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Rapid uptake and retention of neonicotinoids in nursery citrus trees as a safeguard against Asian citrus psyllid (*Diaphorina citri*) infestation

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ABSTRACT

There are specific regulations in the USA for the inter-state shipment of containerized citrus from production nurseries located within *Diaphorina citri* Kuwayama (Asian citrus psyllid; ACP) quarantine zones. To ensure trees are protected from the insect, nurseries must treat trees with an approved systemic neonicotinoid at least 30 days, and not more than 90 days, before trees can be shipped. The objective of this study was to reevaluate the necessity for a 30-day pre-shipment restriction by providing regulators with further data on the uptake of imidacloprid, thiamethoxam and dinotefuran, during the days immediately following treatments. In previous studies, ACP-effective thresholds were determined for 1-year old containerized citrus trees by correlating residue concentrations in leaf tissue with ACP efficacy. In this study, we used target concentrations to compare the uptake and retention of these neonicotinoids in 4 cultivars of 1-year old containerized citrus trees. ACP-effective thresholds were achieved within 3 days of treatment with the current lowest label rate (6.43 g AI/m^3 soil) of generic formulations of imidacloprid, and within 1 day of treatment with the maximum label rate (97 g AI/m^3 soil) of the name brand formulation Admire Pro®. The establishment of dinotefuran at ACP-effective thresholds was erratic in citrus cultivars, and residues of both dinotefuran and thiamethoxam had largely dissipated before the 30-day pre-shipment period had expired. The uptake and retention of all three neonicotinoids were significantly compromised under excessive watering (400% ET), although ACP-effective thresholds were still achieved with all imidacloprid treatment rates and thiamethoxam within 3 days. Overall, our results strongly support shortening the 30-day pre-shipment restriction to at most 3 days for trees treated with either imidacloprid or thiamethoxam. And, dinotefuran should be removed from the list of systemic insecticides approved for quarantine treatments.

1. Introduction

The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is one of the most important pests of citrus in the world (Bové, 2006). It is the vector of the deadly plant-pathogenic bacterium *Candidatus* Liberibacter asiaticus (*C*Las), the causal agent of huanglongbing (HLB), also known as citrus greening (Lafleche and Bové, 1970). There is no cure for HLB, and the disease can kill an infected tree within as little as 5 years. When the ACP was first detected in California in 2008 at a residence in San Diego county (CDFA, 2008), the California Department of Food and Agriculture (CDFA) established a quarantine zone to regulate the movement of ACP host plants from areas known to be infested with the insect (CDFA, 2017; Grafton-Cardwell et al., 2013). Since the initial find, ACP has spread throughout southern California, where it is now well-established in residential and commercial citrus. The devastating impacts of the insect and disease have already been experienced in Florida, where the ACP was first documented in 1998 (Halbert et al., 2000; Halbert and Manjunath, 2004). The unrestricted movement of infested citrus nursery stock was a major factor in the dispersal of this insect to all citrus growing areas within Florida (Halbert et al., 2010). When HLB was subsequently detected in 2005, the pervasiveness of the insect was attributed as a major contributing factor in the rapid establishment of the disease in commercial groves. Estimates of the cost of the HLB epidemic to the Florida citrus industry exceeded \$4.5 billion for the five seasons between 2006/07 and 2010/11 (Hodges and Spreen, 2012).

In an effort to avert a similar scenario in California to that which occurred in Florida, state and federal regulators implemented restrictions on the trade of ACP host plants both within and outside of ACP quarantine zones, including inter-state (USDA, 2019; Grafton-Cardwell

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Table 1
Citrus tree production for the neonicotinoid field trial.

Cultivar	Rootstock	Budding Date	Repotting Date
'Parent Washington' navel orange*Citrus sinensis* (L.) Osbeck		June 30, 2017	June 15–20, 2018
'Frost Owari' Satsuma mandarin*C. unshiu* Marcovitch	C-35 citrangeX *Citroncirus* spp		
'Rio Red' grapefruit*C. paradisi* Macfadyen			
'Limoneira 8 A' lemon*C. limon* L. Burm. F.	Carrizo citrangeX *Citroncirus* spp.		

et al., 2013). Currently, all citrus nursery stock must be treated no more than 90 days prior to shipping with an approved foliar contact insecticide and a soil systemic insecticide in order to receive a 90-day certification (CDFA, 2017). During this certification period, plants may be shipped from production facilities to retail outlets. However, in addition to the maximum 90-day pre-shipment requirement, all shipments destined for regions outside of quarantine areas, including inter-state shipments, must be treated no less than 30 days prior to the shipment date (CDFA, 2017). The 30-day restriction was mandated by the USDA-APHIS at a time when nursery trees were produced outdoors rather than in approved insect-proof screenhouses, and when imidacloprid was the predominant systemic neonicotinoid used by nurseries (Byrne et al., 2018). The decision to implement the restriction was based on the best knowledge available at that time on how quickly imidacloprid treatments could disinfest trees of psyllids. No data were available for the uptake and persistence of neonicotinoids in containerized citrus trees, which are typically sold when they are 1 year old. In mature citrus trees, however, imidacloprid uptake was known to take several weeks before it became fully systemic (Castle et al., 2005). This delay likely influenced the decision to instigate a 30-day hold on nursery stock, despite their smaller size and protection in screenhouses, to ensure that the insecticide was distributed throughout the tree, and all potential life stages of ACP were killed prior to shipment.

