Evaluation of systemic neonicotinoid insecticides for the management of the Asian citrus psyllid *Diaphorina citri* on containerized citrus

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Abstract

BACKGROUND: Studies were conducted to evaluate uptake and retention of 3 systemic neonicotinoid insecticides, dinotefuran, imidacloprid, and thiamethoxam, in potted citrus nursery plants treated at standard label rates. Infestation of these plants placed at a field site with moderate levels of Asian citrus psyllid (ACP) was monitored for 14 weeks following treatments and insecticide residues in leaf tissue were quantified using ELISA. Bioassays were conducted using leaves harvested on various dates post-treatment to compare the efficacies of residues against adult ACP.

RESULTS: Residues of the 3 neonicotinoids were detected in leaf tissues within 1 week after treatment. Peak concentrations established at 1 week for imidacloprid and dinotefuran and at 2 weeks for thiamethoxam. Imidacloprid and thiamethoxam outperformed the control and dinotefuran treatments at protecting trees from infestations by ACP eggs and nymphs. For a given insecticide concentration in leaf tissue, thiamethoxam induced the highest mortality of the 3 insecticides, and dinotefuran was the least toxic.

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CONCLUSION: If the time needed to achieve effective thresholds of a systemic neonicotinoid is known, treatments at production facilities could be scheduled that would minimize unnecessary post-treatment holding periods and ensure maximum retention of effective concentrations after the plants have shipped to retail outlets. The rapid uptake of the insecticides and retention at effective concentrations in containerized citrus suggest that the current 30-day post-treatment shipping restriction from production facilities to retail outlets outside of quarantine could be shortened to 14 days. Thiamethoxam should be added to the list of approved nursery treatments.

Keywords: *Diaphorina citri*, Asian citrus psyllid, neonicotinoid, imidacloprid, thiamethoxam, dinotefuran, containerized citrus, citrus production nursery

1 INTRODUCTION

The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is one of the most devastating pests of citrus worldwide. The insect was detected in southern California in 2008 on citrus at a residential property in San Diego County,[1] and is now widespread in southern California on both residential and commercial citrus. Damage from ACP occurs due to preferential feeding by the nymphal stages on developing citrus shoots,[2] which causes sooty mold production, shoot deformation, and plant stunting.[3] Of far greater significance, ACP is a serious vector of the bacterial pathogens (*Candidatus* Liberibacter spp.) responsible for citrus greening disease (Huanglongbing or HLB). HLB produces progressive mottling of leaves, deformed and off-flavor fruit,[4] foliar dieback, and eventual tree death.[5] Currently, there is no known cure for HLB, and infected trees typically die within 10 years. In 2012, the first HLB-positive tree in southern California was discovered in a residential neighborhood in Los Angeles County.[6] Since that initial discovery, there have been further detections in Los Angeles County, all of them in private residences to date.

The cumulative economic impact of HLB on the Florida citrus industry has been estimated to be $4.54 billion for the five seasons between 2006/07 and 2010/11 (Hodges AW and Spreen TH (http://ufdc.ufl.edu/IR00005615/00001)). The unregulated movement of psyllid-infested nursery stock is believed to have played a pivotal role in the spread of both ACP and HLB throughout the state,[7] and has also been implicated in the interstate spread of ACP. The establishment of the psyllid in the Rio Grand Valley of Texas in 2001 was traced back to infested nursery plants originating from Florida.[8] In an attempt to minimize the risk of this occurring in California, quarantine restrictions were implemented by the California Department of Food and Agriculture (CDFA) in regions where ACP was detected and, in addition, a chemical eradication program was implemented for residential citrus. As part of the quarantine, production nurseries are required to treat all citrus nursery stock prior to shipment to retail outlets (CDFA (http://phpps.cdfa.ca.gov/PE/InteriorExclusion/pdf/acptreatments.pdf)). In the case of within-quarantine shipments, plants must be treated under regulatory supervision with both an approved foliar insecticide and a systemic neonicotinoid insecticide in order to receive a 90-day certification. Plants must then ship within 90 days of treatment. Similarly, for shipments outside of the quarantine zone, including inter-state movement, nurseries must apply both foliar and systemic insecticides no more than 90 days, and no less than 30 days, prior to shipment (CDFA (http://phpps.cdfa.ca.gov/PE/InteriorExclusion/pdf/acptreatments.pdf)). The purpose of the foliar treatment is to disinfest trees of ACP through direct contact activity prior to shipment from the production nursery and to provide additional short-term protection to trees from re-infestation while the systemic insecticide is establishing within the tree. Once the residual effects of the foliar treatment have diminished, the protection of the trees from infestation by ACP is dependent entirely upon the activity of the systemic insecticide. Following treatments at the nurseries, trees are individually tagged with a unique identifier. In addition to showing that the trees have been treated according to quarantine requirements, the tagging system permits accurate tracking of all treated trees from the original production facility to the point of sale.

Currently, options for systemic insecticide treatments required under quarantine are limited to three active ingredients (CDFA (http://phpps.cdfa.ca.gov/PE/InteriorExclusion/pdf/acptreatments.pdf)) – dinotefuran, imidacloprid, and thiamethoxam (thiamethoxam is available for intra-quarantine treatments only) – all of which belong to the neonicotinoid Group 4A insecticide class (IRAC (http://www.irac-online.org/modes-of-action/)). The neonicotinoid insecticides act as nicotinic acetylcholine receptor agonists[9,10] and are used extensively in production nurseries throughout the United States to protect young container-grown plants from direct feeding damage and disease transmission by a broad range of sucking insect pests, including aphids, whiteflies, and psyllids.[11,12] Persistence within the plant is a key determinant in the decision to use neonicotinoids for the management of sucking insect pests,[11] because residues circulating within the vascular system of a plant directly exploit the feeding behavior of these insects and, unlike foliar treatments that rely exclusively on contact activity, can move into new tissues during normal plant growth and provide continued protection. The latter attribute is absolutely essential for effective psyllid control whose population dynamics is inextricably linked with the flushing cycles of citrus hosts. Lengthy persistence within plants has the added advantage of reducing the need for multiple applications. This is important because all existing ACP control requirements for containerized citrus apply only to production nurseries. There are currently no treatment requirements for retail nurseries and garden centers, a decision that was motivated by the expectation that plants would reside at these retail nurseries for a short time, and that the treatments applied at production nurseries would provide adequate protection until plants were purchased by homeowners or landscapers. Extensive surveys of nursery plants in retail outlets have subsequently shown that trees treated with imidacloprid according to quarantine regulations regularly reside at retail outlets for periods well beyond the 90-day certification period provided by the neonicotinoid treatments (MPD, unpublished data). In addition, imidacloprid concentrations in a large proportion of trees were often below the 220 ng g^{-1} threshold level required to prevent the establishment of ACP populations,[13] and consequently many of the trees were infested with live stages of the insect (MPD, unpublished data).

