

# Insecticide Residues in Pollen and Nectar of a Cucurbit Crop and Their Potential Exposure to Pollinators

Galen P. Dively

Department of Entomology, University of Maryland, College Park, Maryland 20740, United States

Alaa Kamel\*

Analytical Chemistry Branch, Biological and Economic Analysis Division, Office of Pesticide Programs, U.S. Environmental Protection Agency, Fort George G. Meade, Maryland 20755, United States

**ABSTRACT:** Neonicotinoids are systemic insecticides widely used on many pollinated agricultural crops, and increasing evidence indicates that they move to some extent into pollen and nectar. This study measured levels of neonicotinoid residues in pollen and nectar from a pumpkin crop treated with formulated products containing imidacloprid, dinotefuran, and thiamethoxam using different timings and application methods. Environmental conditions have a significant effect on overall residue levels; nectar residues were 73.5–88.8% less than pollen residues, and metabolites accounted for 15.5–27.2% of the total residue amounts. Foliar-applied treatments and chemigated insecticides applied through drip irrigation during flowering resulted in the highest residues of parent insecticide and metabolites, which may reach average levels up to 122 ng/g in pollen and 17.6 ng/g in nectar. The lowest levels of residues were detected in treatment regimens involving applications of insecticides at planting, as either seed dressing, bedding tray drench, or transplant water treatment.

**KEYWORDS:** neonicotinoid residues, pollen, nectar, pollinators, Cucurbitaceae

## INTRODUCTION

The role that pesticides play in pollinator decline has been the focus of intensive debate and research in recent years. Agricultural-applied pesticides have been hypothesized as a stress factor affecting honey bee health, although there is no conclusive scientific evidence to support this claim.<sup>1</sup> In particular, neonicotinoid insecticides, such as imidacloprid, thiamethoxam, clothianidin, and dinotefuran, have been conjectured to potentially expose pollinators due to their wide uses as soil- and foliar-applied systemics on many agricultural crops. They are very effective against a broad spectrum of insect pests and also highly toxic to most pollinators.<sup>2</sup> Testing for effects in nontarget insect pollinators, such as honey bees, is required by the U.S. EPA when the end-use product is intended for outdoor use and honey bees may be exposed to the pesticide. Honey bee acute contact toxicity is measured by topical applications to individual bees in a laboratory study designed to measure the quantity of pesticide that will cause 50% mortality (LD<sub>50</sub>). A pesticide is classified highly toxic to honey bees if the LD<sub>50</sub> is <2 μg/bee.<sup>3</sup> Contact LD<sub>50</sub> doses for imidacloprid, thiamethoxam, clothianidin, and dinotefuran are all below 0.1 μg/bee.<sup>4,5</sup> Once applied to a crop, neonicotinoids are absorbed by the roots or leaves and then xylem transported in the vascular system through the plant, where they can persist for weeks following application.<sup>6–8</sup> Generally, they are less likely to move translatorally from leaves to the fruiting structures; however, there is increasing evidence that neonicotinoids move to some extent into pollen and nectar.<sup>9,10</sup>

Several studies of residues in bee-collected pollen and bee products have shown that honey bees are exposed to

neonicotinoids.<sup>11</sup> In these studies, the average imidacloprid residue levels in positive pollen samples ranged between 0.9 and 3.1 ng/g, whereas residues of the imidacloprid metabolite, 6-chloronicotinic acid, barely exceed the limit of detection, with average concentrations of 1.2 ng/g both in pollen and in honey.<sup>11</sup> A few studies have measured residue levels of neonicotinoids and the major metabolites in pollen and nectar collected from seed-treated crop plants. Seed treatments represent the largest single use of neonicotinoids, yet residue levels of imidacloprid ranged from 2 to 5 ng/g in pollen and are >1.5 ng/g in nectar of seed-treated corn, sunflower, and rape.<sup>9,12</sup> Clothianidin residues have also been detected in pollen and nectar samples from honey bee colonies foraging in seed-treated canola fields.<sup>13</sup> The maximum residue level was 2.6 ng/g in pollen and 2.2 ng/g in nectar, although the majority of samples had no detectable residues.

To the best of our knowledge, no information has been published on residues of neonicotinoids in pollen and nectar collected directly from crops that are treated after planting and closer to flowering. For many crops such as cucurbits, neonicotinoids can be applied through drip irrigation or as foliar applications anytime during the crop cycle, except within the days-to-harvest restriction (preharvest) intervals, which vary from 0 to 30 days depending on the product. Thus, label

**Special Issue:** Florida Pesticide Residue Workshop 2011

**Received:** January 3, 2012

**Revised:** March 21, 2012

**Accepted:** March 27, 2012

**Published:** March 27, 2012

directions allow treatments during flowering provided that bees are not active at the time of application. In the present study, we measured levels of insecticide residues in pollen and nectar collected from a pumpkin crop treated with label rates of several neonicotinoids using different timings and application methods. We hypothesize that higher residues will be present in pollen and nectar if treatments are applied closer to flowering, but will depend on the particular insecticide, the application rate used, and the time and method of application. The results of this study address the potential exposure to pollinators through pollen and nectar associated with several neonicotinoid insecticides.

## METHODS AND MATERIALS

**Experimental Design.** The study involved a field experiment and residue analysis repeated in 2009 and 2010. The field experiment was located at the Central Maryland Research and Education Center, Beltsville Facility, Beltsville, MD, USA. We used pumpkin (*Cucurbita pepo* L. var. 'Howden') as a surrogate crop system to represent the cucurbit crop grouping because the staminate flowers are relatively large and numerous, produce nectar in the easily accessible cavities at the base of the stamens (63–79  $\mu\text{L}/\text{day}/\text{flower}$ ), and produce copious pollen (47  $\mu\text{g}/\text{flower}$ ).<sup>14</sup> In both years, seedling plants were grown with untreated seed under typical greenhouse conditions without exposure to pesticides and then transplanted onto plastic mulch beds during early June. A replicate treatment plot consisted of a single row 15 m long, containing 18 plants spaced 0.9 m apart. Rows were spaced 15 m apart to avoid overlap of vines and potential contamination between treatments. Treatment plots were arranged in a randomized complete block design with four replicates. The pumpkin crop received a broadcast application of fertilizer prior to laying of the plastic mulch, and later plants were fed additional nitrogen by drip chemigation. Foliar applications of the fungicide chlorothalonil (Bravo; Helena Chemical Co.) were applied several times during the crop cycle to prevent leaf diseases. With the exception of the wide row spacing, all cultural practices were implemented according to commercial production recommendations.

