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INFLUENCES OF A NEONICOTINOID INSECTICIDE ON CANTALOUPE PLANTS (CUCUMIS MELO L.) AND BEMISIA TABACI

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Abstract

The cantaloupe (*Cucumis melo* L.) and whitefly (*Bemisia tabaci)* were used as a system to explore quantitative biological and biochemical methods involving pesticide/plant/pest interactions with the neonicotinoid insecticide, imidacloprid. Toxicity to whiteflies was characterized using plants treated through hydroponic, systemic uptake of imidacloprid versus residual via residues on the surfaces of leaves that had been dipped in insecticide. LC_{50} estimates of toxicity were not statistically different for systemic versus residual routes of exposure to whiteflies. However, residual exposure to imidacloprid was significantly more toxic to whiteflies at concentrations of 10 ppm or greater, resulting in >60% mortality. Influences of residues on plant physiology were noted by observations of differential opening status of cantaloupe stomates in the treatments evaluated. Stomatal opening on cantaloupe plants was significantly altered when imidacloprid was applied systemically with 2 ppm treatments, but not when applied as residual 30 ppm treatments. Systemically treated plants had a greater proportion of closed stomates. Measurement of residues of hydrolyzed imidocloprid on leaf surfaces with Gas Chromatography/Mass Spectroscopy demonstrated benefits of reduced chances of worker exposure to residues when leaves are treated systemically. Hydrolyzed active ingredient was not detected on the surface of leaves treated with systemic or residual treatments of 2.0 ppm imidacloprid but were detected on 30 ppm residual treatments. These findings are discussed in the context of previously published observations of the impact of imidacloprid on whitefly landing and movement behavior.

Introduction

Pest managers in the Southwestern United States have aggressively pursued alternatives to conventional chemical control of *Bemisia argentifolii*, including attempts to incorporate parasitoids into pest control strategies (Hu *et al.*, 2003), tactical use of crop rotation, consideration of whitefly migration within and between crops (Byrne *et al.*, 1996), improved sampling and decision-making (Naranjo *et al.*, 1997), conservation of natural enemies (Hagler & Naranjo, 1994; Gould *et al.*, 1992), fungal pathogens, and insect growth regulators (Dennehy & Williams, 1997). Yet, conventional chemicals continue to be essential elements of whitefly management in the low deserts of Arizona and California, where this pest can cause extensive damage to cotton, vegetable, and melon crops (Byrne & Bellows, 1991; Perring *et al.*, 1993; Li *et al.*, 2003). Characteristics of host plants have proven to be important elements in understanding whitefly biology, with some host plants being associated with elevated development and survival (Naranjo *et al.*, 2004) slightly higher levels of susceptibility to pesticides (Riley & Tan, 2003) and host plant pubescence influencing whitefly instars and hormonal timing (Gelman & Gerling, 2003).

The neonicotinoid insecticide imidacloprid has served a critical role in whitefly control since 1993 (Palumbo *et al.*, 1996). Two formulations of this insecticide are commonly used in Arizona; Admire® for systemic soil treatments, and Provado® for dilute foliar sprays. In Arizona, Admire® was used first in winter vegetables and fall melons, where it has been shown to effectively decrease whitefly populations on a regional basis (Palumbo, 1999, Dennehy & Williams, 1997). Studies have revealed that imidacloprid is highly effective against the whitefly in all stages of growth, and its toxicity surpasses that of many organophosphates and pyrethoids in whitefly control (Stansly *et al.*, 1998).

Given a choice situation, *Bemisia argentifolii* has been shown in the laboratory to land less often on cantaloupe leaves with residual treatment of 30 ppm or systemic treatments of 2 ppm imidacloprid (Marklund *et al.*, 2003). Thus, the systemic and foliar treatments of imidacloprid used in agriculture (e.g. Palumbo *et al*., 1996) may result in repellency of whiteflies from treated plants. In contrast, lower levels of systemic or foliar residues of imidacloprid appeared to have no repellency (Marklund *et al*,. 2003). This reflects the concentration-dependency of repellency. Thus, whiteflies must make landing decisions prior to coming into contact with treated plants, and we hypothesize that imidacloprid treatments modified stimuli emanating from the host plants.

Repellancy of imidacloprid to whiteflies is of interest because it indicates that the protection afforded treated plants includes the combined action of physiological toxicity (Palumbo, 1999; Li *et al*., 2000) plus behavioral effects (e.g., Isaacs *et al*., 1999). However, to our knowledge, there is no evidence that repellency of imidacloprid against whiteflies comprises a major role in the mode of action of this insecticide in field settings. However, repellency can have multiple confounding implications for laboratory-based bioassays of pest susceptibility and must be taken into considerations for optimal design of resistance monitoring methods.

The cantaloupe (*Cucumis melo* L.) and whitefly (*Bemisia tabaci)* system was used to explore quantitative biological and biochemical methods involving pesticide/plant/pest interactions with the neonicotinoid insecticide, imidacloprid. Toxicity to whiteflies was characterized using plants treated through hydroponic, systemic uptake of imidacloprid versus residual uptake via residues on the surfaces of leaves that had been dipped in insecticide. Impacts of imidacloprid on plant physiology were identified by observing differences between treatments in the opening status of cantaloupe stomates. Differences in imidacloprid residues on leaf surfaces were measured using gas chromatography/mass spectroscopy. Lastly, findings were related to earlier published work on the impact of imidacloprid residues on whitefly landing and movement behavior on cantaloupe leaves.

