

Article

Concentration Levels of Imidacloprid and Dinotefuran in Five Tissue Types of Black Walnut, *Juglans nigra*

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Abstract: Black walnut, a valuable economic and environmentally important species, is threatened by thousand cankers disease. Systemic imidacloprid and dinotefuran applications were made to mature black walnut trees to evaluate their translocation and concentration levels in various tissue types including leaf, twig, trunk core, nutmeat, and walnut husk. The metabolism of imidacloprid in plants produces a metabolite, olefin-imidacloprid, which has been documented to have insecticidal properties in other systems. Trunk CoreTect (imidacloprid) soil pellets and a trunk spray of dinotefuran were applied to mature black walnuts in spring 2011. Imidacloprid concentrations were detected in both the lower and upper strata in all tissue types tested and progressively increased through month 12 post-treatment in twig and leaf tissue. Olefin-imidacloprid was detected in the nutmeat and walnut husk. Dinotefuran was only detected in the first sampling period and was found in low concentration levels in leaf and twig tissue types, and was not detected in the trunk, nutmeat or the walnut husk.

Keywords: black walnut; imidacloprid; dinotefuran; chemical concentration; thousand cankers disease

1. Introduction

Black walnut, *Juglans nigra* L., is native to eastern North America, where it grows in forest coves and well-drained river bottoms [1,2]. In the United States, its distribution extends from New England to Florida and from the east coast to the western delineation of the Great Plains [2]. These native trees are prized for their economic, ornamental, and ecological importance in the eastern United States [3].

The establishment of thousand cankers disease (TCD) in the eastern United States, once limited to tree hosts in the western states, threatens the economic industries and ecological roles associated with black walnut within the region. In Tennessee, an estimated 26 million black walnut trees exist on public and private timberland with a value estimated at around \$1.47 billion [4]. Due to black walnut's fine, straight-grained wood, it is considered premium lumber for timber, gunstock, furniture, cabinetry, and other finished wood products [1,2]. Homeowners and landowners harvest this valuable nut crop for personal use or income. Additionally, there is a high economic potential for this crop [5,6]. Industrial use of black walnuts include ground walnut shells used as jet engine cleaner, filler in dynamite, nonslip agent in automobile tires, and as a flour-like carrying agent in various insecticides [1].

Environmentally, it is difficult to assess the potential loss of these trees on wildlife within the various habitats, as well as the negative impact on the aesthetic quality of the areas they inhabit. More than 300 insect species have been documented on this valuable tree [7], but few are considered pests [1]. Despite the small number of major pests associated with black walnuts, these trees face some severe health risks. TCD is a serious insect/disease complex that poses a significant threat to black walnut within its native range. The discovery of this disease in July 2010 in eastern Tennessee represents the first documentation of this pest complex east of the Mississippi River [8]. Tree deaths from TCD are attributed to the fungal species, *Geosmithia morbida* Kolařík *et al.* [9–11]. The only known vector of *G. morbida* is the walnut twig beetle (WTB), *Pityophthorus juglandis* Blackman [5,11]. Like several other bark beetles, WTB bores beneath the bark into the phloem region of twigs, branches, or trunks of black walnuts. Initially, the primary means of managing TCD within the infested areas were rapid detection and removal of infected trees [12]. Various control methods, including biological controls and systemic chemicals, are currently under investigation to prevent further spread of TCD.

Two chemicals used to manage bark beetle populations are imidacloprid and dinotefuran. Imidacloprid is used against a variety of forest pests, including *Tomicus piniperda* L., a bark beetle that attacks the shoots of young scotch pine, *Pinus sylvestris* L. [13]. Dinotefuran and imidacloprid are successful tools in reducing numbers of the wood-boring larvae of the Asian longhorned beetle, *Anoplophora glabripennis* (Motschulsky) [14–16]. In addition, larvae of the emerald ash borer, *Agilus planipennis* Fairmaire, feed within the phloem and phloem-xylem regions of the ash tree and are treated with systemic insecticides including imidacloprid, emamectin benzoate, and dinotefuran [17,18]. Topical applications of imidacloprid are often used in nut-producing trees due to the low mammalian toxicity and reduced systemic movement within plants to suppress such pests [19]. However, systemic applications have not been approved for black walnuts since it is considered a group 14 nut tree by the Office of the Federal Register, which includes other crops such as pecans, almonds, and hickory [20,21].