Imidacloprid is the most widely used neonicotinoid within California ornamental and nursery production systems, and has been the predominant choice of citrus producers for the protection of containerized citrus from ACP infestation (MPD, unpublished data). Researchers in Florida have shown imidacloprid has excellent efficacy against the ACP.[14,15] Relatively little is known about the efficacy of dinotefuran and thiamethoxam against ACP, and with the paucity of efficacy data for these chemicals on citrus, growers may be reluctant to choose these chemicals over imidacloprid given the success of imidacloprid in both nursery and field production. The inclusion of dinotefuran and thiamethoxam on the quarantine treatment roster likely stems from their use in the management of a vast array of sucking pests associated with ornamental production,[11,12] and the perception that they would also be effective against the ACP because of their systemic activity.

In formulating strategies for protecting containerized citrus, the main concern of regulators has been that the systemic insecticides have sufficient time to establish within the trees prior to shipment from the production nursery. There is concern within the nursery industry, however, that the 30-day post-treatment shipping restriction for shipments outside of quarantine zones is prohibitive, with the main argument being that if the systemic treatment has established well in advance of the 30-day limit, then trees would become vulnerable to infestation sooner at retail outlets once the original 90-day post-treatment certification period expired. In this study, we aimed to address certain knowledge gaps related to the efficacy of neonicotinoids against ACP on containerized citrus. Specifically, we compared the uptake and retention of dinotefuran, imidacloprid, and thiamethoxam in containerized citrus trees by quantifying residues in leaf tissue over time. Such work would provide regulators and nursery producers with important information on the relative efficacies of the three neonicotinoids, and the effective period of protection provided. In particular, regulators could optimize treatment protocols in relation to the timing of treatments before trees are shipped.

In addition to quantifying residues of insecticides within leaf tissue, we determined the toxicity of insecticide residues in bioassays by exposing adult ACP to leaf tissue sampled from the treated trees. And finally, treated and untreated trees were monitored weekly to determine the relative effectiveness of the three neonicotinoids at preventing the establishment of ACP populations in the field.

2 METHODS

2.1 Trees

Parent Washington navel oranges (*Citrus sinensis* L.) were budded on Carrizo citrange rootstock in June 2013 and grown in 12.7 cm diameter pots at the Lindcove Research and Extension Center, near Exeter, California (LREC). Trees were transferred from LREC on June 14, 2014 and maintained in a shade house at Agricultural Operations (Ag-Ops), on the campus of University of California Riverside, until they were repotted for the main study. At the start of the study, the trees ranged in height between 75 and 90 cms, with rootstock diameters of *ca.* 2.5 cms.

Trees were repotted in 18.9-liter containers on June 24, 2014 in a potting mix consisting of 10% sand, 15% peat moss, 15% coconut fiber, and 60% redwood. The potting mix was prepared by staff at the Ag-Ops farm and steam sterilized at 100°C. Once the trees were repotted, they were transferred to a field location at Ag-Ops where the irrigation was set up.

Trees were watered daily through a newly established drip irrigation system with emitters placed on spaghetti tubing feeding from the main water line into each pot. Prior to the experiment the evapotranspiration (ET) rate was determined for the trees in this potting mix over the course of three days for which daily high temperatures were above 35°C. These evapotranspiration values were used to define the volume of water needed for "replacement watering" (approximately 1.2 L per day; 120% ET) – sufficient water to support plant growth during peak summer temperatures while minimizing the potential for leaching of insecticide from the pot. During the course of the study, the trees received monthly 150 g applications of Nurserymans Citrus and Fruit Tree Food (Red Star Fertilizer Co., Corona, CA; 10/10/4 N/P/K).

2.2 Neonicotinoid treatments

Admire Pro (imidacloprid; 0.55 kg $AI.liter^{-1}$ suspension concentrate), Flagship 25 WG (thiamethoxam; 25% AI water dispersible granule) and Safari 20 SG (dinotefuran; 20% AI soluble granule) were applied to trees at recommended label rates. The final concentrations of active ingredients per tree were 182 mg imidacloprid, 118 mg thiamethoxam, and 170 mg dinotefuran.

The three neonicotinoid insecticides were applied on July 10, 2014. Treatments were randomly assigned to trees after they were arranged in a 25 x 4 grid pattern. There were 25 trees for each insecticide treatment and 25 untreated controls. Pots were pre-irrigated to ensure adequate wetting of the potting media prior to insecticide application. The insecticide treatments were administered to each pot using a measuring cylinder, followed by further watering from a watering can to ensure the insecticide had permeated below the potting media surface into the root zone. The replacement watering regimen was implemented at 24-h after the insecticides had been applied.

2.3 Chemical quantification of neonicotinoid residues

Residues of imidacloprid (QuantiPlate Kit for Imidacloprid; Envirologix, Portland, ME, USA; cat # EP 006), dinotefuran and clothianidin (SmartAssay Series Dinotefuran Test Kit; cat # 3100176146; Horiba Scientific, Irvine, CA, USA), and thiamethoxam (SmartAssay Series Thiamethoxam Test Kit; Horiba Scientific; cat # 3100184669) were quantified using commercially available ELISA (Enzyme-Linked ImmunoSorbent Assay) kits. The lower limits of quantitation (LOQ) of residues in citrus leaves for the three ELISAs were 200 ng dinotefuran, 75 ng imidacloprid, and 90 ng thiamethoxam per gram of leaf tissue. We used the dinotefuran ELISA to quantify clothianidin residues in trees that were treated with thiamethoxam (clothianidin is a metabolite of thiamethoxam). Due to structural similarities between dinotefuran and clothianidin, the antibody used in the dinotefuran ELISA cross-reacts with both chemicals (FJB, unpublished data), although the lower limit of quantitation for clothianidin was only 300 ng per gram of leaf tissue. Clothianidin standards were prepared from technical material that was purchased from a commercial supplier (https://www.chemservice.com; product #: N-11493-100MG). LOQs for each ELISA system were determined empirically by spiking citrus leaf extracts with known concentrations of insecticide and then determining the required dilution to eliminate matrix effects and optimize recovery.[16,17] The ELISAs are colorimetric assays, and absorbance (450 nm) readings were determined using a SpectraMax250 microplate reader (Molecular Devices, 1311 Orleans Dr, Sunnyvale, CA).