Once trees have shipped to a retail outlet, there are no requirements that trees be retreated once the 90-day certification has elapsed, since the certification rules only apply at production facilities. However, recent data showed that citrus trees often remain in retail for periods well in excess of the certification period (Byrne et al., 2018). During these long residencies, there is a serious risk of trees becoming infested with ACP and acting as conduits for the further spread of the insect and disease when the trees are eventually sold to homeowners or landscapers. Clearly, it is imperative that the protective effects of the two mandated pre-shipment insecticide treatments are maximized in order to limit the possibility of plants becoming infested by ACP while awaiting sale at retail. While the approved foliar contact treatments are highly effective against all ACP life stages (Bethke et al., 2015, 2017; Morse et al., 2016), they do not provide long residual control (Tofangsazi et al., 2017); the bulk of the long-term protection is provided by the systemic treatment (Byrne et al., 2017, 2018; Rogers and Shawer, 2007). Therefore, unnecessary delays in shipping after the treatments have been applied at the production nurseries will shorten the period of time that trees at retail are protected by those systemic treatments. Previous research (Byrne et al., 2017, 2018) has shown that containerized citrus trees treated systemically with imidacloprid could be shipped within two weeks of treatment, when peak residues were established within trees. Since those studies were completed, USDA-APHIS has updated quarantine regulations (USDA, 2019), primarily in response to the detection of HLB in California in 2012 (Kumagai et al., 2013). All citrus produced within an area quarantined for ACP and/or HLB, and destined for another citrus-producing state, must now be produced within a USDA-APHIS-approved structure, and must undergo stringent inspection and permitting measures before shipment is approved. However, despite the requirement that all citrus be produced within insect-proof structures, pre-shipment treatment requirements for production nurseries have not changed, and the 30-day restriction remains in place. Before considering any further changes, additional studies were required that focused on early uptake within the first week following treatment of imidacloprid, since this information was lacking (Byrne et al., 2018). Furthermore, evaluations of additional imidacloprid treatment rates were needed. Several generic formulations of imidacloprid are approved for use as pre-shipment treatments (CDFA, 2017). However, the treatment rates for many of the generic products are lower than the name brand product, Admire Pro®. Therefore, the use of different products by separate production nurseries could potentially result in different levels of protection for citrus trees.

The overall goals of this study were to address the concerns of state and federal regulators regarding how rapidly imidacloprid and other approved neonicotinoid quarantine treatments become fully systemic within nursery trees at concentrations known to be effective in preventing the colonization of trees by ACP (Sétamou et al., 2010). The uptake and retention of imidacloprid were compared at four treatment rates that span the recommended label rates for the name brand and generic imidacloprid formulations. In addition, we conducted further evaluations of the neonicotinoids thiamethoxam and dinotefuran. Based on previous research, we expressed concerns on the use of dinotefuran as a quarantine treatment, and the exclusion of thiamethoxam from the inter-state treatment schedule (Byrne et al., 2017). Therefore, we included these two chemicals in our studies and evaluated their early uptake under two irrigation regimes. Over-watering could be especially problematic for the efficacy of these highly water-soluble compounds if insecticides were leached from the potted trees. In particular, excessive irrigation at retail outlets could lessen the protective effect of the treatments applied by production nurseries (Byrne et al., 2018).

2. Materials and methods

2.1. Trees

Details on the production of the 4 citrus cultivars used in this study are summarized in Table 1. Four hundred citrus trees were propagated on June 30, 2017 by budding one of four cultivars on C35 or 'Carrizo' citrange rootstocks growing in 12.7 cm diameter treepots (Stuewe & Sons; cat # CP512CH). The trees were maintained in a protective structure free of any insecticide treatments at the Lindcove Research and Extension Center (LREC) in Exeter, CA until they were approximately 1 yr old. Trees were transported to a lathe house at the University of California's Citrus Research Center-Agricultural Experiment Station (CRC-AES) on May 21, 2018, and were replotted on June 15–20, 2018 into 18.9 L pots. The latter is the predominant pot size used by California nurseries for the production of citrus trees for sale at retail outlets. The soil mix consisted of a modified formulation of UC Soil Mix #1 (http://agops.ucr.edu/soil/) with 10% sand, 60% redwood bark, 15% moss, 15% coconut fiber. All other constituents were included at the standard level of UC Soil Mix #1. The trees were top-dressed as needed with a granular fertilizer (Vigoro® Citrus & Avocado Plant Food) over the course of the experiment. Two weeks after replotting (July 3, 2018), the trees were transferred from the lathe house to a field plot, where they were laid out in a 16 × 25 grid pattern with 1.5 m spacing between potted trees.

2.2. Irrigation

Each tree was provided with drip irrigation designed to deliver one of two watering levels. The irrigation regimes were implemented using

Table 2
Neonicotinoid insecticides and treatment rates.

Insecticide	Active Ingredient	Treatment Rate
Admire Pro 0.55 kg AI/l (suspension concentrate)	Imidacloprid	6.4 g AI/m^3 soil (121 mg/tree) 19.4 g AI/m^3 soil (368 mg/tree) 64.3 g AI/m^3 soil (1.21 g/tree) 97 g AI/m^3 soil (1.84 g/tree)
Flagship 25 WG25% water dispersible granule	Thiamethoxam	5.90 g AI/m^3 soil (118 mg/tree)
Safari 20 SG20% soluble granule	Dinotefuran	8.50 g AI/m^3 soil (170 mg/tree)

adjustable DIG® emitters, whose output was verified at the outset of the study. The watering levels were established based on measures of daily evapotranspiration (ET) on a subset of 5 trees from each cultivar that were chosen randomly from the experimental trees. This involved watering them to capacity, then weighing the entire potted tree within the next hour and again 24 h later to calculate the daily change in mass. ET measurements were repeated three times over consecutive days. Based on this value we selected two irrigation rates that we refer to as replacement watering (100% ET) and overwatering (400% ET). There were no appreciable differences in ET measurements between cultivars.

Dinotefuran and thiamethoxam uptake and retention were evaluated at both irrigation levels in all 4 cultivars, since there were no data available for containerized citrus showing the impact of over-watering on these insecticides. In previous work, we showed that overwatering did impact imidacloprid performance (Byrne et al., 2018); however, we were interested in determining whether the effects were consistent across a range of treatment rates, so we tested this in the mandarin cultivar.

2.3. Insecticide treatments

On Aug 27, 2018, trees were treated with one of three systemic applications of neonicotinoids - Admire Pro (imidacloprid; 0.55 kg active ingredient (AI) L^{-1} suspension concentrate), Flagship 25 WG (thiamethoxam; 25% AI water-dispersible granule) and Safari 20 SG (dinotefuran; 20% AI soluble granule). Imidacloprid was evaluated at 4 rates, while thiamethoxam and dinotefuran were applied at their recommended label rates. For each treatment, or treatment rate, 10 replicate trees for each cultivar were treated. In Table 2, treatment rates are summarized in terms of active ingredient (AI) per m^3 of soil for easy comparison between chemicals used in this study, and other products available on the market. Pots were pre-irrigated for 15 min to ensure adequate wetting of the soil mix prior to insecticide application. The formulated insecticides were diluted in water, and then administered to each pot in a final volume of 250 mls using a measuring cylinder, followed by an additional 1 L from a watering can to ensure the insecticide permeated below the soil mix surface into the root zone. The daily drip irrigation regime at each of the two water volume levels was implemented 24 h after the insecticides were applied.