**Insecticide Treatment Regimens.** As part of the 2009 study, we conducted a telephone survey of crop consultants and entomologists in major production areas and had open discussions with growers to determine how neonicotinoid insecticides are used on cucurbit crops. Survey results showed that the application methods used by growers varied widely, depending on the insect pest complex. For production areas where only early-season pests are a problem, bedding-tray drenches and transplant water treatments at planting were the most common practices. For areas with season-long insect pest pressure, foliar treatments and chemigation through drip irrigation during the crop cycle were used more often.

On the basis of survey results, the 2009 experiment included nine treatment regimens consisting of different neonicotinoid insecticide application methods (total of 36 plots) as follows: (1) bedding-tray drench of imidacloprid (Admire Pro, 55% active ingredient (ai); Bayer CropSciences, Durham, NC, USA) applied at a reduced rate of 0.005 g per plant (or 30 g ai/ha); (2) transplant water treatment of imidacloprid (Admire Pro) applied during planting (low label rate of 281 g ai/ha); (3) transplant water treatment of imidacloprid (Admire Pro) applied during planting (high label rate of 422 g ai/ha); (4) split treatments of imidacloprid (Admire Pro) applied as half rate in transplant water (211 g ai/ha) and the remaining half rate applied 3 weeks later by drip irrigation; (5) split treatments of dinotefuran (Venom, 70% ai; Valent U.S.A., Walnut Creek, CA, USA) applied as a half rate (151 g ai/ha) in transplant water and the remaining half rate applied 3 weeks later by drip irrigation; (6) two foliar treatments of dinotefuran (Venom), each 151 g ai/ha at 4 and 6 weeks after transplanting; (7) split treatments of thiamethoxam (Platinum, 21.6% ai; Syngenta Crop Protection, Greensboro, NC) applied as a half rate (96 g ai/ha) in transplant water and the remaining half rate applied 3 weeks later by drip irrigation; (8) two foliar treatments of

thiamethoxam (Actara, 25% ai; Syngenta Crop Protection), each 96 g ai/ha, oz/acre, at 4 and 6 weeks after transplanting; and (9) an untreated control. The same treatments were evaluated in 2010, except treatment 3 was deleted and three additional treatments were added (11 treatments for a total of 44 plots). We evaluated two- and three-way split applications of the systemic carbamate insecticide oxamyl (Vydate L, 24% ai; DuPont Co., Wilmington, DE, USA), each applied at the rate of 140 g ai/ha by drip irrigation. In one regimen, three weekly treatments were applied commencing 1 week after transplanting, and the other involved two treatments applied one and three weeks after transplanting. We also evaluated a new seed dressing application (FarMore Technology by Syngenta Crop Protection), which delivered 0.75 mg ai of thiamethoxam per seed (or 4.5 g ai/ha) and three fungicides (i.e., fludioxonil, mefenoxam, azoxystrobin). This treatment was direct-seeded by hand into plots 10 days prior to the establishment of the other plots. Seed dressing is a more convenient and economical control strategy, with a lower dose per hectare to minimize environmental exposure.

In both years, transplant treatments were applied by drenching 0.25 L of solution around each seedling immediately after transplanting. Foliar treatments were applied with a CO<sub>2</sub> backpack sprayer and 3 m boom with six hollow cone nozzles, delivering 188 L/ha of diluted spray at 40 psi. Drip treatments were applied using CO<sub>2</sub> pressure to first force 8 L of water through the drip line to prime emitters, followed by 8 L of the treatment solution, and then 8 L of water to flush out the line. All transplant and drip treatment rates were based on a plant population of 5977/ha, typical of a commercial field with the recommended row and plant spacing.

**Flower and Leaf Sampling.** During flowering (ca. 5 weeks after transplanting), wax-coated paper bags were placed over staminate flower buds that had not yet bloomed to prevent pollinator visits. Bags were twisted at the lower open end to secure the bag around the peduncle or stem of the flower. Bags with open flowers were removed the following day and brought to the laboratory to extract nectar and pollen. Each flower was dissected by removing the calyx and corolla to expose the single filament of anthers and receptacle base. Nectar was secreted from a layer of tissue lining the bottom cavity at the base of the filament. Nectar was collected with a 1 mL syringe by drawing liquid from this cavity. Extractions were made on multiple flowers (usually 40–50 per replicate) until 1.5 mL had been collected from each treatment plot. After nectar was extracted, pollen from the anther stalk of flowers was dislodged to collect at least 3 g per plot. At all steps in sample collection and processing, separate tools for each treatment were used, disposable gloves were changed between samples, and other quality assurance measures were deployed to minimize cross-contamination.

In both years, the flower bagging and extraction process was repeated two and sometimes three times over a period of 7–10 days to collect the required quantities for analysis. Residues detected in these cumulative quantities of nectar and pollen represented the average level present during the entire collection period. One exception was that a separate sample of nectar and pollen was extracted from flowers after each foliar treatment of dinotefuran and thiamethoxam in 2010. Leaf samples were also collected in 2010 from all plots but after the second foliar treatments were applied (ca. 7 weeks after transplanting). Fully expanded leaves (10–15) were randomly removed for each plot and subsampled to obtain a composite sample of ca. 5 g of tissue for analysis. All samples were stored in 15 mL centrifuge tubes at  $-80\text{ }^{\circ}\text{C}$  until ready for residue analysis.