Young leaf flush tissue was collected from each tree on multiple sampling dates after the trees were treated. The samples (n=4 for each tree) were taken from each cardinal direction. The height of the samples was not considered an important parameter, given that the imidacloprid distribution in 25-30 feet tall citrus trees was shown not to be significantly affected by height or cardinal direction.[18] The tissue (0.5 g) was placed in a vial, chopped into small pieces using scissors, and then extracted by the addition of 5 ml of 100% methanol. Extracts were shaken for 12 h at 25°C. An aliquot (10 µl) of each extract was dried completely in a TurboVap LV evaporator (Caliper Life Sciences, Hopkinton, MA, USA) and then dissolved in a 0.05% aqueous solution of Triton X-100 prior to analysis by ELISA.

A purification step for imidacloprid was required to determine whether there was any contamination of extracts with imidacloprid metabolites[19,20] that could potentially cross-react with the ELISA kit antibody.[16,17] An aliquot (100 µl) of each imidacloprid extract was spotted directly on the concentrating zone of LK6DF silica gel 60 TLC plates (Whatman, Inc., Florham

Park, NJ, USA) and then chromatographed in a mobile phase of methylene chloride+methanol+ammonium hydroxide (45+5+1 by volume). The position of imidacloprid was determined by co-chromatographing an imidacloprid standard (ChemServices, West Chester, PA, USA; cat # N-12206-500MG) with the citrus extracts. The imidacloprid bands were cut from the plate, washed from the silica with 100% methanol (1 ml), and then quantified directly by ELISA.

2.4 Insect monitoring in the field

All trees used in the experiment were examined for the presence of ACP life stages up to 14 weeks after the trees had been treated. On each census date, signs of ACP were recorded based on visual inspection of the entire tree, using DA10 OptiVISOR Optical Glass Binocular Magnifiers (Donegan Optical Company, Inc., Lenexa, KS, USA) to facilitate detection of the smaller life stages. For each census, plants were recorded as having, categorically, any live ACP eggs or nymphs, or ACP adults. The densities of ACP adults or immature stages (including eggs) were not recorded because, from a production nursery standpoint, the occurrence of a single live individual of any life stage (egg, nymph or adult) can trigger a regulatory action that prohibits the shipment of the infested tree from the facility.

2.5 Insect bioassays

A series of bioassays was conducted in which adult ACP from the "Azusa" UCR colony were exposed to young leaf flush that was collected periodically from a subset of the control (n=12) and insecticide-treated trees (n=12 for each treatment) beginning at 1 week after the trees were treated. The ACP "Azusa" colony was established in the Insectary and Quarantine facility at UC Riverside in 2012 from insects collected on multiple occasions between Feb 24 and April 27 from untreated curry plants at a private residence in Azusa, CA.[21] The colony is maintained on curry plants (*Murraya koenigii*) and young Mexican lime (*Citrus aurantifolia*) seedlings in an insecticide-free environment, and is used as our reference susceptible strain in insecticide efficacy studies.

In bioassays, the young leaf tissue was placed adaxial side down on a bed of agar (1.5%) in Falcon® 60 x 15 mm Petri Dishes (Corning, Inc., Corning, NY, USA; cat # 351007). Adult ACP (n=5 for each replicate) were aspirated directly from the colony and transferred to the leaflets (two leaflets in each of the 12 replicates per treatment). The bioassay was maintained in a controlled environment room (27°C and 16:8 L:D photoperiod) within the insectary, where mortality was assessed at 24, 48, 72, and 96 h. At 96 h, only psyllids capable of normal motor activity (flight and jumping) upon probing were considered survivors in the bioassay, whereas insects incapable of movement or unable to remain in an upright position, were recorded as dead. Upon termination of the bioassay, the concentrations of insecticides were determined within the bioassay leaflets using the ELISA methods described earlier.

2.6 Statistical analyses

Imidacloprid, dinotefuran, and thiamethoxam residues were compared to each other using a linear mixed-effects analysis of variance model with fixed effects of insecticide type and time since application, and a random effect of plant identity to account for repeated measures made on individual replicate trees (lmer() in the R programming language).[22] Concentrations (ng/g of plant) were $\log_{10}$ x+1 transformed prior to analysis but are back-transformed to original units on all graphical representations of the data. Clothianidin is a metabolite of thiamethoxam,[23] and is insecticidal to a broad range of arthropod pests, including ACP.[24] Clothianidin residues in trees treated with thiamethoxam were analyzed over time using a separate linear mixed-effects model that included a fixed effect of days since application.

We compared ACP mortality in bioassays among imidacloprid, dinotefuran, thiamethoxam, and control treatments using a generalized linear mixed-effects model with binomial error, fixed effects of insecticide type and time since application, and a random effect of plant identity to account for repeated measurements on replicate trees.[25] We also compared the effects of insecticide concentration on ACP mortality rate over 48 h using separate generalized linear mixed-effects models for each of the three insecticides with binomial error, with fixed effects of concentration, and a random effect of plant identity. For both analyses we initially explored both logit and probit link functions in the model, whose results were congruent with each other. Based on information theory criteria (i.e. AIC values), the logistic versions slightly outperformed the probit models. Therefore, we report the results from the logistic models, only, including their use in estimating LC50 values for each of the insecticides.

A Cox proportional hazards model was used to compare the effect of treatment type on the first occurrence of ACP, with tests for significance between insecticide treatments conducted using the Wald test.[22] Eggs or nymph establishment, and adult establishment were analyzed separately.

3 RESULTS

3.1 Neonicotinoid residues over time

Residues of the three neonicotinoids were detected in leaf tissues within 1 week of treatments, with peak residues of imidacloprid and dinotefuran occurring at that time. Despite very similar application rates (182 mg imidacloprid per pot, 170 mg dinotefuran per pot), peak dinotefuran concentrations were 4.3-fold higher than peak levels of imidacloprid. Thiamethoxam residues peaked at two weeks after treatment, and concentrations were 1.7-fold higher than those of imidacloprid at peak uptake, despite a 1.5-fold lower application rate (Figure 1).

There were highly significant effects of insecticide type ($\chi^2 = 72.64$, df = 2, $p < 0.0001$) and time since application ($\chi^2 = 1840.72$, df = 7, $p < 0.0001$), with all three insecticides showing steady declines over time. There was also a significant interaction between insecticide type and time since application ($\chi^2 = 167.74$, df = 14, $p < 0.0001$), which may be attributible to the extremely high peak levels of dinotefuran relative to imidacloprid and thiamethoxam at week 1, followed by a rapid decline in dinotefuran concentration to levels that were similar for all three compounds by week 4.