2.4. Chemical quantification of neonicotinoid insecticides

Residues of imidacloprid (QuantiPlate Kit for Imidacloprid, cat. # EP 006; Envirologix, Portland, ME, USA), dinotefuran (SmartAssay Dinotefuran Test Kit, cat. # 306–33989; FUJIFILM Wako Chemicals USA Corp, Richmond, VA) and thiamethoxam (SmartAssay Thiamethoxam Test Kit, cat. # 300–34009; FUJIFILM Wako Chemicals USA Corp, Richmond, VA) were quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kits. The lower limits of quantitation (LOQs) of residues in citrus leaves for the three ELISAs were set at 850 ng dinotefuran, 75 ng imidacloprid, and 90 ng thiamethoxam g^{-1} leaf tissue. 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 (Byrne et al., 2005). ELISA absorbance (at 450 nm) readings were determined using an accuSkan GO microplate reader (Fisher Scientific Company, Hanover Park, IL, USA). Samples of young leaf flush tissue were collected from each tree at each cardinal direction, immediately prior to treatment, and then on 13 sampling days after the trees were treated (1, 3, 5, 7, 10, 14, 21, 28, 60, 90, 120, 150 and 180 d). Tissue samples of 0.5 g from each tree were placed in vials, chopped into small pieces using scissors and then extracted by the addition of 5 mL of absolute methanol. Extracts were shaken on an orbital shaker 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 TLC purification step for imidacloprid was used to eliminate imidacloprid metabolites from the extracts (Nauen et al., 1998) that could potentially cross-react with the ELISA kit antibody (Byrne et al., 2005).

2.5. Data analysis

Imidacloprid uptake and retention for all replicate trees irrigated at the 100% ET level over the full (180 d) duration of the study were compared using a linear mixed-effects model (LMM; Crawley, 2009). Specifically, we analyzed imidacloprid concentrations, that were log (x + 1) transformed to meet test assumptions, with fixed effects of citrus cultivar (navel orange, lemon, grapefruit, mandarin), imidacloprid treatment rate (6.43, 19.4, 64.3, and 97 g AI/m^3 potting media), and sampling day post-treatment (from 1 through 180 d). A random effect of tree replicate identity was included to account for autocorrelation stemming from repeated measurements of the same trees on each sampling day (Crawley, 2009).

A similar model was used to analyze imidacloprid residues in the potted mandarin trees irrigated at the two different irrigation levels. We used a LMM on log (x + 1) transformed imidacloprid concentrations that included fixed effects of irrigation regime (100% or 400% ET), imidacloprid treatment rate, sampling day post-treatment, all interactions, and a random effect of replicate identity due to multiple measures of each tree.

We compared the frequency of ACP-effective residues for each of the three neonicotinoids at 100% ET watering for the 60-d duration of measurement for dinotefuran and thiamethoxam. We had anticipated sampling the dinotefuran and thiamethoxam trees for 180 d, but terminated sampling of these trees at 60 d, when analysis of the 30-d and 60-d samples showed titers in most samples were either well below ACP-effective thresholds or at non-detectable levels. Rather than include presumptive data that would zero-inflate the thiamethoxam and dinotefuran datasets, imidacloprid data for the four treatment rates were truncated to allow direct comparison of the three insecticides over the same timeframe. Effective residues for imidacloprid were based on prior research showing 220 ng imidacloprid/g leaf tissue were required for high ACP nymphal mortality (Sétamou et al., 2010). We used previously determined thresholds for dinotefuran and thiamethoxam of approximately 900 ppb and 150 ppb, respectively, that were associated with low ACP colonization rates in a field study (Byrne et al., 2017). We used a generalized linear mixed-effects model (GLMM) with binomial error (Pinhero and Bates, 2009) on the fraction of samples with ACP-effective residues, with fixed effects of citrus cultivar, sampling day post-treatment, and insecticide treatment (4 imidacloprid rates, thiamethoxam, dinotefuran), and a random effect of replicate identity. Due to problems with model convergence, the maximum model evaluated included main effects and no interactions.

In a final analysis, the effects of watering regime on dinotefuran and thiamethoxam were examined in the four citrus cultivars. This analysis

Table 3
Statistical results for effects of citrus cultivar, imidacloprid treatment rate (rate), sampling day post-treatment (sampling day), and their interactions, on imidacloprid concentration.

Source	χ^2	df	P
citrus cultivar	4.705	3	0.1947
imidacloprid treatment rate	1607.07	3	<0.0001
sampling day post-treatment	28046.5	12	<0.0001
cultivar*rate	12.049	9	0.2106
cultivar*sampling day	353.644	36	<0.0001
rate*sampling day	2874.18	36	<0.0001
cultivar*rate*sampling day	1720.80	108	<0.0001

again considered the fraction of trees with ACP-effective residues using a binomial GLMM, with fixed effects of treatment (insecticide type), citrus cultivar, irrigation level (100 or 400% ET), sampling day post-treatment, and a random effect of tree replicate identity. The maximum model considered for the GLMM included all main effects and the two-way interactions cultivar x treatment and treatment x sampling day post-treatment. Replication within the mandarins treated with dinotefuran and irrigated at 400% ET was limited to 3 trees due to mortality or poor plant growth during the experiment. Therefore, some caution should be used when interpreting the results of that treatment combination.