Spatial and temporal distribution of imidacloprid [22,23] and dinotefuran [24] has been evaluated in ash sp. as a control for emerald ash borer. Additionally, it has been documented in several tissue types of ash [23]. Ash species provide a good homology for predicting systemic insecticide distribution due to similar grain structure and ring porosity. However, little is known regarding the movement of these two systemically applied pesticides, imidacloprid and dinotefuran, within the black walnut tissue. Additionally, little is known about the imidacloprid metabolite, olefin-imidacloprid, which is reported to be more toxic than imidacloprid [25], and it is unknown at what levels and for how long olefin-imidacloprid can remain within deciduous plant material, including nutmeat. Although research has been conducted on the spatial and temporal movement of imidacloprid in eastern hemlocks, *Tsuga canadensis* L., [26], it is important to understand the movement of imidacloprid and its derivative chemicals, *i.e.*, olefin-imidacloprid, through black walnuts if they are going to be considered for control of wood-boring insects associated with TCD. Therefore, the objective of this project is to document the translocation and within-tree concentrations of imidacloprid and dinotefuran within various types of black walnut tissue, including leaves, twigs, trunk core, nut husk, and nutmeat, as well as track the movement and concentration levels of olefin-imidacloprid within nut husk and nutmeat tissue.

2. Materials and Methods

2.1. Study Sites

Mature black walnuts ($n = 21$) were selected from Strong Stock Farm (36°3'7.1526" N; 83°47'23.3802" W), Knoxville, TN, USA, in April 2011. Trees selected were located on either the edge of the forest, individually in open fields or clumped in open fields. Tree heights averaged 17.8 m (± 3.49 m SE) with an average diameter at breast height (DBH) of 51.6 cm (± 4.29 cm SE). Trees were tagged with yellow flagging tape to mark their location. Selected trees were arranged in a complete randomized block design, consisting of seven blocks of three trees per block. Samples were taken from two locations (lower and upper strata) on each tree with the lower stratum delineated at regions of trunk below 5.18 m, while the upper stratum was delineated above 5.18 m. Samples represented composites taken from representative sides (north, south, east, and west) within each designated stratum. Treatments consisted of imidacloprid (CoreTect, Bayer Environmental Science, Research Triangle Park, NC, USA, 20% imidacloprid), a trunk spray of dinotefuran (Safari, Valent U.S.A. Corporation, Walnut Creek, CA, USA, 20% dinotefuran), and no treatment (control). Soil pellets were chosen due to ease of application since they do not require water as other systemic soil applications do of imidacloprid. Chemicals were applied in the spring on 20 April 2011. CoreTect, a systemic soil pellet, was applied to the soil at 1 pellet per 2.5 cm of tree DBH. Pellets were placed 30.5 cm away from the trunk and buried 5.1–12.7 cm deep in the soil encircling the tree. A trunk spray of dinotefuran was applied at 1.13 AI per 3.8 liters of water and sprayed on the tree trunk from base to a height of 3 m until saturation using an 18.9 liter hand held sprayer. Multi-stem tree rates were based on cumulative centimeters of DBH of all stems for both treatment types.

2.2. Sampling

Samples were collected one (18 May 2011), three (26 July 2011), five (27 September 2011), eight (10 December 2011), and 13 (9 May 2012) months post-treatment. Samples were taken from the lower and upper tree strata to monitor the translocation of imidacloprid and dinotefuran. Samples consisted of two leaf, two twig, two trunk core, and two nut (when present) samples from each tree stratum for a total of 16 samples per tree per collection period. Two 24-cm branch and two nut (when present) samples were collected from each tree stratum using a 3-m pole pruner. Two 5-cm trunk core samples were collected at breast height (1.5 m) from each tree using a 5-mm increment borer. Trunk core samples were offset horizontally among sampling dates. All samples were immediately sealed in plastic bags, packed in ice and taken to the laboratory to be stored in a freezer at -18°C until chemical extraction. To detect the concentration levels of dinotefuran, imidacloprid and its metabolite, olefin-imidacloprid, within the nutmeat, samples were separated into nutmeat and walnut husk. All tissue types were divided into 1-g units, cut, crushed using a scalpel, and added to 10.0 mL of histological grade acetone in 10-dr glass vials.

2.3. Extraction, Clean-Up and Quantification of Chemicals

Extraction and clean-up of imidacloprid and dinotefuran were conducted following the protocol established by Schöning and Schmuck [27] and Kamel [28], respectively. High pressure liquid chromatography coupled with tandem mass spectroscopy (HPLC/MS/MS) was used to quantify imidacloprid and dinotefuran concentrations in tree matrices (leaf, twig, trunk core, nutmeat, and walnut husk tissue from black walnut trees).