**Residue Analysis. Standards and Reagents.** Analytical reference materials of imidacloprid (99.4%), imidacloprid olefin (97.9%), imidacloprid, 5-hydroxy (99.3%), imidacloprid urea (99.4%), desnitro imidacloprid olefin (97.5%), desnitro imidacloprid HCl (97.9%), 6-chloronicotinic acid (99.3%), thiamethoxam (98.9%), thiamethoxam metabolite CGA-322704 (clothianidin, 97.4%), dinotefuran (99.8%), dinotefuran UF (99.7%), and oxamyl (99.3%), as well as the internal standard <sup>13</sup>C-*d*<sub>3</sub>-imidacloprid (1  $\mu\text{g}/\mu\text{L}$  solution), were all obtained from the U.S. EPA National Pesticide Standard Repository (Ft. Meade, MD, USA). Working standard solutions

**Table 1.** Residue Levels of the Neonicotinoid Insecticides Imidacloprid, Dinotefuran, Thiamethoxam, and Their Respective Metabolites Detected in Pollen Collected from Flowers of a Pumpkin Crop Treated with Different Rates and Application Methods, 2009

insecticide	treatment regimen	parent compound (ng/g)			metabolites <sup>a</sup> (ng/g)		
		mean <sup>b</sup>	min	max	mean <sup>b</sup>	min	max
imidacloprid, LOD = 0.2 ng/g	bedding drench	4.9 c	3.3	6.7	0.7 b	0.1 <sup>c</sup>	2.7
	transplant (low)	36.7 b	30.1	40.1	11.4 a	8.3	16.6
	transplant (high)	60.9 ab	40.5	86.6	17.5 a	10.6	21.9
	transplant-drip	80.2 a	52.3	101.0	19.1 a	13.2	27.5
dinotefuran, LOD = 0.2 ng/g	transplant-drip	57.5 a	44.0	69.2	10.3 b	8.1	12.0
	two foliar	88.3 a	36.0	147.0	17.1 a	14.6	21.1
thiamethoxam, LOD = 0.2 ng/g	transplant-drip	68.0 a	54.8	90.4	21.0 a	13.8	41.2
	two foliar	95.2 a	60.7	127.0	26.8 a	9.8	35.1

<sup>a</sup>Imidacloprid metabolites included imidacloprid olefin, 5-OH imidacloprid, imidacloprid urea, imidacloprid desnitro olefin, imidacloprid desnitro HCl, and 6-chloronicotinic acid; dinotefuran metabolite is UF (1-methyl-3-(tetrahydro-3-furylmethyl)urea); thiamethoxam metabolite is clothianidin. <sup>b</sup>Means within a column followed by the same letter are not statistically significant (Tukey  $p < 0.05$ ). <sup>c</sup>Nondetected (ND) samples were scored a value of half the LOD for statistical analysis.

were prepared from stock solutions in acetonitrile (ACN). Calibration standards were prepared in each matrix by fortifying extracted control samples at the final stage, at four to five concentration levels ranging from the limit of detection (LOD) to 10 times the limit of quantitation (LOQ). Control samples of pumpkin foliage were obtained from untreated pumpkin field trials, whereas control pollen samples were obtained from the local market (Organic Bee Pollen, Dietary Supplement, Y. S. Organic Bee Farms, Sheridan, IL, USA). The laboratory prepared control nectar samples by mixing a sugar solution composed of 60% sucrose + 20% glucose + 10% fructose to mimic the sugar composition of cucurbit nectar.<sup>15</sup> The LOD was determined by obtaining a signal-to-noise (S/N) ratio of >3:1 for two monitored precursor/product ion transitions in the liquid chromatography–tandem mass spectroscopy (LC-MS/MS) process. The LOQ was estimated as 3.33 times the LOD achieving a S/N ratio of 10:1 for the quantification ion transition and at least 3:1 for the confirmation ion transition. The LOD for the investigated parent neonicotinoid insecticides imidacloprid, dinotefuran, and thiamethoxam was 0.2 ng/g (ppb) and was 7 ng/g for the carbamate insecticide oxamyl, whereas the LOD ranged from 0.2 to 3 ng/g for the metabolites.

**Sample Preparation.** Extraction and cleanup of pumpkin foliage, pollen, and nectar were performed using a refined method previously developed and published.<sup>16</sup> Briefly, homogenized samples (3 g) were extracted with 12 mL of water and 15 mL of 2% triethylamine (TEA) in ACN by shaking in a Geno/Grinder (SPEX CertiPrep Inc., Metuchen, NJ, USA) for 2 min at 1200 strokes per minute. To each sample were added 6 g of magnesium sulfate anhydrous (MgSO<sub>4</sub>) and 1.5 g of sodium acetate (NaOAc) (UCT, Bristol, PA, USA), and the samples were shaken again using the Geno/Grinder at 1200 strokes per minute for another 2 min. Samples were then centrifuged (Jouan Inc., VA, USA) for 5 min at 2500 rpm. The organic supernatant was transferred to a 15 mL tube containing 0.5 g of MgSO<sub>4</sub> to remove moisture and shaken by hand or with a vortex. An aliquot of the extract was passed through a C<sub>18</sub> solid phase extraction (SPE) cartridge (1 g, Phenomenex, Torrance, CA, USA) preconditioned with 3 mL of 2% TEA in ACN with the aid of a vacuum or positive pressure and rinsed with an additional 10 mL of 2% TEA/ACN. The combined eluants were evaporated to dryness in a water bath under a stream of N<sub>2</sub>. Samples were then reconstituted to 1 mL of water/methanol (75:25) by adding 1 mL of the internal standard solution (10 ng/mL in water/methanol (75:25)). Samples were filtered through 0.7 μm glass microfiber filter disks (GF/F, Whatman, Maidstone, U.K.) followed by 0.2 μm nylon filter discs (Pall Life Sciences, Ann Arbor, MI, USA) into liquid chromatography (LC) vials.

**Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS) Instrument.** The LC-MS/MS instrument consisted of a Waters Acquity ultraperformance liquid chromatograph (UPLC) equipped with a 10 cm × 2.1 mm (i.d.), 1.8 μm particle size, Acquity HSS T3 column (Waters, Milford, MA, USA) coupled to a Waters Xevo TQ triple-quadrupole mass spectrometer operated in the positive electrospray ionization mode. The LC was operated under gradient conditions with mobile phases of water/methanol (95:5) + 5 mM ammonium formate + 0.1% formic acid (A) and water/methanol (5:95) + 5 mM ammonium formate + 0.1% formic acid (B) at a flow rate of 0.35 mL/min and 40 °C. The initial mobile phase composition was 95% A, which was held for 3 min, followed by a linear gradient to 40% B in 12 min and then to 95% B in 1 min, and was held for 1 min to rinse the column. The analytical column was then equilibrated at the initial conditions for 2 min for a total run time of 19 min. The injection volume was 5 μL. The MS source temperature was set at 120 °C with nitrogen flow rates of 50 and 1000 L/h for the cone and desolvation gases, respectively. The desolvation temperature was 450 °C. Argon was used as the collision gas with a flow of 0.15 mL/min, which produced a pressure of 4 × 10<sup>-3</sup> mbar in the collision cell. The mass spectrometer was operated in multiple reaction monitoring mode (MRM) with monitoring of two precursor/products ion transitions for each analyte. The target ion transition with highest intensity (primary ion transition) was used for quantitation, whereas the second target ion transition was used for confirmation. Further confirmation was obtained through the ratio between the two ion transitions for each analyte, which was within 20% of the ratio between the same ion transitions in standards. The instrument uses Target Lynx software version 4.1 (Waters Corp.) for quantitation and confirmation calculations. Ion transitions, cone voltages, and collision energies for the analytes are the same as those published earlier.<sup>16</sup>