3. Results

3.1. *Effect of treatment rate on imidacloprid residues in 4 citrus cultivars under replacement irrigation*

Results for imidacloprid concentration in all four citrus cultivars receiving replacement watering (100% ET) showed significant main effects for treatment rate and sampling day post-treatment, and significant cultivar x sampling day, cultivar x treatment rate, and cultivar x treatment rate x sampling day interactions (Table 3). In general, imidacloprid residues increased sharply after 1 day post-treatment, peaked at 3–4 weeks post-treatment, and declined sharply after 2 months for all 4 citrus cultivars (Figs. 1 and 2). Indeed, mean imidacloprid residues for all cultivars at all rates were above the ACP-effective concentration (220 ppb; 2.34 on a $\log_{10}$ scale) between at least 3 days and 2 months post-treatment (Figs. 1 and 2b). One day after treatment, mean residues exceeded the ACP-effective concentration at the highest two treatment rates in navel orange, lemon and mandarin, and all but the lowest rate in grapefruit (Fig. 2a). From 3 months onward, differences in imidacloprid concentrations were more apparent among cultivars and treatment rates. In grapefruit and mandarin, concentrations declined to zero at the lowest two rates while concentrations at the highest two rates exceeded the ACP-effective concentration through 6 months post-treatment (Fig. 1). For navel orange and lemon, concentrations also declined to zero at the lower two rates, while effective concentrations were maintained for 5 months at the second highest rate, and for 6 months at the highest rate (Fig. 1).

3.2. *Uptake and retention of four rates of imidacloprid in mandarins under two irrigation regimes*

Results for imidacloprid concentration in mandarins irrigated at two levels showed significant main effects and all interactions except for the interaction irrigation level x sampling day post-treatment (Table 4).

In general, the higher watering level resulted in reduced imidacloprid residues, although the magnitude of reduction depended on treatment rate and sampling day post-treatment (Figs. 2 and 3). After 1 day post-treatment, imidacloprid residues increased sharply and stayed relatively high for approximately 2 months, regardless of the irrigation regime (Fig. 3). Under both irrigation regimes, mean ACP-effective concentrations were reached within 3 days at all treatment rates (Fig. 2). At the two lowest treatment rates (6.43 g and 19.4 g AI/m^3), imidacloprid was not detected from 3 months post-treatment under either irrigation regime (Fig. 3). At the two higher treatment rates (64.3 g and 97 g AI/m^3), from 3 months post-treatment onward the higher

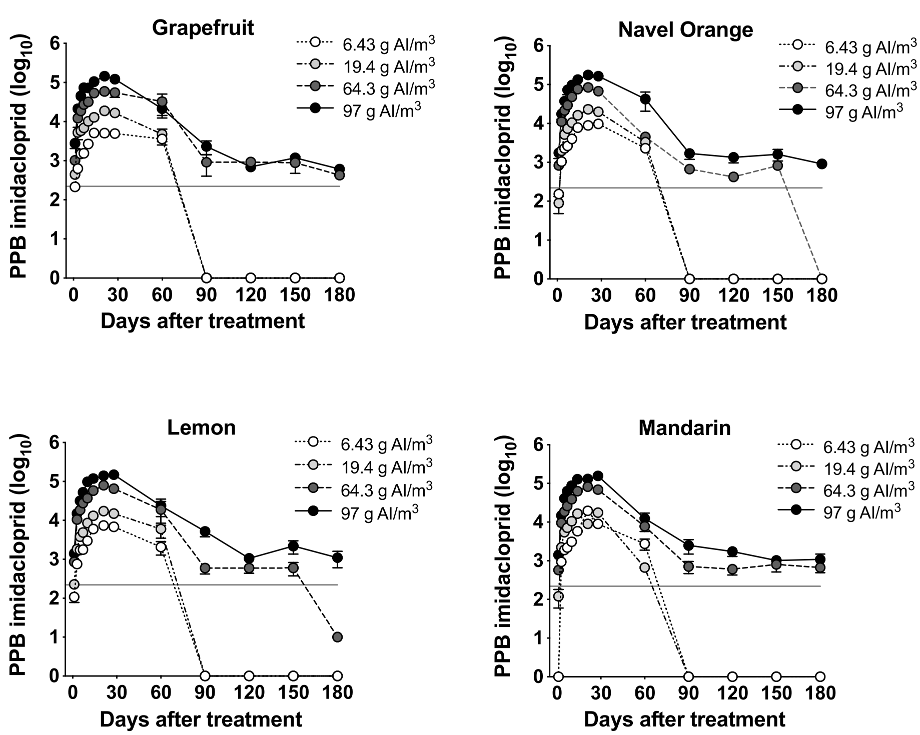

Fig. 1. Imidacloprid concentrations (log10 ($\bar{x}$) ±SE) in leaf tissue samples collected from 4 citrus cultivars (10 tree replicates per cultivar) treated at 4 different treatment rates (Table 2) and sampled for up to 6 months post-treatment. The horizontal line denotes the ACP-effective concentration (2.34 on a log10 scale). Residue data for 1 day and 3 days post-treatment are shown in more detail in Fig. 2.