HPLC/MS/MS was conducted using a Hewlett Packard HP 1100 high pressure liquid chromatography coupled with a tandem triple quadrupole Applied Biosystems API 3000 mass spectrometer. The HPLC was fitted with a Phenomenex Luna C18 reversed phase column (15 cm length \times 4.6 mm i.d.). Parameters for the HPLC/MS/MS were as follows: injection volume: 50 μL , oven temperature: 40 $^{\circ}\text{C}$, mobile phase A: water + 0.1 mL acetic acid per liter, mobile phase B: acetonitrile + 0.1 mL acetic acid per liter, gradient: 0–10 min 20% mobile phase B, 11–15 min 90% mobile phase B, 16–19 min 20% mobile phase B, stop time: 19 min, flow (column/MS): 1.0/0.15 mL/min, retention time for imidacloprid: 9.1 min, olefin-imidacloprid: 4.4 min, dinotefuran: 3.3 min, interface: electrospray, turbo-ion spray potential: +5000 V, temperature: 300 $^{\circ}\text{C}$, scan type: multiple reaction monitoring (MRM), polarity: positive, and collision gas: nitrogen 5.0 (99.999% purity), 0.87 L/min. Solvents were obtained from Sigma Aldrich (St. Louis, MO, USA) and consisted of HPLC grade water (99.9%) and acetonitrile (99.9%). Standards of imidacloprid (96.9%) and dinotefuran (99.7%) were obtained from Sigma Aldrich (St. Louis, MO, USA), and standards of olefin-imidacloprid (97.5%) were obtained from Crescent Chemical Company (Islandia, NY, USA).

2.4. Data Analysis

Using the formula outlined by Schöning and Schmuck [27], imidacloprid, olefin-imidacloprid, and dinotefuran residuals in parts per billion (ppb) were determined using the average peak areas of imidacloprid and dinotefuran conversions for each analyte [25]. Imidacloprid, olefin-imidacloprid, and

dinotefuran concentration data were converted from ng g^{-1} of each analyte (dry weight) to ppb and placed into an Excel[®] file and analyzed using Proc Mixed ANOVA in SAS [29]. Imidacloprid concentrations represented data from multiple time points with the exception of nutmeat and walnut husk tissue, since these values are taken from only one time point. Imidacloprid data were analyzed as a repeated measures analysis of variance (ANOVA). Treatment is the fixed factor, block is the random factor, and time is the repeated measures factor. Mauchly's test of sphericity indicated that the assumption of sphericity was met. Where significance was determined from repeated measures ANOVA for differences in imidacloprid concentrations, Tukey's honestly significant differences (HSD) test was performed for mean separation. Olefin-imidacloprid and dinotefuran concentrations represent data taken from one time point and were analyzed using ANOVA. To verify that olefin-imidacloprid and dinotefuran concentration data conformed to the assumptions of ANOVA, Shapiro-Wilks *W* test for normality and Levene's test of homogeneity of variances were used and met. Means were separated using Tukey's honestly significant differences (HSD) procedures ($P < 0.05$).

3. Results

3.1. Concentration Levels of Imidacloprid in Walnut Tissue Types

Mean imidacloprid concentration levels differed significantly (Tukey's HSD test; $P < 0.05$) by stratum, tissue type, and time. Imidacloprid concentrations were highest in leaf tissue taken from the lower stratum for all sampling periods and lowest in trunk core tissue for all sampling periods, except in September samples when nuts were present. Imidacloprid concentrations were significantly higher (Tukey's HSD test; $P = 0.0001$) in samples from the lower stratum within each tissue type (leaf, twig, trunk core, husk, and nutmeat) (Table 1). Within each stratum, leaves had significantly higher (Tukey's HSD test; $P = 0.0021$) concentrations of imidacloprid than either twig or trunk core tissue. In September, when nuts were present, leaf and twig tissue had higher concentrations than the nuts. In all cases, the highest concentration of imidacloprid was present in the leaves, followed by twig, nutmeat, and either trunk core or husk tissue. In the lower stratum, husk tissue had a higher concentration than trunk core tissue, while in the upper stratum the opposite was true.

Concentration levels within nutmeat samples exceeded the established nut crop acceptable tolerance level (50 ppb) for imidacloprid [21] (Table 1). Therefore, a spring treatment of CoreTect on black walnut trees will likely cause imidacloprid concentrations to exceed the acceptable level in nut crops. It is possible that a fall treatment may result in concentrations below the acceptable level for imidacloprid. However, it is unknown how long imidacloprid would persist in the black walnut nut crops over time. Concentration levels of imidacloprid were highest in the last sampling period 13 months after treatment (May 2012). During this 13 month study, imidacloprid concentrations progressively increased (Table 1).