**Sample Analysis and Quality Assurance.** A typical analytical set run on the LC-MS/MS included four to five calibration standards in matrix, a reagent (procedural) blank sample, a control sample, a matrix spike sample fortified at 2 × LOQ with all analytes, followed by 10 samples, then a calibration check standard injection. This sequence was repeated for more samples, and the sample set ended with at least one calibration check standard injection. Quality assurance/quality control (QA/QC) criteria were met for the majority (>95%) of the samples including a relative percent difference (RPD) not to exceed 20% for calibration checks and for recoveries for the matrix spike to fall within the recoveries achieved during method validation.<sup>16</sup> Analyte concentration was calculated using the internal standard method, by dividing the analyte peak area by the internal standard (IS) area (<sup>13</sup>C-*d*<sub>3</sub>-imidacloprid) and multiplying by the IS concentration (10 ng/mL).

**Statistical Analysis.** Residue data for the parent and metabolite compounds were averaged by year to calculate means and ranges for

**Table 2. Residue Levels of the Neonicotinoid Insecticides Imidacloprid, Dinotefuran, Thiamethoxam, and Their Respective Metabolites Detected in Nectar Collected from Flowers of a Pumpkin Crop Treated with Different Rates and Application Methods, 2009**

insecticide	treatment regimen	parent compound (ng/g)			metabolites <sup>a</sup> (ng/g)		
		mean <sup>b</sup>	min	max	mean <sup>b</sup>	min	max
imidacloprid, LOD = 0.2 ng/g	bedding drench	0.4 c	0.3	0.5	0.1 c	0.1 <sup>c</sup>	0.2
	transplant (low)	5.7 b	3.8	7.3	1.8 bc	0.1	4.0
	transplant (high)	7.4 ab	4.7	11.9	3.4 ab	0.2	5.9
	transplant-drip	11.2 a	9.0	13.7	6.4 a	5.0	9.4
dinotefuran, LOD = 0.2 ng/g	transplant-drip	9.2 a	7.1	10.6	4.1 a	3.5	4.8
	two foliar	7.5 a	5.3	10.8	6.5 a	1.8	10.8
thiamethoxam, LOD = 0.2 ng/g	transplant-drip	9.5 a	7.8	12.2	4.0 a	2.4	6.4
	two foliar	8.2 a	6.7	9.1	1.9 ab	0.7	3.3

<sup>a</sup>Imidacloprid metabolites included imidacloprid olefin, 5-OH imidacloprid, imidacloprid urea, imidacloprid desnitro olefin, imidacloprid desnitro HCl, and 6-chloronicotinic acid; dinotefuran metabolite is UF (1-methyl-3-(tetrahydro-3-furylmethyl)urea); thiamethoxam metabolite is clothianidin. <sup>b</sup>Means within a column followed by the same letter are not statistically significant (Tukey  $p < 0.05$ ). <sup>c</sup>Nondetected (ND) samples were scored a value of half the LOD for statistical analysis.

each treatment regimen (Tables 1–5). Nondetected (ND) samples were scored a value of half the LOD, whereas samples with detected traces between LOD and LOQ were scored 0.2 ng/g (LOD) for the parent neonicotinoids and the LOD value of each metabolite. Differences between treatment regimens were determined by the mixed model procedure of ANOVA, and Tukey's procedure tested multiple mean comparisons at a significance level of  $p < 0.05$ . We did not include control data in the analyses because the primary aim was to test for differences among treatment regimens. Correlations between matrices and data by year were evaluated with Pearson's coefficient ( $r$ ). All statistical analyses were performed using SAS (version 9.1.3) software.

## RESULTS AND DISCUSSION

The parent neonicotinoids and several metabolites were identified and quantified in 92.1, 88.2, and >98% of the pollen, nectar, and leaf samples from the treated plots, respectively. We also detected low levels of residues exceeding the LOD in 38% of the pollen and nectar samples from untreated control plots, of which the majority were thiamethoxam and clothianidin. These background levels could increase the total residue amounts but to a minor extent and only the thiamethoxam treatments. Means and ranges of parent and metabolite residues in pollen and nectar for each treatment regimen by year are presented in Tables 1–4. Nectar residues were consistently lower than pollen residues by 73.5–88.8%, depending on the neonicotinoid and treatment regimen. Overall residue levels were significantly higher in 2009 than those levels in 2010 on the basis of pooled data from treatment regimens included in both experiments. Differences in mean residue levels of parent compounds among treatment regimens between years were highly correlated ( $r = 0.94$ ,  $p < 0.001$ ), so relative residue amounts were consistent, even though overall levels of imidacloprid, dinotefuran, and thiamethoxam in 2010 were 53, 62, and 64% less than levels in 2009, respectively. Although insecticide rates and application methods were the same, lower residue levels in 2010 were likely due to extreme environmental conditions. In 2010, pumpkin plants were heat and moisture stressed during most of the period from transplanting through flowering, and drip irrigation had to be applied several times each week to maintain plant growth and flower production. Maximum daily temperatures were significantly higher in 2010, with 43 days over 32 °C during June, July, and August

compared to 11 days for the same period in 2009. Rainfall amounts were suboptimal during both years, but the higher temperatures in 2010 increased plant transpiration, thus requiring more frequent irrigation. It is possible that the increased need for drip irrigation, which was soil-applied beneath the plastic mulch, may have resulted in enhanced leaching (dissipation) of the transplant and chemigation treatments, given that the neonicotinoid insecticides are comparatively water-soluble.<sup>17,18</sup> Plant stress also is known to reduce leaf absorption of foliar-applied systemic insecticides due to induced epicuticular wax accumulation and rapid desiccation of insecticide spray droplets,<sup>19</sup> and to increase the dissipation rate due to higher photodegradation and evaporation.<sup>20,21</sup>

Results showed that pollen and nectar residues were significantly different among imidacloprid treatment regimens within each year for the parent compound and combined metabolites. In both years, the bedding tray drench resulted in the lowest residue levels of imidacloprid, ranging from 0.1 to 6.7 ng/g in pollen (Tables 1 and 3) and from ND to 0.5 ng/g in nectar (Tables 2 and 4). This treatment was applied to trays of seedling plants prior to transplanting at a rate 6 times higher than the label rate for planthouse application but 21 times less than the low label rate for field application. Many cucurbit growers treat transplants using this low-cost, prophylactic approach, particularly in eastern U.S. production areas where protection is needed for early-season pests only.