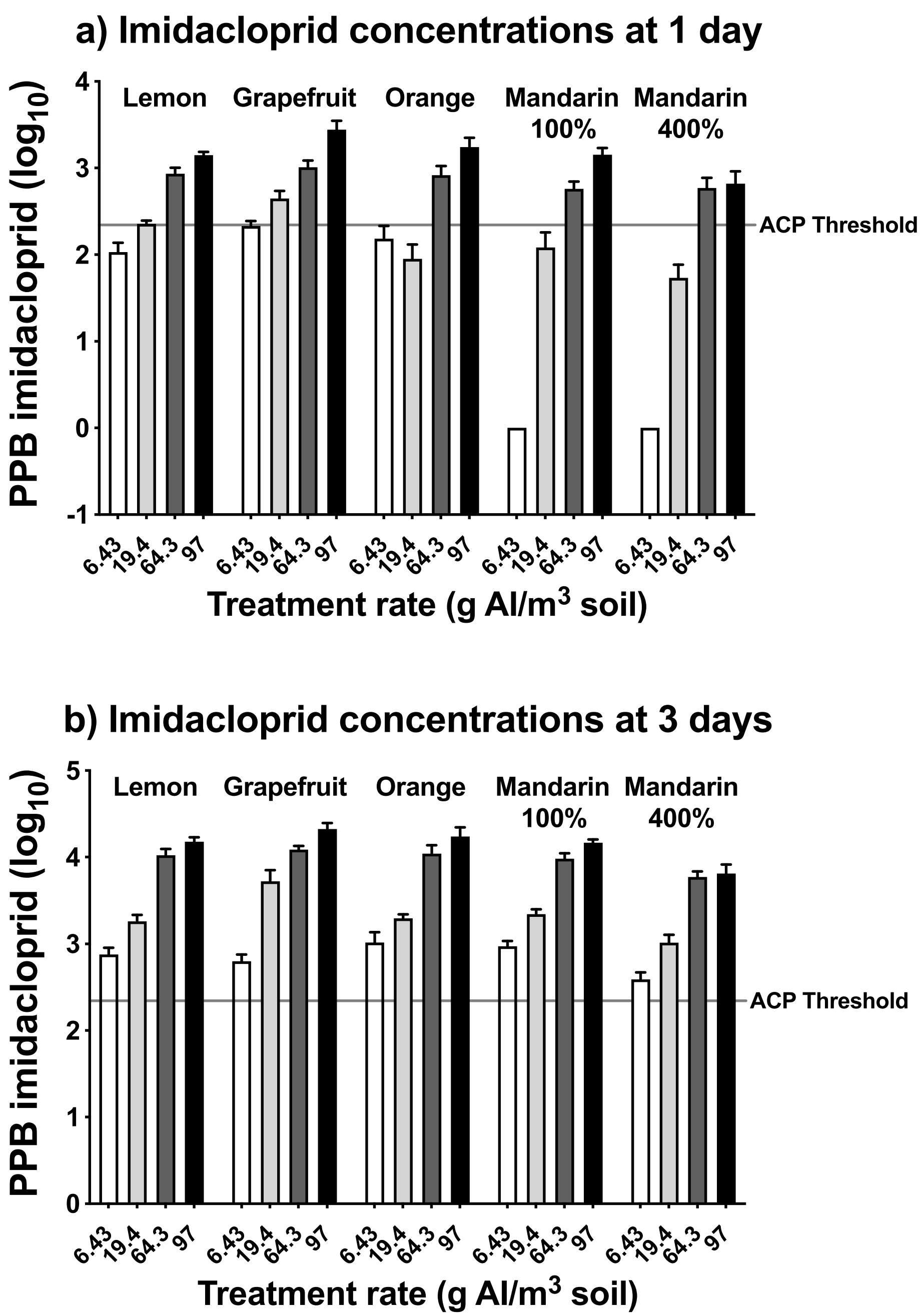

Fig. 2. Imidacloprid concentrations (log_{10} ($\bar{x}$) ±SE) in 4 citrus cultivars (10 tree replicates per cultivar) at a) 1 day, and b) 3 days after treatment with 6.43, 19.4, 64.3, and 97 g AI imidacloprid/m^3 soil. All cultivars other than mandarins were watered at 100% ET. Residue data for the entire 180 day monitoring period are shown in Fig. 1.

Table 4
Statistical results for effects of irrigation level (water), imidacloprid treatment rate (rate), sampling day post-treatment (sampling day), and their interactions, on imidacloprid concentrations in young mandarin trees.

Source	χ^2	df	P
water	51.818	1	<0.0001
imidacloprid treatment rate	484.11	3	<0.0001
sampling day post-treatment	7249.9	12	<0.0001
water*rate	10.513	3	0.0147
water*sampling day	19.516	12	0.0768
rate*sampling day	659.93	36	<0.0001
water*rate*sampling day	74.985	36	0.0001

irrigation rate generally reduced imidacloprid residues near or below ACP-effective concentrations compared to the lower irrigation rate (Fig. 3).

3.3. Uptake and retention of imidacloprid, dinotefuran and thiamethoxam in 4 citrus cultivars under replacement irrigation

The comparison of the uptake and retention of the three insecticides over 60 d post-treatment showed significant effects of cultivar, treatment, and days since treatment (Table 5). In general, thiamethoxam resulted in the most consistently rapid uptake, dinotefuran exhibited substantial variability in both uptake and retention, and imidacloprid resulted in the longest retention over time (Fig. 4).

More specifically, for all cultivars, the lower 3 imidacloprid rates did

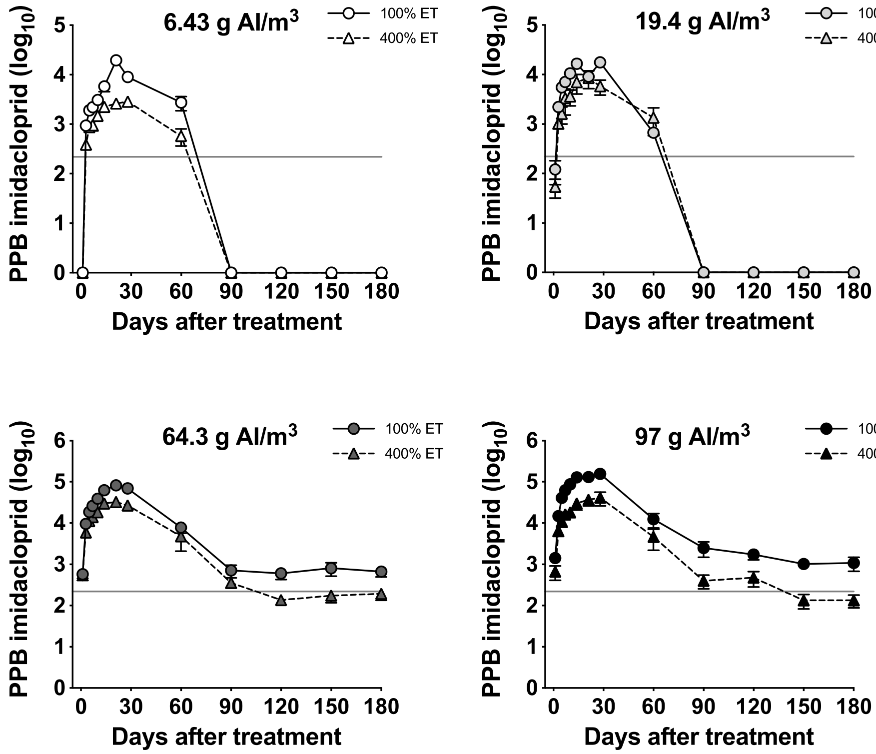

Fig. 3. Imidacloprid concentrations ($\log_{10}$ ($\bar{x}$) ±SE) in leaf tissue samples collected over 6 months from young mandarin trees following treatment with 6.43, 19.4, 64.3, or 97 g AI imidacloprid/m^3 soil and irrigated at either 100% or 400% evapotranspiration rate (ET). The horizontal line denotes the ACP-effective concentration.

