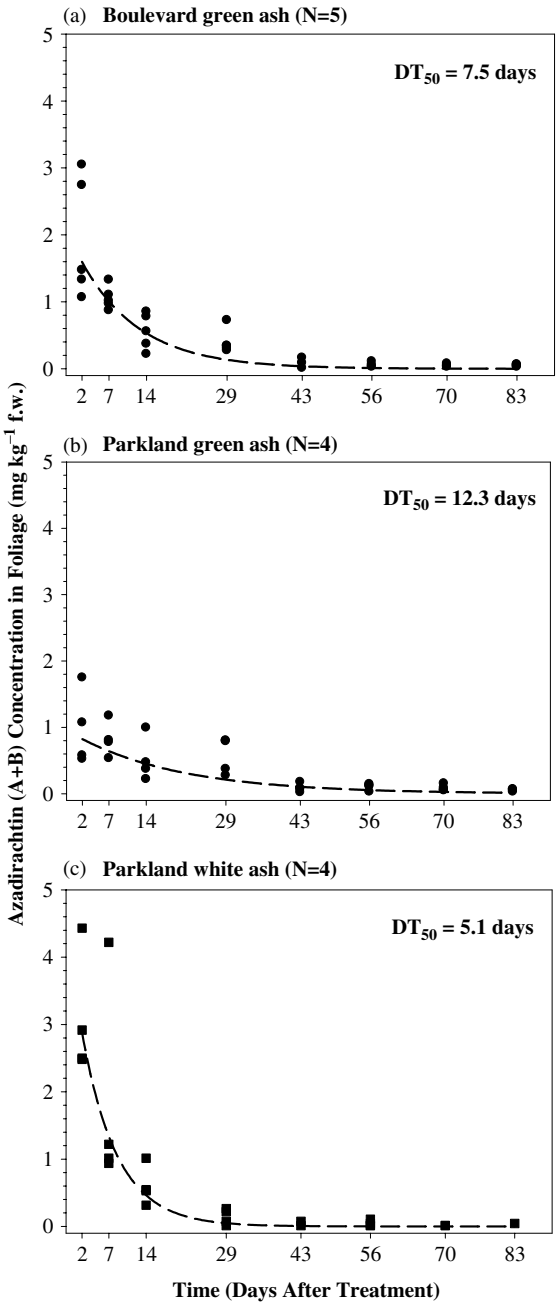

Figure 3. Dissipation of total azadirachtin A + B foliar residues following direct stem injection of TreeAzin™ at a rate of 0.2 g AI cm^{-1} dbh into green or white ash in various urban settings.

the significantly reduced number of completed larval galleries, the reduced number of new exit holes and the greater crown density estimates that have been observed in previous studies by the present authors,[19] involving similar injection rates, uptake and persistence dynamics, it is postulated that similar levels of efficacy on larval EAB would have been likely in this study.

3.4 Dissipation kinetics and persistence estimates for foliar residues

Azadirachtins A and B are known to be relatively labile natural compounds, susceptible to photolysis, hydrolysis and microbial degradation.[23] In this study, total azadirachtin A + B residues were observed to be non-persistent in foliage of either green or white ash. Foliage samples taken from either species at 7 and 14 DAT showed progressively lower proportional concentrations of Aza-A compared with Aza-B. By 14 DAT, the proportion of the total foliar concentrations attributable to Aza-B had increased to 60% and 52% in green and white ash, respectively, compared with initial values approximating 27–29%, thus suggesting that Aza-B was slightly more persistent than Aza-A in ash tree foliage.

Foliar residues dissipated rapidly in both species, following exponential decline kinetics, as shown in Fig. 3. Statistical data pertaining to the fitted models and derivative persistence endpoints of time to 50 and 90% dissipation are provided in Table 4. The rapid initial uptake and dissipation patterns observed in this study on trees growing under typical urban conditions are very similar to those previously reported for nursery- and plantation-grown ash.[19] This suggests that generally good uptake and translocation of azadirachtins following direct stem injection with the EcoJect® system may be anticipated irrespective of ash tree size or growth conditions. The coefficient of determination (r^2) values, as reported in Table 4, indicate that the exponential model accounted for 64–82% of the observed variation in residue dissipation patterns, depending on the test scenario. DT_{50} values ranged from 5.1 days in parkland white ash to 12.3 days in parkland green ash, reflecting the generally rapid dissipation of total azadirachtin foliar residues over time. While residues in white ash dissipated relatively more rapidly than those in green ash under either growing condition, more than 90% of total azadirachtin residues had dissipated by 44 DAT in all cases.