Clothianidin is a known metabolite of thiamethoxam,[23] and was detected in leaf tissue samples from trees treated with thiamethoxam. Clothianidin concentrations were strongly correlated with thiamethoxam concentrations among samples (r = 0.886), and were also significantly affected by time since application ($\chi^2 = 969.91$, df = 5, $p < 0.0001$). Over the first 90 days post application, as thiamethoxam residues declined substantially, clothianidin concentrations remained consistently higher, only declining at the final assay conducted at 16 weeks post application (Figure 1).

3.2 Time to first establishment of ACP life stages in the field

Census data for the presence of all ACP life stages, including eggs and nymphs versus adults, are summarized in Figure 2. Despite being situated at the field site for 3 weeks before treatments were applied, no ACP life stages were detected on the trees during this interval. In fact, the first psyllid life stage was not detected until the Sept 16 census, which coincided with the main Fall flush of tender new foliage that is highly attractive to adult psyllids for feeding and oviposition, and is required for nymph development. Over the 7 census dates conducted after the trees were treated, 35% of trees were found at least once to have ACP nymphs or eggs present, and more than half (56%) had adults present on at least one census. There was a significant effect of insecticide treatment on the time to the first appearance of nymphs or eggs (Wald test = 12.81, df = 3, p = 0.0051) and adults (Wald test = 11.06, df = 3, p = 0.0114). For nymphs and eggs, infestations on control and dinotefuran treatments occurred earliest, at 82 and 68 days post treatment, respectively (Figure 2A). Imidacloprid and thiamethoxam treatments outperformed the control and dinotefuran treatments, with infestations occurring later and at approximately half the rate by the end of the study (Figure 2A). For adults (Figure 2B), control trees were infested earlier (starting at 68 days post treatment) and were at the highest rate by the end of the study (76% at 4 months post treatment). Thiamethoxam-treated trees were infested with adults later (starting at 96 days post treatment) and at the lowest rate overall (32% at 4 months post treatment), while infestations on dinotefuran- and imidacloprid-treated trees were intermediate between levels on control and thiamethoxam-treated trees (Figure 2B).

A comparison of insecticide residues in trees at the time of first presence of any life stage showed that ACP establishment only occurred on imidacloprid-treated trees after residues had dropped below detection levels of the ELISA (75 ng g^{-1} of plant tissue; Figure 3). In contrast, ACP established on thiamethoxam-treated trees at concentrations of 163 ng g^{-1} of plant tissue for adults (Figure 3A) and 121 ng g^{-1} for nymphs or eggs (Figure 3B). On dinotefuran-treated trees, ACP (all life stages) established at concentrations as high as 900 ng g^{-1} of plant tissue (Figure 3).

3.3 Laboratory bioassays

3.3.1 Efficacy of neonicotinoid treatments over time

Laboratory bioassays were conducted to compare the efficacies of the three neonicotinoids against adult ACP (Figure 4). In the control group, mortality was consistently low, with a mean of 0.6% over the 16-week duration of the study. ACP mortality in the dinotefuran, imidacloprid, and thiamethoxam treatments were simlarly high at 1 week after treatment, averaging 60-80%. Mortality in dinotefuran and imidacloprid treatments declined relatively quickly, and averaged levels similar to those in the control bioassays before 90 days had elapsed following treatments. Thiamethoxam induced significant mortality levels for slightly longer than imidacloprid and dinotefuran (Figure 4).

There were significant effects of treatment type ($\chi^2 = 103.75$, df = 3, $p < 0.0001$) and time since application ($\chi^2 = 255.13$, df = 1, $p < 0.0001$) on mortality, and there was a significant interaction between treatment type and time since application ($\chi^2 = 20.92$, df = 3, $p < 0.0001$). Analyses on individual treatments showed that there was a significant decline in mortality rate over time for imidacloprid ($\chi^2 = 80.943$, df = 1, $p < 0.0001$), dinotefuran ($\chi^2 = 90.365$, df = 1, p <

0.0001), and thiamethoxam ($\chi^2 = 104.68$, df = 1, $p < 0.0001$), but not the control group ($\chi^2 = 0.222$, df = 1, $p = 0.6372$).

3.3.2 *Effects of insecticide concentration on mortality*

Separate analyses were conducted to evaluate the effects of insecticide concentration on ACP mortality in the bioassays (Figure 5). The concentrations of insecticide residues in samples significantly affected adult ACP mortality in bioassays for imidacloprid ($\chi^2 = 19.985$, df = 1, $p < 0.0001$), dinotefuran ($\chi^2 = 70.291$, df = 1, $p < 0.0001$), and thiamethoxam ($\chi^2 = 56.065$, df = 1, $p < 0.0001$). In all three cases, higher concentrations were associated with greater mortality. For any given concentration, however, thiamethoxam induced the highest mortality rate of the three insecticides, whereas dinotefuran was the least toxic (Figure 5A). Based on the model fit, values for 50% mortality (LC_{50}) of ACP adults would require concentrations of 1100, 2500, and 5500 ng g^{-1} of plant tissue for thiamethoxam, imidacloprid, and dinotefuran, respectively. These concentrations were only briefly achieved in the field data (Figure 1).

In plants with the thiamethoxam treatment, the relationship between clothianidin residues and ACP mortality was evaluated from week 4 onwards using a binomial general linear mixed model. Clothianidin residues were significantly associated with adult ACP mortality ($\chi^2 = 39.76$, df = 1, $p < 0.0001$), with higher mortality rates at higher concentrations (Figure 5B). However, due to a strong correlation between thiamethoxam and clothianidin concentrations among the samples ($r = 0.87$), it is difficult to determine whether the effects on adult ACP mortality in the thiamethoxam treatment are attributable primarily to thiamethoxam residues, clothianidin residues, or both.

4 DISCUSSION

The requirement for both foliar and systemic insecticide treatments prior to shipment of containerized citrus trees from production nurseries located within California's established quarantine areas (to destinations within or outside quarantine areas) is to ensure the trees are free of ACP (which might harbor the huanglongbing bacterium) upon leaving the production facility and to protect the trees while awaiting sale at a retail outlet. Although the foliar treatment can provide protection while the systemic treatment is establishing within the tree, and could conceivably do so after the trees have shipped if treatments are timed close to the shipment date, quarantine regulations mandate a 30-day pre-shipment holding period at the nursery to ensure that the systemic treatments are actively protecting the trees at the time of shipment to areas not within quarantine. However, if the systemic treatments have established well in advance of the 30-day post-treatment shipping restriction, and data in our study indicate they do, trees will become vulnerable to infestation sooner at retail outlets once the protective effect of the treatments diminishes. By definition, shipments outside quarantine (including inter-state) are to areas where the psyllid has not been detected, and therefore shipments of clean plants should be a priority. However, ACP continues to expand its range in California, and together with the long residency times of trees at retail outlets, it is important to maximize the post-shipment protective period by eliminating unnecessary delays in shipping. In addition, the 30-day restriction presents a complicated logistical problem for production facilities because new orders cannot be supplied to buyers until the trees have been treated for the required period.