Applications of the low label rate of imidacloprid in transplant water resulted in significantly higher ( $p < 0.05$ ) residue amounts in pollen, ranging from 13.2 to 40.1 ng/g, than in nectar, ranging from 3.8 to 7.3 ng/g. Although not statistically different, residues resulting from the high label rate in transplant water were 30–66% higher than those at the low rate of imidacloprid. These prophylactic treatments are widely used in cucurbit crops grown from transplants, and the high label rate is often required for season-long control of insect pests. The highest residues of imidacloprid were found in samples from plots receiving the high label rate but as split applications in transplant water followed 3 weeks later by drip chemigation. Residue levels ranged from 23.9 to 101 ng/g in pollen and from 6.7 to 16 ng/g in nectar but were not significantly different from the transplant water treatments

**Table 3. Residue Levels of the Neonicotinoid Insecticides Imidacloprid, Dinotefuran, and Thiamethoxam and of the Carbamate Insecticide Oxmyl with Their Respective Metabolites Detected in Pollen Collected from Flowers of a Pumpkin Crop Treated with Different Rates and Application Methods, 2010**

insecticide	treatment regimen	parent compound (ng/g)			metabolites <sup>a</sup> (ng/g)		
		mean <sup>b</sup>	min	max	mean <sup>b</sup>	min	max
imidacloprid, LOD = 0.2 ng/g	bedding drench	0.1 <sup>c</sup>	0.1	0.1	0.1 d	0.1	0.1
	transplant (low)	18.2 ab	13.2	23.9	0.1 d	0.1	0.1
	transplant-drip	31.8 a	23.9	44.0	0.1 d	0.1	0.1
dinotefuran, LOD = 0.2 ng/g	transplant-drip	15.2 ab	11.6	19.3	5.7 abc	4.0	7.6
	one foliar	11.2 b	8.0	13.5	7.9 ab	7.0	8.6
	two foliar	34.7 ab	7.6	79.5	0.1 a	7.5	34.7
thiamethoxam, LOD = 0.2 ng/g	seed treatment	0.1 c	0.1	0.1	0.1 d	0.1	0.1
	transplant-drip	24.8 ab	17.3	33.2	3.0 cd	0.1	8.8
	one foliar	15.3 ab	13.9	16.8	2.3 bcd	2.2	2.3
	two foliar	25.2 ab	18.1	29.6	3.7 bcd	0.1	8.2
oxamyl, LOD = 7 ng/g	two drip	3.5 c	3.5	3.5	NA <sup>d</sup>	NA	NA
	three drip	3.5 c	3.5	3.5	NA	NA	NA

<sup>a</sup>Imidacloprid metabolites included imidacloprid olefin, 5-OH imidacloprid, imidacloprid urea, imidacloprid desnitro olefin, imidacloprid desnitro HCl, and 6-chloronicotinic acid; dinotefuran metabolite is UF (1-methyl-3-(tetrahydro-3-furylmethyl)urea); thiamethoxam metabolite is clothianidin. <sup>b</sup>Means within a column followed by the same letter are not statistically significant (Tukey  $p < 0.05$ ). <sup>c</sup>Nondetected (ND) samples were scored a value of half the LOD for statistical analysis. <sup>d</sup>NA, not analyzed.

applied alone at the same seasonal rate. Split applications are a common treatment strategy in western U.S. production areas where systemic protection against late-season sucking insects is required. However, drip treatments applied later in the crop cycle when plants were beginning to flower increased levels of pollen and nectar residues.

Residue analysis identified five metabolites of imidacloprid in the 2009 pollen and nectar samples, representing 26.3% of the total residue amounts (Tables 1 and 3). The olefin and hydroxy metabolites were most predominant, accounting for 35.6 and 52.3%, respectively, of the total metabolite residues. These metabolites have roughly similar toxicity to honey bee adults on an acute exposure basis as imidacloprid. The urea and desnitro metabolites were present in trace amounts and are practically nontoxic to bees on an acute exposure basis. The chloronicotinic acid metabolite (LOD = 3 ng/g) was not detected in any of the samples, and no metabolite residues were found in the 2010 samples. Differences in metabolite residues in pollen and nectar were proportional to those of the parent compound in 2009 but at much lower amounts.

Samples from plots receiving foliar treatments of dinotefuran contained residues of the parent compound and UF metabolite (1-methyl-3-(tetrahydro-3-furylmethyl)urea) at higher levels than levels in the transplant-drip regimen; however, residues levels from the two methods of application were not statistically different. The UF metabolite accounted for 21.3–27.2% of the total residual amounts. Pollen residues in 2009 ranged from 44 to 69.2 ng/g of dinotefuran and from 8.1 to 12 ng/g of UF for the transplant-drip regimen and from 36 to 147 ng/g of dinotefuran and from 14.6 to 21.1 ng/g of UF for foliar treatments (Tables 1). Amounts of dinotefuran and UF in nectar ranged from 7.1 to 10.9 ng/g and from 3.5 to 4.8 ng/g for the transplant-drip regimen and from 5.3 to 10.8 ng/g and from 1.8 to 10.8 ng/g for foliar treatments, respectively (Table 2). Residues of dinotefuran in 2010 were 28.8 and 65.8% lower for nectar and pollen samples (Tables 3 and 4), but relative differences were consistent with the 2009 results. In both years,

foliar treatments of dinotefuran were applied during the flowering period and thus resulted in the highest residues of the parent and metabolite in both floral matrices. To determine the residues present after each application, samples were collected during the week following each foliar treatment in the 2010 study. Results showed that the second foliar treatment applied at 6 weeks after transplanting resulted in about 3 times more residue of dinotefuran than the first foliar treatment applied 2 weeks earlier, that is, at 4 weeks after transplant. Due to variability in the residue data, differences between the two foliar treatments were not statistically significant. However, it is clear that the second foliar treatment timing resulted in a greater likelihood of exposure to pollinators because the application occurred during peak flower production at 6 weeks after transplanting.