Table 5
Statistical results for analyses on the proportion of trees with ACP-effective residues of imidacloprid, thiamethoxam or dinotefuran (treatment), among 4 citrus cultivars, and sampling day post-treatment. Data for all treatments were included in the analysis up to 60 d post application.

Source	χ^2	df	P
citrus cultivar	12.902	3	0.0049
treatment	195.98	5	0.0001
sampling day post-treatment	322.79	8	<0.0001

not result in 100% uptake to ACP-effective concentrations after 1 day. However, after 3 days at these rates, all trees had ACP-effective residues except for 1 lemon tree treated with the second lowest rate and 1 mandarin tree treated with the second to highest rate. Once ACP-effective imidacloprid residues were reached in all trees, they were maintained for at least 60 d post-treatment at all rates in orange and grapefruit, and at the two higher rates in lemons and mandarins. At the highest imidacloprid rate all trees reached ACP-effective concentrations within 1 d post-treatment.

Dinotefuran showed more substantial variation in the fraction that reached ACP-effective concentrations. For 3 of the 4 cultivars, uptake was not at 100% after 1 d. Indeed, in lemons, no more than 80% of trees ever attained effective residues (Fig. 4). Moreover, dinotefuran retention was generally poorer than the other neonicotinoids, with noticeable declines approximately 30 d after treatment.

Thiamethoxam applications resulted in 100% of trees with effective residues after 1 d, and remained at that level for approximately 21 d in all cultivars, after which a clear decline occurred (Fig. 4).

3.4. Uptake and retention of dinotefuran and thiamethoxam in 4 cultivars of citrus under two irrigation regimes

The final analysis of the effects of irrigation regime on dinotefuran and thiamethoxam uptake and retention showed significant main effects and significant interactions between cultivar x treatment and treatment x sampling day post-treatment (Table 6). Trees treated with dinotefuran showed substantial variability within the first month following treatment, with a relatively high fraction of lemon and grapefruit trees having residues that were below the ACP-effective concentration (Figs. 4 and 5). In contrast, thiamethoxam treatments resulted in a consistently higher proportion of trees with ACP-effective concentrations over the first three weeks, after which the proportion dropped sharply (Figs. 4 and 5). Higher irrigation levels were associated generally with reduced dinotefuran uptake and retention during the first 2 weeks following treatment, and reduced thiamethoxam uptake during the first few days following treatment (Fig. 5).

4. Discussion

This study provided a comprehensive assessment of how different treatment rates of imidacloprid and irrigation regimens affect insecticide uptake and retention in containerized citrus. The purpose of the treatments is to protect trees from infestation by ACP, and thereby disrupt the spread of psyllids on nursery trees. Therefore, it is essential to interpret residue levels in a manner appropriate to that goal. Although we did not establish the efficacy of the treatments directly against ACP in this investigation, prior studies provide sufficient data to allow an assessment of the relevancy of residue levels at preventing the establishment of colonies. Byrne et al. (2017) showed that 2 varieties of 1-year old containerized citrus trees treated with the 6.43 g rate of imidacloprid did not become infested with ACP until residues had declined to at least 75 ng g^{-1} of leaf tissue, whereas untreated control trees became infested much sooner. There are no other data available relating residue and efficacy data for ACP on containerized citrus. However, in an orchard study conducted on 3- and 4-year old non-bearing 'Rio-Red' grapefruit trees (the same grapefruit variety used in this study), imidacloprid residues of 220 ng g^{-1} prevented the establishment of ACP colonies on trees (Sétamou et al., 2010). Both of these studies rated treatment efficacy on the ability of ACP to establish

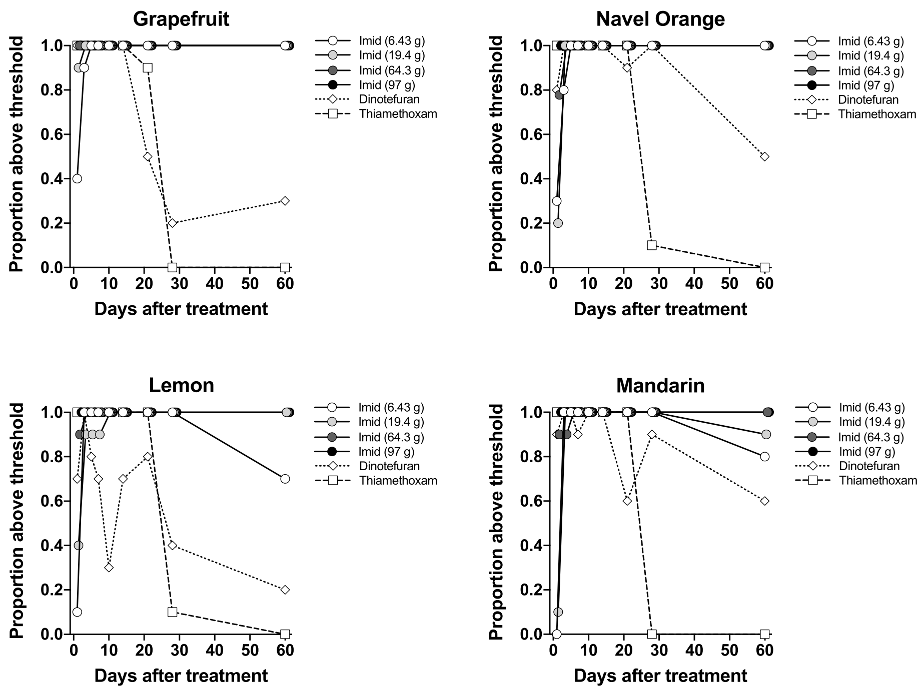

Fig. 4. Proportion of citrus trees (4 cultivars) with ACP-effective concentrations of imidacloprid (treated at four different treatment rates; Table 2), dinotefuran, or thiamethoxam over 60 d post-treatment. All trees were irrigated at 100% ET. Symbols at each time-point are offset slightly for clarity.