Two key elements to consider when utilizing systemic insecticide treatments to protect shipments of citrus are the rate of uptake and the retention time of effective residues within trees. The initial rate of uptake of insecticide will determine how quickly the insecticides are established within trees, while the period of retention of effective concentrations will determine the time frame over which trees are adequately protected. A major complicating factor is that the thresholds for activity against ACP are not known for all the chemicals, although our bioassay and census data now provide some insight into levels that are required. For a population to establish on a tree, adult ACP must oviposit on newly flushing leaf tissue to ensure the survival and development of their offspring. Therefore, the detection of viable nymphal stages on a treated tree should indicate that the threshold concentrations of insecticide are no longer present. A concentration of 220 ng imidacloprid g^{-1} leaf tissue was required to prevent the establishment of ACP nymphs on young grapefruit trees.[13] In our study, ACP life stages were not detected on imidacloprid-treated containerized trees until residues of imidacloprid had fallen below the detection limit of the ELISA (75 ng imidacloprid g^{-1} leaf tissue). The concentration of thiamethoxam at the time of first detection of nymphal stages was 121 ng g^{-1} leaf tissue, while that of dinotefuran was 900 ng g^{-1} leaf tissue. These threshold levels were readily observed in the containerized citrus. In Southern California, ACP population densities are driven by seasonal flushing patterns of the citrus trees. Therefore, the low pest densities during the first 4-6 weeks of the trial can be attributed to the lack of flush on the trees (both our study trees and the trees in the surrounding area). A Fall growth flush is typical of citrus trees in Southern California (a second main flush occurs during the Spring), and the first detections of ACP coincided with the appearance of the young leaf tissue which is required to sustain the development of ACP nymphs. While the pattern of colonization of trees might have been different under higher pest pressure, the concentrations of insecticides measured in the trees at the time of first infestation were significant, and provide a clear indication of the relative efficacies of the three compounds. The goal of this study was to establish a safe holding period for trees at production facilities while the systemic treatments established within the trees. Once that period has been determined, uninfested trees could then be safely shipped to areas outside quarantine where ACP has not been detected. Therefore, the establishment of effective concentrations should be a key determinant in how soon after treatment, treated trees could leave a production facility. In this study, peak concentrations of the three neonicotinoids occurred within 2 weeks of application, with imidacloprid and dinotefuran peaking at week 1 when the first samples were taken from the treated trees. Once the peak concentrations were attained, the measureable levels of all insecticides began a steady decline in subsequent weeks, but remained above the thresholds for up to 8 weeks. Based on these data, the 30-day post-treatment shipping restriction imposed by the quarantine regulations could be shortened significantly, a measure that would extend the period of protection for trees at retail locations.

The LC_{50} data generated from bioassays with adult ACP were 1100, 2500, and 5500 ng g^{-1} of plant tissue for thiamethoxam, imidacloprid, and dinotefuran, respectively, and clearly indicate major differences in the inherent toxicity of the 3 neonicotinoid insecticides recommended for use in quarantine treatments. At the time the first life stages of ACP were detected on treated trees, residues had declined to levels substantially lower than these LC_{50}s. In fact, the LC_{50} concentrations were only briefly achieved during the field study (Figure 1). It is important to understand the apparent disconnect between the acute mortality levels measured under the artificial conditions of a laboratory bioassay, in which the insects are required to feed over a (relatively) short period of time on a treated substrate, and the time to first detection of ACP on treated trees when the residues were much lower. ACP adults survived extremely high concentrations in bioassays, yet natural infestations of trees occurred when concentrations in leaf tissue were at least an order of magnitude lower. Two key contributing factors to this discrepancy are likely first, the low natural populations that existed in the mature citrus trees surrounding the study site, which would decrease the likelihood of psyllids encountering the treated trees, and second, the greater toxicity of insecticides to immature ACP stages (FJB, unpublished data). The former would be compounded by the lack of flush on the trees which is necessary for nymph survival. Additional contributing factors may include sublethal and antifeedant effects of the treatments, and the effects of toxic metabolites formed within the trees after the uptake of the parent material. Sublethal and anti-feedant effects of imidacloprid are well documented for many different species of insects,[26,27] including ACP.[28] At the time ACP were detected on our study trees, the imidacloprid residues were below the detectable levels of the ELISA, suggesting that antifeedant effects of the insecticide could have been a contributing factor in preventing colonization. In bioassays, antifeedant effects artificially inflate the acute toxicity of the insecticides, and are thus highly problematic for deriving accurate threshold concentrations of efficacy. It appears from our data that a more reliable method of determining imidacloprid efficacy against ACP would be to monitor tree colonization in conjunction with residue measurements because such a measure would represent the combined contributions of toxicity and antifeedant effects. In addition to antifeedant effects, the contribution of toxic imidacloprid metabolites would also be accounted for by gathering field data. Although we did not quantify metabolites in our study trees, imidacloprid-olefin and 5-hydroxy imidacloprid have been detected in mature citrus trees two months after soil application at combined concentrations ranging between 18 and 25% of the total residue complement (imidacloprid, imidacloprid olefin and 5-hydroxy imidacloprid).[29] If this level of metabolism holds true for smaller trees, then failure to establish a colony after the parent imidacloprid concentrations have declined below our detection limit, may reflect an increase in the concentrations of metabolites. Such an effect is not unprecedented, as sustained (up to 7 years) control of hemlock woolly adelgid, *Adelges tsugae* [Annand] on eastern hemlock trees by imidacloprid treatments has recently[30] been shown to rely heavily on the presence of both imidacloprid and the imidacloprid-olefin metabolite which is 14-fold more toxic than imidacloprid.[31] In the same way, thiamethoxam efficacy may be enhanced by the presence of clothianidin residues, providing a combined toxic effect after the individual chemicals are no longer within the detection range of the ELISA. Finally, trees treated with dinotefuran became infested with nymphs before the control trees, suggesting that sublethal and antifeedant effects of dinotefuran had little impact on the colonization rate, and that dinotefuran is not a suitable systemic treatment for use in quarantine control of ACP.