Residue analysis detected thiamethoxam and its major metabolite clothianidin in all samples collected in 2009. Clothianidin represented 15.5 and 20.4% of the total residue amounts in nectar and pollen samples, respectively, in 2010. In 2010, due to overall lower levels of residues found, most pollen samples contained thiamethoxam and clothianidin, but only the parent compound was detected in nectar. Because both parent and metabolite residues are potentially toxic to bees, we combined the 2009 data to summarize ranges of residues that would represent the worst-case levels for each treatment regimen. Pollen residues ranged from 68.6 to 131.6 ng/g for the transplant-drip regimen and from 70.5 to 162.1 ng/g for foliar treatments (Table 1). Amounts of thiamethoxam and clothianidin in nectar ranged from 10.2 to 18.6 ng/g for the transplant-drip regimen and from 7.4 to 12.4 ng/g for foliar treatments (Table 2). Similar to dinotefuran, total residue levels of thiamethoxam and clothianidin in pollen were highest in the foliar-treated plots, but residues were not significantly different from the transplant-drip treatments. By comparison, the opposite was true for nectar residues, which were consistently higher in the transplant-drip treatments. It is possible that the translocation of insecticide residues to nectar and pollen may

**Table 4. Residue Levels of the Neonicotinoid Insecticides Imidacloprid, Dinotefuran, and Thiamethoxam and of the Carbamate Insecticide Oxamyl with Their Respective Metabolites Detected in Nectar Collected from Flowers of a Pumpkin Crop Treated with Different Rates and Application Methods, 2010**

insecticide	treatment regimen	parent compound (ng/g)			metabolites <sup>a</sup> (ng/g)		
		mean <sup>b</sup>	min	max	mean <sup>b</sup>	min	max
imidacloprid LOD = 0.2 ng/g	bedding drench	0.1 <sup>c</sup>	0.1	0.1	0.1	0.1	0.1
	transplant (low)	6.1 ab	4.8	6.7	0.1	0.1	0.1
	transplant-drip	9.1 a	6.7	16.0	0.1	0.1	0.1
dinotefuran, LOD = 0.2 ng/g	transplant-drip	4.8 abc	0.1	10.9	0.1	0.1	0.1
	one foliar	2.1 bc	0.1	5.0	0.1	0.1	0.1
	two foliar	7.0 ab	0.1	16.0	0.1	0.1	0.1
thiamethoxam, LOD = 0.2 ng/g	seed treatment	0.1 c	0.1	0.1	0.1	0.1	0.1
	transplant-drip	10.7 a	9.0	15.1	0.1	0.1	0.1
	one foliar	1.6 bc	0.1	2.5	0.1	0.1	0.1
	two foliar	4.3 ab	3.0	7.0	0.1	0.1	0.1
oxamyl, LOD = 7 ng/g	two drip	3.5 c	3.5	3.5	NA <sup>d</sup>	NA	NA
	three drip	3.5 c	3.5	3.5	NA	NA	NA

<sup>a</sup>Imidacloprid metabolites included imidacloprid olefin, 5-OH imidacloprid, imidacloprid urea, imidacloprid desnitro olefin, imidacloprid desnitro HCl, and 6-chloronicotinic acid; dinotefuran metabolite is UF (1-methyl-3-(tetrahydro-3-furylmethyl)urea); thiamethoxam metabolite is clothianidin. <sup>b</sup>Means within a column followed by the same letter are not statistically significant (Tukey  $p < 0.05$ ). <sup>c</sup>Nondetected (ND) samples were scored a value of half the LOD for statistical analysis. <sup>d</sup>NA, not analyzed.

**Table 5. Residue Levels of the Neonicotinoid Insecticides Imidacloprid, Dinotefuran, and Thiamethoxam and of the Carbamate Insecticide Oxamyl with Their Respective Metabolites Detected in Leaf Tissue of a Pumpkin Crop Treated with Different Rates and Application Methods, 2010**

insecticide	treatment regimen	parent compound (ng/g)			metabolites <sup>a</sup> (ng/g)		
		mean <sup>b</sup>	min	max	mean <sup>b</sup>	min	max
imidacloprid, LOD = 0.2 ng/g	bedding drench	5.0 cd	4.0	6.0	8.0 bc	5.0	10.5
	transplant (low)	22.7 cd	18.3	31.0	39.1 ab	18.0	77.5
	transplant-drip	39.5 bc	22.0	53.0	34.4 ab	27.5	40.5
dinotefuran, LOD = 0.2 ng/g	transplant-drip	41.2 bc	25.0	65.6	22.3 abc	7.4	37.2
	two foliar	102.2 ab	74.1	142.7	72.4 a	63.4	90.7
thiamethoxam, LOD = 0.2 ng/g	seed treatment	6.5 cd	4.0	12.0	0.1 <sup>c</sup>	0.1	0.1
	transplant-drip	174.8 a	87.8	279.6	9.5 bc	5.0	19.4
	two foliar	142.2 a	80.9	173.5	70.3 ab	14.0	219.2
oxamyl, LOD = 7 ng/g	two drip	33.6 cd	4.8	70.2	NA <sup>d</sup>	NA	NA
	three drip	12.9 cd	3.5	24.7	NA	NA	NA

<sup>a</sup>Imidacloprid metabolites included imidacloprid olefin, 5-OH imidacloprid, imidacloprid urea, imidacloprid desnitro olefin, imidacloprid desnitro HCl, and 6-chloronicotinic acid; dinotefuran metabolite is UF (1-methyl-3-(tetrahydro-3-furylmethyl)urea); thiamethoxam metabolite is clothianidin. <sup>b</sup>Means within a column followed by the same letter are not statistically significant (Tukey  $p < 0.05$ ). <sup>c</sup>Nondetected (ND) samples were scored a value of half the LOD for statistical analysis. <sup>d</sup>NA, not analyzed.

be different depending on whether the insecticide enters the plant through leaves or roots. Residues of thiamethoxam were much lower in the 2010 study (Tables 3 and 4) but the additional treatment regimens provided information on the relative exposure risks associated with the different ways that this insecticide is used in cucurbit crops. The FarMore seed treatment is a relatively new crop protection technology that resulted in the lowest exposure of residues because the low dose of thiamethoxam per seed (21 times less than the foliar application) is taken up by the plant 5–6 weeks prior to flowering. None of the samples collected from the seed-treated plots contained detectable levels of thiamethoxam (LOD = 0.2 ng/g) or clothianidin (LOD = 0.6 ng/g). Results showed that the second foliar treatment applied at 6 weeks after

transplanting resulted in about 1.6–2.7 times more residue of thiamethoxam and clothianidin than the first foliar treatment applied 2 weeks earlier, that is, at 4 weeks after transplanting.