In the present study, only the dynamics of Aza-A and Aza-B foliar residues were investigated. There was no attempt to monitor metabolites from azadirachtin A and B or other similar nortriterpenoid compounds known to exist in formulations derived from neem seed extracts and that may contribute to

Table 3. Mean foliar residues of total azadirachtins (A + B) observed in green and white ash trees growing under different conditions in urban scenarios and at various times after stem injection of TreeAzin™ at a rate of 0.2 g AI cm^{-1} dbh

				Mean foliar residue[b] (mg kg^{-1})		
Species	Type	DAT[a]	*N*	f.w.[c]	d.w.[d]	CV[e] (%)
Green ash	Boulevard	2	5	1.93	3.35	46
Green ash	Park	2	4	0.98	1.70	58
White ash	Park	2	4	3.08	5.35	30
Green ash	Boulevard	70	5	0.05	0.08	41
Green ash	Park	70	4	0.09	0.15	56
White ash	Park	70	4	≤0.01	≤0.02	22
Green ash	Boulevard	365	5	≤0.01	≤0.02	23
Green ash	Park	365	4	0.02	0.03	58
White ash	Park	365	3	≤0.01	≤0.02	43

[a] DAT: days after treatment.
[b] Kurskal–Wallis ANOVA on ranks followed by Dunn's multiple comparison method performed on initial (2 DAT) data showed a significant difference ($P < 0.05$) between residue levels observed in white ash and green ash growing in the park, but not among green ash growing under park and boulevard conditions. Values shown with a ≤ symbol indicate that all are at or below analytical limits of quantitation on a fresh weight basis.
[c] f.w.: fresh weight.
[d] d.m.: dry mass.
[e] CV: coefficient of variation.

Table 4. Non-linear regression statistics for models of total azadirachtin (A + B) residue dissipation in green and white ash following direct stem injection of TreeAzin™

Species Site	Green ash Boulevard	Green ash Park	White ash Park
Y_i	1.93	0.98	3.59
Y_{i50}	0.965	0.49	1.795
Y_{i10}	0.193	0.098	0.359
a	1.92	0.91	3.92
b	−0.090	−0.050	−0.153
r^2	0.770	0.635	0.820
SEE	0.332	0.342	0.390
P	<0250001	<0.001	<0.001
DT_{50}	7.5	12.3	5.1
DT_{90}	24.9	44.1	15.6

Exponential decline models take the form $y = a^* \exp(-b^*x)$.
Y_i = initial mean foliar concentration of total azadirachtins (A + B).
Y_{i50} = calculated value for 50% of the initial concentration.
Y_{i10} = calculated value for 10% of the initial concentration.
a = model intercept estimate.
b = model slope estimate.
r^2 = coefficient of determination.
SEE = standard error of estimate.
DT_{50} = estimated time for residues to decline to 50% of initial value.
DT_{90} = estimated time for residues to decline to 90% of initial value.

biological activity. Given that analysis of azadirachtin metabolites and related nortriterpenoids in complex environmental matrices requires highly sophisticated analytical techniques, there is relatively little information available on the potential role of these compounds in biological activity on either EAB or other target and non-target organisms. However, with recent advances in hybrid quadrupole time of flight (QTOF) LC-MS techniques, this is an area where further research may now be logistically feasible. Future studies undertaken to investigate this aspect should also include a sampling regime that allows for assessment of within-tree variation in residue levels in foliage, as pertinent to effects on maturation of feeding adults, and in xylem, cambium and phloem tissues, as pertinent to larvae.

3.5 Late-season and 1 year post-treatment foliar residue levels

The dissipation curves (Fig. 3), as well as data presented for mean foliar concentrations of azadirachtins A + B (Table 3), indicate that these analytes are at very low levels approximating analytical limits of quantitation (LOQ) at any time beyond 70 DAT. In this regard, sampling at the 70 DAT time point was invoked specifically to correspond to the time when leaf senescence and abscission are beginning to occur in ash trees throughout southern Ontario. Mean residue levels for samples taken at 83 DAT were consistently at or below 0.05 mg kg^{-1} in all three test cases. Overall, the continuous decline in residues throughout the treatment year and the exceedingly low-level residues observed both in the fall and in the early summer of the year subsequent to treatment indicate an optimal temporal pattern likely to induce significant effects in EAB adults and larvae, with minimal potential for effects in non-target organisms, except in those feeding during the same timeframes and having sensitivity equivalent to that of EAB. Organisms whose feeding activity increases in late summer and fall, for example during prehibernation periods, are thus very unlikely to be exposed to biologically meaningful levels of azadirachtins. Similarly, the potential magnitude of exposure for biota in soil or surface water compartments receiving leaf litter inputs may be estimated as 0.05 mg kg^{-1} or less, a value well below any published biological effect threshold levels of which the authors are aware.

4 CONCLUSIONS

Results of this study demonstrate rapid translocation of azadirachtins following stem injection of TreeAzin™ into white and green ash trees growing in typical urban scenarios of southern Ontario. The persistence of foliar residues was limited, with concentrations declining exponentially with time in all cases. Based on comparative assessment with previous and recent studies, the magnitude and duration of exposures observed following these treatments are considered to be in excess of the thresholds required to induce significant effects in both foliar-feeding adult EAB and larvae feeding in the stem tissues, and thus protective of tree health. Essentially complete dissipation of foliar residues prior to leaf abscission with no quantifiable levels of azadirachtin in foliage in the year subsequent to treatment indicates an exceedingly low potential for non-target effects associated with transfer of residues through leaf fall into receiving soil or aquatic systems. Results of this study add incrementally to the weight of scientific evidence supporting the use of stem injections of azadirachtin as an environmentally acceptable and effective method for controlling emerald ash borer and protecting the health of high-value ash trees in urban environments.