Imidacloprid concentrations in ash trees progressively increased through time and were highest in leaf tissue [23]. In a two-year study monitoring the translocation and concentrations of imidacloprid in eastern hemlock, significantly higher concentrations of imidacloprid were found in the lower stratum compared to the upper [26]. In addition, 293 insect species were documented to be associated with eastern hemlock and at least 33 species were impacted initially by systemic imidacloprid

treatments [30]. Furthermore, two non-target predaceous species that feed on hemlock woolly adelgids *Adelges tsugae* Annand that had consumed imidacloprid from treated trees exhibited both lethal and sublethal effects [31]. The distribution of various nutrients, including nitrogen and potassium, has been documented in both evergreen and deciduous trees. Deciduous trees had higher nutrient translocation rates compared to evergreen trees [32]. As such, the translocation of imidacloprid may be dependent on the type of tree or plant material to which they are applied. Additionally, translocation of systemic insecticides can be greatly affected by application timing, tree health, weather conditions, soil conditions, insecticide formulation, and application method.

Table 1. Imidacloprid concentrations (ppb)* (mean \pm SE), determined using HPLC/MS/MS from tree tissue of *Juglans nigra* L., Knoxville, TN, 2011–2012.

	Sampling Time				
	20 May 2011	20 July 2011	20 September 2011	20 December 2011	20 April 2011
Tissue Types					
Upper Twig	11.98 \pm 1.74 dE **	63.62 \pm 2.55 dD	87.30 \pm 1.34 dC	101.23 \pm 2.34 dB	123.11 \pm 2.10 dA
Upper Leaf	12.89 \pm 1.25 cE	68.85 \pm 3.25 cD	97.13 \pm 1.78 cC	123.09 \pm 2.14.0 cB	134.22 \pm 1.09 cA
Upper Nutmeat	–	–	72.19 \pm 1.99 e	–	–
Upper Husk	–	–	11.79 \pm 1.13 h	–	–
Lower Twig	24.79 \pm 1.66 bE	96.79 \pm 2.06 bD	112.97 \pm 1.24 bC	134.45 \pm 2.67 bB	157.43 \pm 3.42 bA
Lower Leaf	31.46 \pm 1.89 aE	99.45 \pm 2.69 aD	129.26 \pm 1.44 aC	157.32 \pm 2.34 aB	171.29 \pm 1.31 aA
Lower Nutmeat	–	–	84.02 \pm 2.56 e	–	–
Lower Husk	–	–	56.40 \pm 2.09 f	–	–
Trunk Core	1.25 \pm 0.54 eC	34.56 \pm 2.13 eB	45.05 \pm 6.45 gA	47.13 \pm 5.39 eA	49.56 \pm 4.97 eA

* ppb = parts per billion; ** Means ($n = 14$) within columns followed by the same lowercase letter(s) and means ($n = 14$) within the same row followed by the same uppercase letter(s) are not significantly different (Tukey's HSD test; $P > 0.05$).

3.2. Concentration Levels of Dinotefuran in Walnut Tissue Types

Concentrations of dinotefuran were only detected in the first sampling period one month post-treatment. No residues of dinotefuran were detected in walnut tissue post 18 May 2011. Although concentration levels were highest in the upper and lower leaf, no significant differences (Tukey's HSD; $P = 0.6775$) in dinotefuran concentrations were documented among tissue types as only trace amounts were detected. The only tissue type that was significantly different occurred during the first sampling period with no dinotefuran detected in the trunk core samples compared to the trace amounts found in the other tissue types. The upper twig and leaf tissue types had an average dinotefuran concentration of 1.13 ± 0.34 (SE) ppb and 1.56 ± 0.68 , respectively. Lower twig and leaf tissue had an average dinotefuran concentration of 1.26 ± 0.56 and 1.41 ± 0.73 ppb, respectively. Walnuts are only present in the fall; thus nutmeat and husk could only be analyzed from September 2011, from which dinotefuran was not detected.