In addition to neonicotinoid use on cucurbit crops, the survey conducted in 2009 found that the carbamate insecticide oxamyl was widely used on cucurbit crops, particularly in the mid-Atlantic area, as a postplant treatment for insect and nematode pests, and is applied through the drip irrigation lines after planting. This systemic carbamate insecticide/nematocide is moderately to highly toxic to honey bee adults on an acute exposure basis. For this reason, we included the two- and three-way split applications of oxamyl in the 2010 study. No residues of oxamyl (LOD = 7 ng/g) were detected in any pollen or nectar samples collected from treated plots.

Leaf analysis was conducted in 2010 to determine if residue levels of neonicotinoids and their metabolites in leaf tissue correlated with residue amounts in pollen and nectar. Samples were collected at 7 weeks after transplanting, after all treatments were applied. Imidacloprid residues ranged up to 53 ng/g and closely corresponded directly with residue levels in pollen ( $r = 0.94$ ,  $p < 0.001$ ) and nectar ( $r = 0.88$ ,  $p < 0.001$ ) (Table 5). However, metabolites of imidacloprid constituted a significantly higher proportion (54.8%) of the total residue amounts in leaf tissue and showed no significant correlation ( $p = 0.58$ ) with metabolite levels in pollen. Residues of 5-OH, desnitro olefin, and desnitro HCl accounted for 8.6, 6.7, and 84.8%, respectively, of the total breakdown products. Leaf residues of dinotefuran and thiamethoxam were significantly higher by 2.7–16.3 times and poorly correlated with amounts in pollen and nectar. The UF and clothianidin metabolites added up to 66 and 76.9% of the total residuals of dinotefuran and thiamethoxam, respectively. Both neonicotinoids have short half-lives, are more soluble than imidacloprid, and appear to translocate more rapidly and more completely throughout the foliage of plants. These properties together probably account for the higher residue levels and greater proportion of metabolites. Leaves from the thiamethoxam seed treatment and oxamyl plots contained the respective parent compounds at relatively low levels but no metabolites. These low levels were consistent with the absence of detectable residues in pollen and nectar. In general, mean levels of leaf residues for each insecticide may be used to provide conservative estimates of the relative levels of pollen and nectar residues but not the absolute amounts.

Some general comments can be made about the overall results. First, the study repeated over two years showed that environmental stresses (heat and moisture) can have a significant effect on the overall residue levels of neonicotinoids in pumpkin flowers. Studies to assess residue levels should be replicated at different locations or over multiple years to determine the range of residues under different environmental conditions. Second, pumpkin, with its large staminate flowers, was used as a surrogate to represent the cucurbit crop grouping, because of the ease in collecting pollen and nectar. However, other cucurbits such as cucumber and melons with higher plant densities, less plant biomass, and smaller and fewer flowers per plant may have different residue profiles. Planting on bare ground may affect the rate of insecticide dissipation in a different way from plastic mulch, which can reduce runoff and minimize percolation of foliar- and soil-applied pesticides.<sup>22</sup> Neonicotinoid metabolites accounted for 15.5–27.2% of the total residue amounts found in pollen and nectar. Breakdown products, such as olefin and hydroxy metabolites of imidacloprid and clothianidin, have acute toxicity to some pollinators similar to that of the parent compound and thus should be considered in an exposure assessment. Nectar residues were consistently 73.5–88.8% lower than pollen residues, suggesting that pollen alone may be used to estimate exposure risk to pollinators and thus eliminating the difficult task of collecting nectar.

We presented here evidence to support our hypothesis that higher residues are present in pollen and nectar if systemic neonicotinoids are applied closer to flowering. Foliar treatments and chemigation through drip irrigation applied during flowering resulted in the highest residues of parent insecticide and metabolites. The three neonicotinoids in the study showed similar residue profiles in pollen and nectar with regard to the

split transplant-drip treatment regimen. The lowest levels of residues were detected in treatment regimens involving applications of insecticides at planting, as either seed dressing, bedding tray drench, or transplant water treatment. The uptake and translocation of residues from these at-planting applications clearly decreased as the growing season progressed and the crop approached flowering. If neonicotinoids are needed for insect control on cucurbits, they should be applied at planting or shortly after to mitigate the exposure to pollinators.

The results of this study showed that potential exposure of neonicotinoid residues in pollen and nectar to pollinators depends on the method and timing of application relative to flowering. Residue levels in pollen and nectar of a treated pumpkin crop were significantly higher than residue amounts published in the open literature from studies of seed-treated agronomic crops.<sup>11</sup> According to this study, residues of neonicotinoids plus metabolites in pumpkin treated with label rates may reach average levels up to 122 ng/g in pollen and 17.6 ng/g in nectar. This worst-case exposure scenario is not acutely lethal to honey bees based on acute oral LD<sub>50</sub> values for neonicotinoids, which vary widely depending on the study protocol and source of bees.<sup>11</sup> Many laboratory and microcolony studies have reported sublethal effects of neonicotinoids on foraging behavior, cognitive abilities of individual honey bees, and brood development, although few effects have been reproduced in field studies using queenright colonies.<sup>11</sup> Chronic exposure of sublethal doses could be short-lived or extended depending upon the period of flowering of the crop and the sequence of treated crops pollinated by bees. Moreover, the actual dose of neonicotinoids exposed to a honey bee colony may be much lower than the residue levels in stored and processed pollen and nectar in the hive. Honey bees forage on a wide range of floral food sources and will visit different kinds of plants during the same trip. Returning bees can be carrying mixed loads of pollen from a treated crop together with uncontaminated pollen, which would dilute the residue levels entering the hive. Furthermore, stored pollen and nectar are subject to multiple steps in food processing, storage, and exposure conditions<sup>23</sup> that can result in rapid neonicotinoid degradation. Further studies are needed to determine the fate of these neonicotinoids and their actual exposure dose in bee broods, workers, and the queen.

## ■ AUTHOR INFORMATION

### Corresponding Author

\*Phone: +1 (410) 305-2925. Fax: +1 (410) 305-3091. E-mail: kamel.alaa@epa.gov.