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REFERENCES

1 Pimental D, Zuniga R and Morrison D, Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecol Econ* **52**:273–288 (2005).
2 Meyerson LA and Mooney HA, Invasive alien species in an era of globalization. *Front Ecol Environ* **5**:199–208 (2007).
3 An invasive alien species strategy for Canada. Government of Canada, p. 46 (2004).
4 Langor DW, DeHaas LJ and Footit RG, Diversity of non-native terrestrial arthropods on woody plants in Canada. *Biol Invasions* **11**:5–19 (2009).
5 Haack RA, Exotic bark- and wood-boring Coleoptera in the United States: recent establishments and interceptions [electronic resource]. *Can J Forest Res* **36**:269–288 (2006).
6 Anulewicz AC, Poland TM, Cappaert DL and McCullough DG, Host range of the emerald ash borer (*Agrilus planipennis* Fairmaire) (Coleoptera: Buprestidae) in North America: results of multiple-choice field experiments [electronic resource]. *Environ Entomol* **37**:230–241 (2008).

7 Liu H, Petrice TR, Haack RA, Zhao T, Bauer LS and Gao R, Exploratory survey for the emerald ash borer, *Agrilus planipennis* (Coleoptera: Buprestidae), and its natural enemies in China. *Great Lakes Entomol* **36**:191–204 (2003).

8 Poland TM and McCullough DG, Emerald ash borer: invasion of the urban forest and the threat to North America's ash resource. *J For* **104**:118–124 (2006).

9 Grimm CW, *The Illustrated Book of Trees*. Stackpole Books, Harrisburg, PA (1983).

10 Cappaert D, Siegert NW, Poland TM and McCullough DG, Emerald ash borer in North America: a research and regulatory challenge. *Am Entomol* **51**:152–165 (2005).

11 Liu H and Bauer LS, Microbial control of *Agrilus planipennis* (Coleoptera: Buprestidae) with *Beauveria bassiana* strain GHA: field applications. *Biocontrol Sci Tech* **18**:557–571 (2008).

12 Yang Z-Q, Wu H, Gould JR and Wang X-Y, Host specificity of *Spathius agrili* Yang (Hymenoptera: Braconidae), an important parasitoid of the emerald ash borer [electronic resource]. *Biol Control Theory Applic Pest Manag* **47**:216–221 (2008).

13 Herms DA, McCullough DG, Smitley DR, Sadof C, Williamson RC and Nixon PL, Insecticide options for protecting ash trees from emerald ash borer. North Central IPM Center (2009).

14 Kreutzweiser D, Good K, Chartrand D, Scarr T and Thompson D, Non-target effects on aquatic decomposer organsims of imidacloprid as a systemic insecticide to control emerald ash borer in riparian trees. *Ecotoxicol Environ Saf* **68**:315–325 (2007).

15 Kreutzweiser DP, Thompson DG and Scarr TA, Imidacloprid in leaves from systemically treated trees may inhibit litter breakdown by non-target invertebrates. *Ecotoxicol Environ Saf* **72**:1053–1057 (2009).

16 Koul O, Isman MB and Ketkar CM, Properties and uses of neem, *Azadirachta indica*. *Can J Bot* **68**:1–11 (1990).

17 Poland TM, Haack RA, Petrice TR, Miller DL and Bauer LS, Laboratory evaluations of the toxicity of systemic insecticides for control of *Anoplophora glabripennis* and *Plectrodera scalator* (Coleoptera Cerambycidae). *Econ Entomol* **99**:85–93 (2006).

18 Poland TM, Haack RA, Petrice TR, Miller DL, Bauer LS and Gao R, Field evaluations of systemic insecticides for control of *Anoplophora glabripennis* (Coleoptera: Cerambycidae) in China. *Econ Entomol* **99**:383–392 (2006).

19 McKenzie N, Helson BV, Thompson DG, Otis G, McFarlane JW, Buscarini T, *et al*, Azadirachtin: an effective systemic insecticide for control of the emerald ash borer, *Agrilus planipennis*. *Econ Entomol* **103**:708–717 (2010).

20 Helson BV, Lyons B and Wanner KW, Control of conifer defoliators with neem-based systemic bioinsecticides using a novel injection device. *Can Entomol* **133**:729–744 (2001).

21 FAO specifications and evaluations for agricultural pesticides – azadirachtin. Food and Agricultural Organization of the United Nations, pp. 1–23 (2007).

22 Schmutterer H, Properties and potential of natural pesticides from the neem tree, *Azadirachta indica*. *Annu Rev Entomol* **35**:271–297 (1990).

23 Thompson DG and Kreutzweiser DP, A review of the environmental fate and effects of natural 'reduced risk' pesticides in Canada. *Am Chem Soc* **947**:245–274 (2007).

24 Operating instructions for tree injections using the EcoJect system and TreeAzin systemic insecticide. BioForest Technologies Inc., Sault Ste. Marie, ON, p. 23 (2009).

25 European Commission Document SANCO/2007/3121, 31 October (2007).