Surveys have shown that 35% of containerized trees can remain at retail nurseries for more than a year following arrival from a production facility (MPD, unpublished data). Of greater concern, in *ca.* 90% of trees tested that were present beyond the 90-day certification period, imidacloprid titers measured below the 220 ng g^{-1} threshold (MPD, unpublished data). For a variety of reasons related to economics, licensing requirements, and most importantly, public safety perceptions, pesticide applications at retail nurseries are unlikely to occur. With the effects of the systemic treatments declining within about 8 weeks, the long residency times at the retail outlets increase the risk that trees could become infested with ACP if the current range of the insect was to expand to areas outside quarantine. Furthermore, the recent detection of HLB at several sites within southern California has raised concerns that trees within a retail outlet could become infested with infected psyllids and serve as a source of inoculum for the disease if moved into an area where ACP has become established, a scenario not unlike that which occurred in Florida. While integrating different measures will be needed to mitigate the problem of extended residency times at retail outlets, shortening the timeframe between treatment and shipping dates should be a priority in order to ensure trees are protected for the maximum period while in retail, even if the insecticide concentrations ultimately decline at the retail facility. Furthermore, while our treated trees remained psyllid-free for extended periods under the low pest pressure conditions in the vicinity of the site at the beginning of the study, this may not be the case under higher pest pressures, which are likely to occur during the main Spring and Fall flushing periods.

The combination of leaf residue concentrations, mortality levels in bioassays, and ACP detection levels on treated trees demonstrates the extent to which systemic neonicotinoids can protect containerized citrus from ACP infestations. Currently, imidacloprid is used almost exclusively by the nursery industry, but our data show that thiamethoxam could be used as an effective alternative. Thiamethoxam was more potent than imidacloprid against adult ACP in bioassays conducted at 4 and 6 weeks after treatment, even though detectable residues were in decline. The continued efficacy of thiamethoxam in bioassays may be partly due to the presence of clothianidin, which was present at high concentrations at weeks 4 and 6 (Figure 1). In bioassays, leaves from trees treated with thiamethoxam were more potent than those from imidacloprid-treated trees against adult ACP, but because of the strong correlation between thiamethoxam and clothianidin concentrations, it was not possible to distinguish between the contributions of the two insecticides. Thus, the 2-fold lower LC_{50} for thiamethoxam compared with imidacloprid may have been due to a combination effect of both thiamethoxam and clothianidin. In contrast to imidacloprid and thiamethoxam, the efficacy of dinotefuran was relatively short-lived, and the data would support removing this insecticide from the list of approved treatments for citrus nursery stock. The high solubility of dinotefuran contributes to the rapid uptake of extremely high titers into trees, so it could prove useful as a rapid clean-up insecticide for infested trees. However, it would have limited benefit as a long-term protectant because of the ability of ACP to colonize trees with titers as high as 900 ng g^{-1} leaf tissue.

5 CONCLUSIONS

The uptake of soil-applied neonicotinoid insecticides in containerized citrus trees occurred rapidly, with peak concentrations establishing within 1 week for imidacloprid and dinotefuran, and 2 weeks for thiamethoxam. Although there was a steady decline in measureable residues during the weeks following peak uptake, no ACP life stages (eggs, nymphs, or adults) were detected until 10 weeks post-treatment. The rapid uptake of the insecticides, together with the retention of residues at effective concentrations, provide strong evidence that the current 30-day post-treatment shipping restriction should be shortened in order to extend the relative protective period of trees once they leave a production facility. Thiamethoxam would seem to be a strong candidate for inclusion in the treatment protocol for shipments outside quarantine areas, with superior toxicity and retention at effective concentrations compared to dinotefuran. Dinotefuran was the least suitable neonicotinoid for protecting trees. Despite its rapid uptake, trees treated with this compound were the first to become infested by ACP.

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Figure Legends

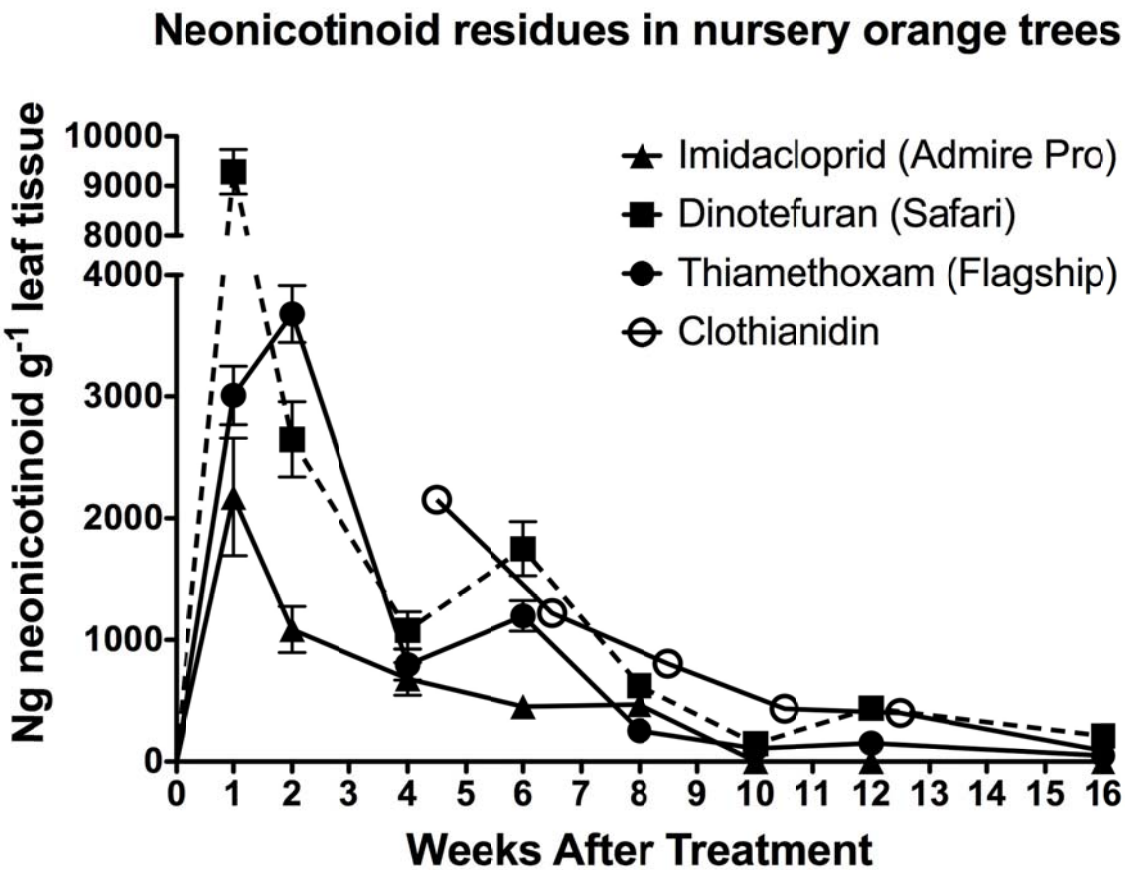
Figure 1. Mean (± SEM) insecticide concentrations in leaf tissue sampled from navel orange citrus trees over the course of the field trial for dinotefuran (solid square), imidacloprid (solid triangle), and thiamethoxam (solid circle). Clothianidin (open circle) is an insecticidal neonicotinoid metabolite of thiamethoxam that was detected in thiamethoxam-treated trees.