### Funding

The field trial part of this work was funded by the Strategic Agricultural Initiative Grants Program, EPA Region III (Project X8-97376101) and the Eastern Apicultural Society Foundation for Honey Bee Research. The views expressed in this paper are those of the authors and do not necessarily represent the opinion of the U.S. EPA. No official agency endorsement should be inferred.

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

We are grateful to Terry Patton for treatment applications, Kevin Conover for preparing farm land for experiments,

Elizabeth Kolbe for laboratory quality assurance, and the many undergraduate students who bagged and processed flowers.

## ■ REFERENCES

- (1) vanEngelsdorp, D.; Evans, J.; Saegerman, C.; Mullin, C.; Haubruge, E. Colony collapse disorder: a descriptive study. *PLoS ONE* **2009**, *4*, 1–17, e6481.
- (2) Kegley, S.; Hill, B.; Orme S.; Choi, A. PAN Pesticide Database, Pesticide Action Network, North America (San Francisco, CA, 2010); <http://www.pesticideinfo.org>.
- (3) U.S. EPA. Technical Overview of Ecological Risk Assessment – Analysis Phase: Ecological Effects Characterization; [http://www.epa.gov/oppefed1/ecorisk\\_ders/toera\\_analysis\\_eco.htm](http://www.epa.gov/oppefed1/ecorisk_ders/toera_analysis_eco.htm).
- (4) Iwasa, T.; Motoyama, N.; Ambrose, J. T.; Roe, M. R. Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. *Crop Prot.* **2004**, *23*, 371–378.
- (5) Decourtye, A.; Devillers, J. Ecotoxicity of neonicotinoid insecticides to bees. In *Insect Nicotinic Acetylcholine Receptors*, 1st ed.; Thany, S. H., Ed. Springer: New York, 2010; pp 85–95.
- (6) Rouchaud, J.; Gustin, F.; Wauters, A. Soil biodegradation and leaf transfer of insecticide imidacloprid applied in seed dressing in sugar beet crops. *Bull. Environ. Contam. Toxicol.* **1994**, *53*, 344–350.
- (7) Westwood, F.; Bean, K. M.; Dewar, A. M.; Bromilow, R. H.; Chamberlain, K. Movement and persistence of [<sup>14</sup>C] imidacloprid in sugar-beet plants following application to pelleted sugar-beet seed. *Pestic. Sci.* **1998**, *52*, 97–103.
- (8) Alsayed, H.; Pascal-Lorber, S.; Nallanthigal, C.; Debrauwer, L.; Laurent, F. Transfer of the insecticide [<sup>14</sup>C] imidacloprid from soil to tomato plants. *Environ. Chem. Lett.* **2008**, *6*, 229–234.
- (9) Maus, C.; Cure, G.; Schmuck, R. Safety of imidacloprid seed dressings to honey bees: a comprehensive overview and compilation of the current state of knowledge. *Bull. Insectol.* **2003**, *56*, 51–57.
- (10) Schmuck, R.; Schoning, R.; Stork, A.; Schramel, O. Risk posed to honeybees (*Apis mellifera* L. Hymenoptera) by an imidacloprid seed dressing of sunflowers. *Pest Manag. Sci.* **2001**, *57*, 225–238.
- (11) Blacquièrre T.; Smagghe G.; van Gestel C. A.; Mommaerts V. Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. *Ecotoxicology* **2012**, DOI: 10.1007/s10646-012-0863-x.
- (12) Bonmatin, J.; Marchand, P.; Charvet, P.; Moineua, I.; Bengsch, E.; Colin, M. Quantification of imidacloprid uptake in maize crops. *J. Agric. Food Chem.* **2005**, *53*, 5336–5341.
- (13) Cutler, G.; Scott-Dupree, C. Exposure to clothianidin seed-treated canola has no long-term impact on honey bees. *J. Econ. Entomol.* **2007**, *100*, 765–772.
- (14) Vidal, M.; Jong, D.; Wien, H.; Morse, R. Nectar and pollen production in pumpkin (*Cucurbita pepo* L.). *Rev. Brasil Bot.* **2006**, *29*, 267–273.
- (15) Ashworth, L.; Galetto, L. Differential nectar production between male and female flowers in a wild cucurbit: *Cucurbita maxima* ssp. *andreana* (Cucurbitaceae). *Can. J. Bot.* **2002**, *80*, 1203–1208.
- (16) Kamel, A. Refined methodology for the determination of neonicotinoid pesticides and their metabolites in honey bees and bee products by liquid chromatography-tandem mass spectrometry (LC-MS/MS). *J. Agric. Food Chem.* **2010**, *58*, 5926–5931.
- (17) Felsot, A.; Cone, W.; Yu, J.; Ruppert, J. Distribution of imidacloprid in soil following subsurface drip chemigation. *Bull. Environ. Contamin. Toxicol.* **1998**, *60*, 363–370.
- (18) Felsot, A.; Evans, R.; Tallman, G. Soil distribution and plant uptake of imidacloprid under drip and furrow irrigation. In *Proceedings of the 4th Decennial National Irrigation Symposium*; Phoenix, A. Z., Evans, R. G., Benham, B. L., Trooien, T. P., Eds.; American Society of Agricultural Engineers: St. Joseph, MI, 2000; pp 416–427.
- (19) Baker, E. A.; Hunt, G. M. Developmental changes in leaf epicuticular waxes in relation to foliar penetration. *New Phytol.* **1981**, *88*, 731–747.
- (20) Moza, P.; Hustert, K.; Feicht, E.; Kettrup, A. Photolysis of imidacloprid in aqueous solution. *Chemosphere* **1998**, *36*, 497–502.
- (21) Wamhoff, H.; Schneider, V. Photodegradation of imidacloprid. *J. Agric. Food Chem.* **1999**, *47*, 1730–1734.
- (22) Leib, B. G.; Jarrett, A. R.; Orzolek, M. D.; Mumma, R. O. Drip chemigation of imidacloprid under plastic mulch increased yield and decreased leaching caused by rainfall. *Trans. Am. Soc. Agric. Biol. Eng.* **2000**, *43*, 615–622.
- (23) Gilliam, M.; Prest, D. B.; Lorenz, B. J. Microbiology of pollen and bee bread: taxonomy and enzymology of molds. *Apidologie* **1989**, *20*, 53–68.