Table 6

Statistical results for analyses on the proportion of trees with ACP-effective residues of thiamethoxam or dinotefuran (treatment), among 4 citrus cultivars (cultivar), 2 irrigation levels (water), and days since treatment (sampling day).

Source	χ^2	df	P
citrus cultivar	13.779	3	0.0032
treatment	6.891	1	0.0087
water	16.339	1	<0.0001
sampling day post-treatment	676.61	8	<0.0001
treatment*cultivar	15.602	3	<0.0001
treatment*sampling day	179.23	8	<0.0001

colonies on treated trees, and are, therefore, the most relevant measures of the efficacy of systemic treatments for production nurseries. Other approaches have accessed residue levels on the basis of their ability to prevent adult feeding, and thereby disrupt the transmission of HLB, or to kill adult ACP. Langdon et al. (2018b) assessed the efficacy of imidacloprid treatments in grove trees based on artificial ingestion bioassays (Langdon and Rogers, 2017), and concluded that the highest leaf tissue concentrations measured in field-treated citrus trees (1000 ng g^{-1}) would be ineffective at reducing ACP feeding activity. Unfortunately, no data were provided in that study on the efficacy of the treatments at preventing the establishment of active ACP colonies. In the same way, Byrne et al. (2017) showed that considerably higher concentrations of imidacloprid were required to kill adult ACP in bioassays when insects were exposed to leaves sampled from systemically-treated trees, compared with concentrations measured at the time insects began to establish colonies. The apparent disconnect between the acute mortality residue levels measured under the artificial conditions of laboratory bioassays, in which the insects are required to feed over a (relatively) short period of time on a treated substrate, and those determined at the time ACP begin to colonize treated trees, has been discussed in some detail (Byrne et al., 2017). In particular, bioassays of imidacloprid systemic activity are difficult to interpret because of confounding sublethal and anti-feedant behavioral effects. Such effects are known to occur in many different species of insects, including ACP (Boina et al., 2009), and could potentially increase efficacy thresholds, if adult mortality was the only acceptable measure of efficacy. The latter would render most field treatments as ineffective, and would necessitate the use of the maximum imidacloprid label rate by production nurseries in order to reach those thresholds.

In this study, the results show that imidacloprid uptake is rapid in four citrus cultivars, with mean residues surpassing required ACP-effective thresholds in trees within as little as 3 days. In fact, based on the ACP-effective concentration of 220 ng g^{-1} leaf tissue (Sétamou et al., 2010), full protection to trees was achieved in all cultivars within 1 day of treatment with the current highest label rate (97 g AI/m^3 soil) for imidacloprid. In mandarins, over-watering of trees dramatically reduced the overall titers of imidacloprid, and slowed the initial rate of uptake. Yet, even with over-watering conditions of 400% ET, uptake of insecticide was rapid enough to protect trees within 3 days at all rates. Protection beyond 60 d was only achieved at the higher treatment rates. Thus, we have provided further evidence that the requirement for a 30-day delay between treatment with imidacloprid and shipping to retail is unnecessarily long, and may be counter-productive to the overall goal of preventing infestations of ACP on containerized citrus. More significantly, the delay in treatment before shipment shortens the protective effect that the systemic treatments will ultimately have on trees awaiting sale in retail. Without any regulation of residency times, treatment rates, or irrigation levels for containerized citrus trees in retail outlets, trees that lose their protection while in retail become vulnerable to infestation by ACP (Byrne et al., 2018), and become potential reservoirs for the HLB pathogen (Halbert et al., 2012).

There are several generic formulations of imidacloprid available on the market. For nursery stock that is destined for markets outside of quarantine areas, including inter-state markets, any generic formulation of imidacloprid is approved for use (CDFA, 2017). One potential problem, however, is that the pesticide label rates on many of the generic products are not consistent, meaning that the choice of product used by a production facility will impact the amount of active ingredient applied to a tree. The lowest rate evaluated in this study is the maximum treatment rate allowed by the labels for the majority of generic formulations. Based on prior estimates of concentrations required to prevent ACP colonization (Byrne et al., 2017; Sétamou et al., 2010), our data

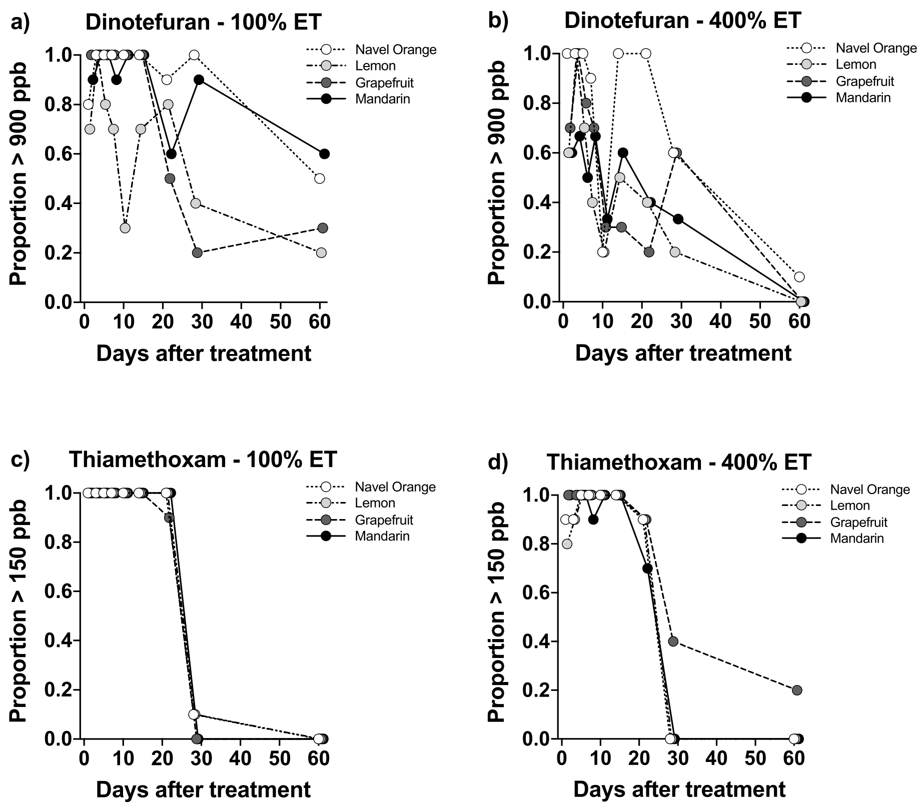

Fig. 5. Proportion of citrus trees over time with residues above approximate ACP-effective concentrations following treatment with dinotefuran [a, b] or thiamethoxam [c, d] and irrigation at 100% [a, c] or 400% of evapotranspiration [b, d]. Symbols at each time-point are offset slightly for clarity.