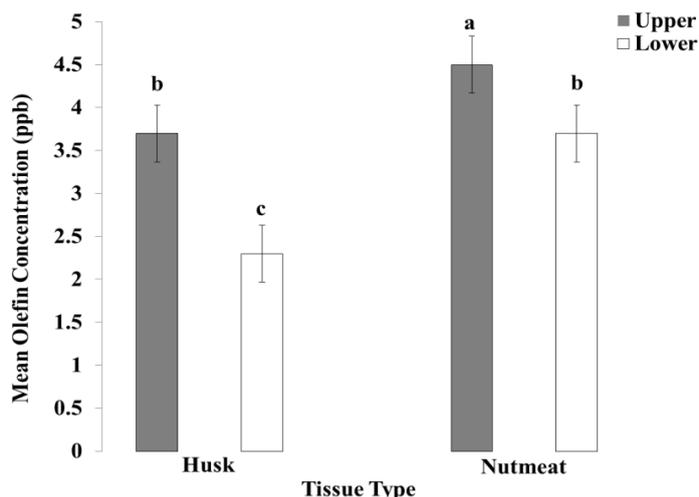
These data imply that with a spring treatment of dinotefuran, this chemical will have degraded before walnuts are ready to harvest in the fall. The absence of detectable dinotefuran concentrations beyond the first month of sampling may be due to dinotefuran moving quickly through the plant and its inability to bind well to organic material. Similarly, in ash trees dinotefuran rapidly translocated and then declined a few months later [24,33]. Since black walnut is labeled as a group 14 crop, chemical

concentration levels in the nutmeat are carefully controlled. Other members of this group include almonds, other *Juglans* species, pecans, *etc.* However, concentration levels of dinotefuran for group 14 have not yet been established by United States federal government [20].

3.3. Concentration Levels of Olefin-Imidacloprid in Walnut Husk and Walnut Nutmeat

Samples of nutmeat and walnut husks obtained during the fifth month post-treatment were tested for the presence of olefin-imidacloprid, a metabolite of imidacloprid. Olefin-imidacloprid concentrations differed significantly (Tukey's HSD; $P = 0.0023$) by stratum and tissue type. Olefin-imidacloprid concentrations were highest in nutmeat tissue from the upper stratum and lowest in walnut husk tissue taken from walnuts collected from the lower stratum (Figure 1). Olefin-imidacloprid concentrations were significantly greater (Tukey's HSD; $P = 0.0001$) in the upper stratum within each tissue type (husk and nutmeat). Within each stratum category, nutmeat had the highest (Tukey's HSD; $P = 0.00132$) concentrations of olefin-imidacloprid followed by walnut husk tissue. Olefin-imidacloprid concentrations were significantly lower (Tukey's HSD; $P = 0.0031$) than imidacloprid concentrations within each respective stratum and tissue type during the time period of this study (Table 1, Figure 1). For example, average imidacloprid concentrations ranged from 8.0 to 134.0 ppb and olefin-imidacloprid concentrations ranged from 2.0 to 4.5 ppb. However, olefin-imidacloprid concentrations have been documented to progressively increase as imidacloprid concentrations decreased over time [25]. The olefin-imidacloprid metabolite, one of 12 known metabolites of imidacloprid, has been reported to be at least ten times more active than its parent compound against the green peach aphid, *Aphis gossypii* Glover [34], and 13 times more active against hemlock woolly adelgid [25]. Another metabolite, *i.e.*, 5-hydroxy-imidacloprid, has been documented to be slightly less active than the parent imidacloprid; however, 5-hydroxy-imidacloprid still retains relatively high toxicological properties [25]. This paper is the first report of olefin-imidacloprid in tissue of any deciduous tree and infers longer-term residual effects that could result in longer control of pest insects due to the toxicity of imidacloprid's metabolites.

Figure 1. Mean \pm SE olefin-imidacloprid concentration (ppb) for each stratum and tissue type, collected on 27 September 2011, Knoxville, TN, USA. Means ($n = 84$) with different letters are significantly different (Tukey's HSD; $P < 0.05$).



4. Conclusions

This study represents the first documentation of the concentration levels of imidacloprid and its metabolite, olefin-imidacloprid, in tissue types of black walnut. The imidacloprid concentration levels were significantly greater in the lower stratum in all tissue types tested, with leaf in the lower stratum containing the highest concentrations of imidacloprid. When testing for olefin-imidacloprid, higher concentrations were found in the upper stratum with nutmeat having the highest concentrations in the tissue types tested (nutmeat and nut husks). In addition, this research implies that on black walnuts, which are treated in the spring with the imidacloprid soil pellet, nutmeat concentration levels (ppb) are above the current established acceptable concentration levels for imidacloprid in nutmeat established by the Office of the Federal Register [21]. Thus, this formulation of imidacloprid may not be an acceptable method to use for controlling pests on black walnut trees due to contamination of nut crops. However, dinotefuran moved quickly through the system and was not detectable in any of the nut tissue or any tissue after the first sampling period. Further research is needed to determine the efficacy of the concentration levels determined in this study on WTB mortality. By studying the movement of imidacloprid and its metabolite, olefin-imidacloprid, along with other chemicals, the effectiveness of these compounds on insect populations can be better understood and potential negative effects on non-target species can be recognized.

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Conflicts of Interest

The authors declare no conflict of interest.

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