Figure 2. Survival curves showing the proportion of trees within each treatment that were infested over time with A) ACP nymphs or eggs, and B) ACP adults.

Figure 3. Frequency histograms of insecticide residues in trees at the time they were first found to harbor live A) ACP nymphs or eggs, and B) ACP adults.

Figure 4. Survivorship of adult ACP in bioassays. On each bioassay date, mortality was assessed after 96 h exposure of insects to leaves sampled from treated and control trees.

Figure 5. Adult ACP mortality as a function of the concentrations of A) the three neonicotinoid insecticides, and B) clothianidin, a metabolite of thiamethoxam detected in thiamethoxam-treated trees. For clarity, mean proportions are shown that are based on binned values associated with natural breaks in ranked insecticide concentration.



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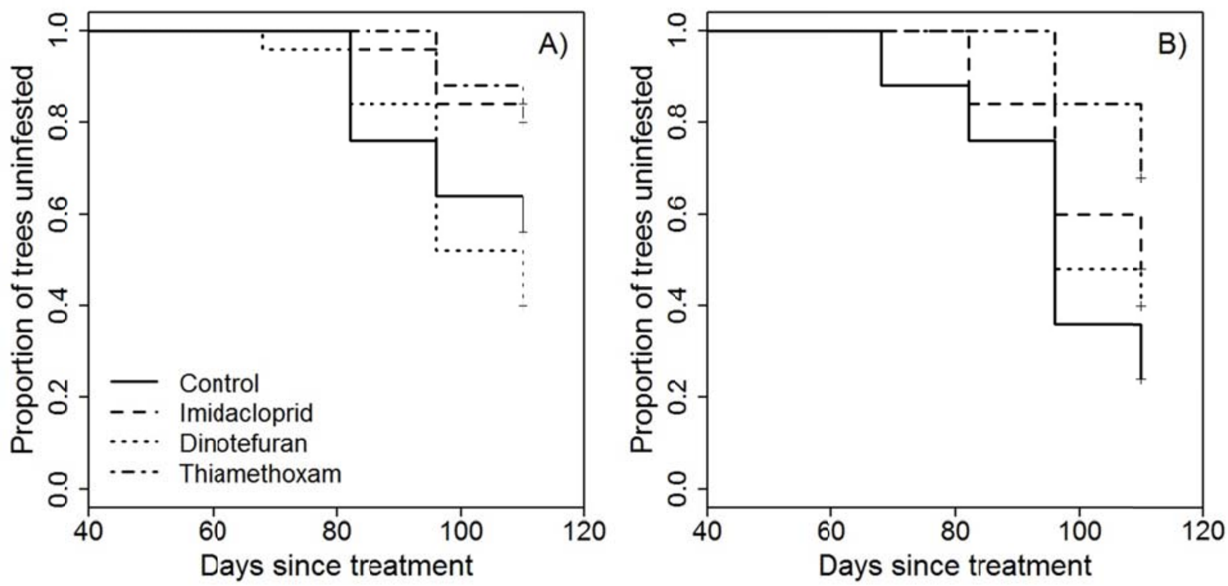

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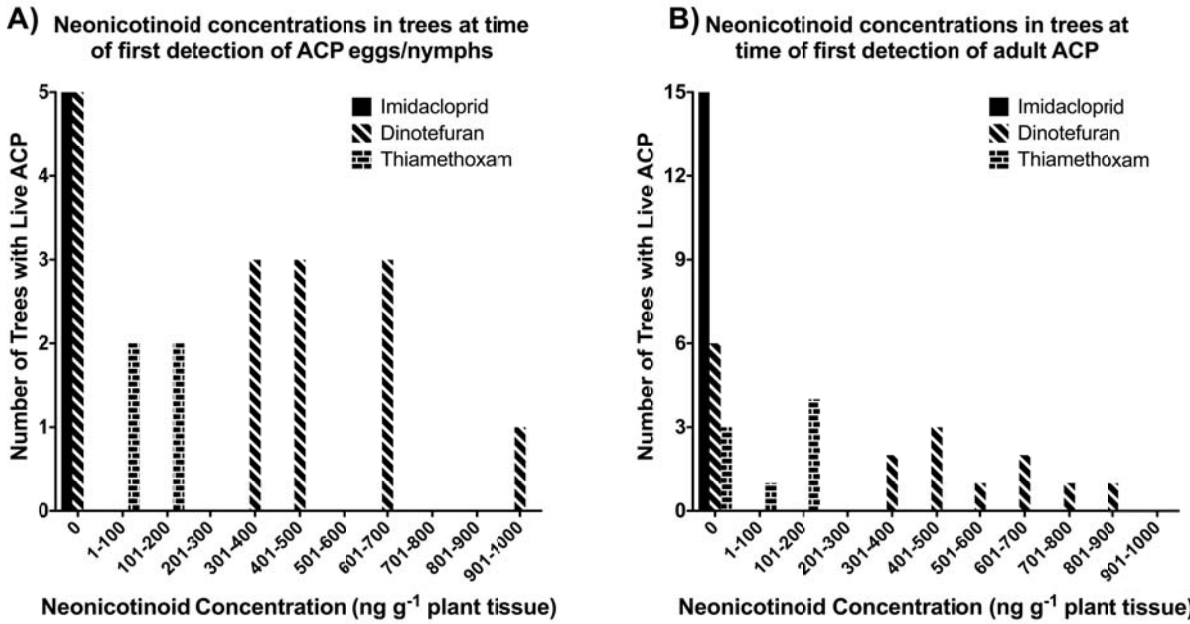