show that this treatment rate (6.43 g AI/m^3 soil) will deliver ACP-effective concentrations to trees within 3 days, although retention of effective residues will be dramatically reduced compared with higher treatment rates permitted by other formulations. There are very few imidacloprid products that permit treatment rates above our lowest test rate. Therefore, while the majority of products may protect trees relatively quickly, they will not protect trees beyond 90 days. One option to consider would be to limit quarantine treatments to products that permit higher treatment rates. By so doing, trees would acquire ACP-effective concentrations more rapidly, as quickly as 1 day, and be protected for a longer period once they are shipped. From a production nursery perspective, the need to wait 30 days before shipping represents a logistical challenge. Once a new order is received, nurseries would prefer not to have to put a 30 day hold on delivering that order while waiting for a treatment to take effect. Therefore, many nurseries have now resorted to the costlier exercise of treating nursery stock every 90 days. By so doing, nurseries have certified stock available that has been treated within the 90-day maximum treatment window, and satisfies the minimum 30-day pre-shipment requirement. However, this strategy complicates the ability to protect nursery trees effectively once they are shipped to retail, since trees could potentially be shipped in as little as 1 day before the expiration of the 90-day certification period, when a large proportion of the insecticide has already dissipated. At lower treatment rates, trees would be vulnerable to infestation sooner once they left the protective environment of the screen house.

Both thiamethoxam and dinotefuran are recommended as approved treatments for the movement of nursery stock within ACP quarantined areas (CDFA, 2017), while dinotefuran is the only active ingredient, other than imidacloprid, that is approved for use on nursery stock that will be shipped outside of quarantined areas. Our data show that dinotefuran is wholly unsuitable as an effective quarantine treatment. Although uptake was very rapid, and higher titers of dinotefuran were established within trees compared with either imidacloprid or thiamethoxam, the lower inherent toxicity of dinotefuran (Byrne et al., 2017) ultimately meant that ACP-effective concentrations were not always reached in trees. Furthermore, over-watering of trees treated with dinotefuran, which is a likely scenario at retail outlets (Byrne et al., 2017), further reduced the efficacy of this treatment, and resulted in extremely erratic residue patterns in the 4 cultivars.

Thiamethoxam was a more effective treatment than dinotefuran. Previous data showed that it is inherently quite toxic to ACP, with significantly lower concentrations needed to protect trees from ACP colonization (Byrne et al., 2017). ACP did not establish on containerized trees until concentrations of thiamethoxam exceeded 163 ng g^{-1}. Again, significantly higher concentrations were required to achieve outright adult mortality in short duration bioassays (Byrne et al., 2017), and to lower the probability of finding an infested leaf flush to practically zero (Langdon et al., 2018a). As with imidacloprid, we regard the more appropriate measure of the efficacy of systemic thiamethoxam for quarantine treatments in terms of their ability to prevent colonization.

The time to achieve ACP-effective concentrations of thiamethoxam was delayed by over-watering; however, residues reached fully effective ACP thresholds within 1 day in trees under replacement irrigation. Interestingly, thiamethoxam was applied at 70% of the treatment rate used for dinotefuran (Table 2), and yet was superior in uptake and marginally so in retention. While the rate of uptake for both thiamethoxam and dinotefuran are equally impressive, both the greater inherent toxicity of thiamethoxam and its rapid establishment at ACP-effective concentrations make this chemical more suitable as a pre-shipment treatment than dinotefuran. For both compounds, however, under current regulations, the 30-day pre-shipment requirement means that neither thiamethoxam nor dinotefuran treatments would protect trees from a potential infestation once the trees leave a production facility, since residues have already dissipated by the time the trees are legally ready to ship.

5. Conclusions

The data generated in this study are in response to a request by federal regulators for additional information on the performance of neonicotinoids during the initial days following the treatment of containerized citrus trees. The results strongly support shortening the current pre-shipment restrictions that have been implemented in California. Although three neonicotinoids are approved for use in quarantine treatments, imidacloprid was the most effective neonicotinoid of those tested in terms of both acquisition and retention of effective thresholds. Even at its lowest treatment rate (6.43 g AI/m^3 soil), which closely matched label rates of thiamethoxam and dinotefuran, imidacloprid out-performed its neonicotinoid counterparts, and was the better of the three options. Irrigation level was clearly influential on the performance of the neonicotinoids, both in terms of initial uptake, and in terms of overall retention of ACP-effective levels within trees.

We suggest the adoption of either a fixed 3-day pre-shipment treatment rate of 6.43 g imidacloprid/m^3 soil, or a 1-day pre-shipment treatment rate of 97 g imidacloprid/m^3 soil. Either of these rates would be highly beneficial to the industry as they would minimize post-treatment delays in shipping, and ensure that all trees were afforded the maximum protection when they left the production facility. Furthermore, without any regulations for retail outlets, the 90-day certification period established at the time of treatment at the production facilities should still be retained as an indication to retail outlets of when unsold trees should be discarded.

CRediT authorship contribution statement

Frank J. Byrne: Conceptualization, Methodology, Investigation, Writing - original draft, preparation, Visualization, Funding acquisition. **Matthew P. Daugherty:** Conceptualization, Methodology, Formal analysis, Writing - review & editing. **Elizabeth E. Grafton-Cardwell:** Conceptualization, Methodology, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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