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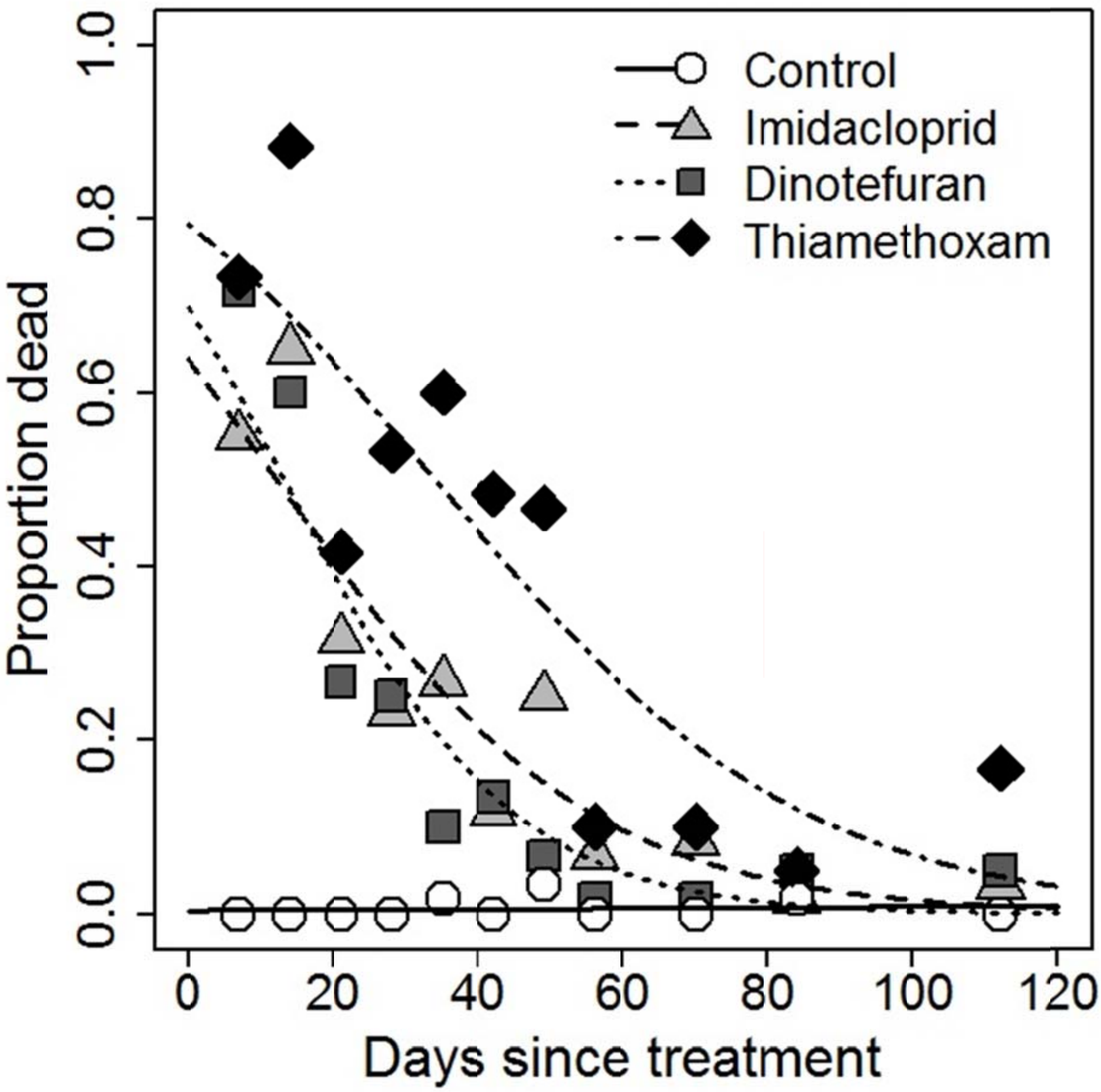

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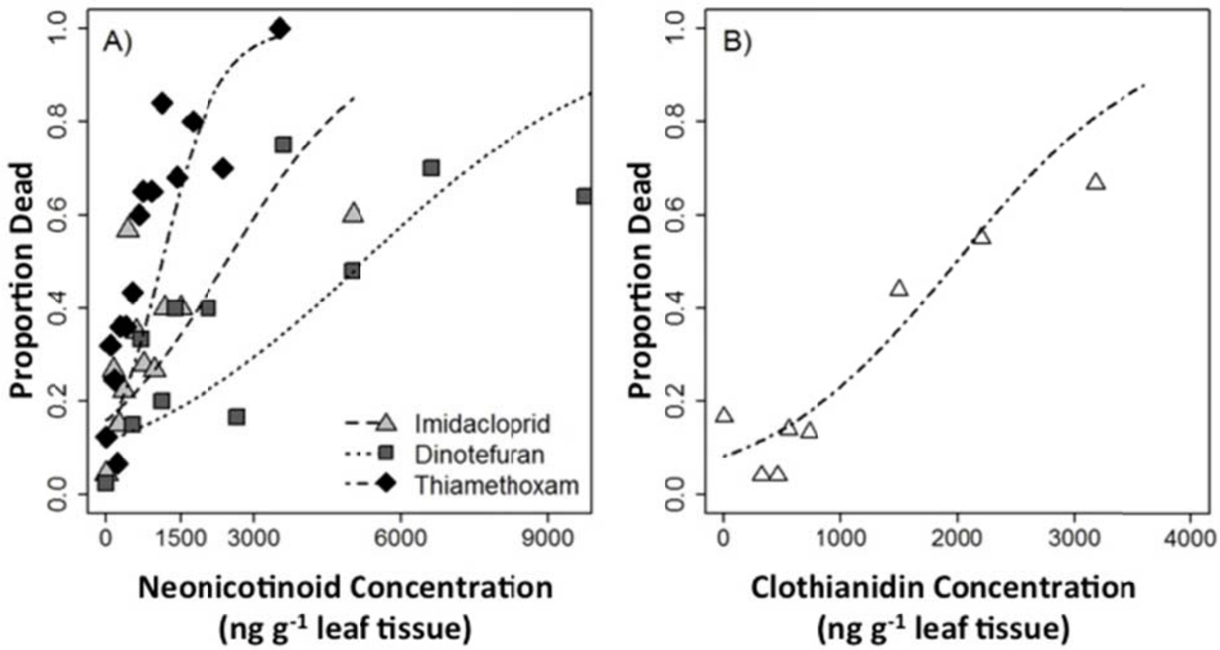

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