Materials and Methods

Whitefly Cultures. Experiments were conducted from 2001 to 2004 on a culture of whiteflies, *Bemisia tabaci,* collected in Arizona in September of 2000. This culture was maintained in the laboratory at the University of Arizona on potted cotton plants and was shipped as necessary to the University of Portland. In Portland, adults were held on cantaloupe plants for one to four days before being used in experiments.

Growing Plants, University of Portland. Cantaloupe plants 2-3 weeks old (to the first true-leaf stage)were used for all tests, and were grown from seed (Topmark®, Hollar Seeds, Rocky Ford, CO) indoors in clay pots. Pots were positioned within 30 cm of two 40-watt, full-spectrum grow lights (GE, F40PL/AQ/WS) on a 16 h photophase. Plants were watered every one to two days with deionized water and one week after planting were fertilized with a dilute (5 ml per 6 liters) solution of Miracle Grow (Stern's Miracle-Gro Products, Inc., Port Washington, NY) in deionized water.

Growing Plants, University of Arizona. Cantaloupe plants were produced in a commercial greenhouse under mist irrigation and ambient light. Seeds of Hale's Best 45 cantaloupe were planted in Scott's commercial potting soil in trays 25 x 50cm in size. After 3 to 5 weeks the flats of plants were brought to the laboratory and leaves were removed for use in bioassays.

Imidacloprid (Admire®) Toxicity Bioassays. We estimated whitefly susceptibility to imidacloprid using systemic-uptake and residual bioassay procedures. The whitefly culture tested was collected in September of 2000 and maintained in laboratory culture on potted cotton plants. The systemic method was described by Cahill *et al.* (1996) It exposed whitefly adults to imidacloprid through their feeding on leaves of young melon, *Cucumis melo* L. (var. DPL-50), plants 21 to 35 days of age. These leaves were detached at the mainstem and placed in 200 ml of the desired concentrations (range 0 to 100 µg imidacloprid/ml) of Admire® 2F for 24 hrs of hydroponic uptake. Leaf disks of 2.5 cm diameter were then excised from the treated leaves and placed on a thin layer of agar gel (1.3%) in 20ml glass scintillation vials. A total of six to twelve replications (vials) were prepared for each concentration over two different testing dates. Twenty to thirty adult whiteflies from the laboratory culture were aspirated into each vial. Vials were capped with dialysis membrane (Spectra/Por*4, Baxter Diagnostics Inc., IL), and then placed in an incubator set at 27 ^{0}C, 16L:8D light cycle for 48 h, after which the assays were read under a binocular dissecting

microscope (Leica). Mortality was assessed by tapping vials on the counter and observing the appendage movement of the whiteflies. Individuals unable to move appendages repetitively (non-reflex) were scored as dead.

Residual bioassays employed a leaf-dip method described by Rowland et al. (1990). Leaf disks of 2.5cm diameter were taken from leaves of 21 to 35 day-old melon plants. The leaf disks were dipped for 10 s in 50 ml of the desired concentrations (0 to 100 µg imidacloprid/ml) of Admire® 2F diluted in water and .01% Triton X-100 surfactant (Sigma-Aldrich Co., St. Louis, MO). The dipped disks were allowed to dry before being placed individually on a base of agar in 20ml scintillation vials, as described above for the systemic uptake assay. A total of six to twelve replications (vials) were prepared for each concentration.

Mean mortality observed with all concentrations evaluated was corrected for control mortality using Abbott's correction (Abbott, 1925) for residual and systemic uptake assays. Statistical differences in population responses to specific groups of concentrations were evaluated by ANOVA using the JMP-IN statistical analysis program (SAS Institute, 2004). Mortality data were subjected to arcsine$\sqrt{x}$ transformations before analysis. Probit analyses of the concentration-dependent mortality were done using POLO-PC (LeOra Software, 1987) to generate lethal concentration statistics.

Stomatal Opening Status. Pairs of treated (systemic or foliar) and untreated cantaloupe plants were made for observation of stomatal opening status. For systemic treatments, 75 ml of an Admire® 2F (Bayer Corporation, Kansas City, MO) solution containing 2 ppm imidacloprid was placed in a test tube into which an intact plant was placed, its roots having been washed to remove potting soil. The untreated plant of the pair was placed in a test tube with water only. For foliar applications, both treated and untreated plants were placed in test tubes containing only water. Thereafter, one plant of each pair was dipped for 5 s into a dilution of Provado® 1.6F formulation containing 30 ppm imidacloprid and .01% Triton X-100 surfactant (Sigma-Aldrich Co., St. Louis, MO). The control plant was dipped in water and .01% Triton X-100 surfactant (Sigma-Aldrich Co., St. Louis, MO). Pairs of plants were then held undisturbed for 24h in darkness at 27 – 34 °C for both the systemic and foliar treatments.

Observations of stomates were carried out on plants with systemic exposure to 2 ppm, or foliar exposure to 30 ppm imidacloprid, plus the corresponding water-treated control groups. These were the concentrations and modes of application previously demonstrated to alter whitefly host plant choice and movement patterns (Marklund *et al*, 2003). Both modes of imidacloprid exposure were held in darkness for 24 h following treatment. After this period, the plants were treated with fixative to create a cast of stomates. The entire lower epidermal surface of both control and treated leaves were brushed with clear Hard as Nails Natural Tint Nail Protection® nail polish (Sally Hansen, Farmingdale, NY). After the nail polish dried, a strip of transparent tape was affixed to the nail polish surface on the lower epidermal surface of the leaf and then pulled off, removing the nail polish cast. The cast was then affixed to a clean microscope slide. The total surface area per leaf from which such casts were made was 4 mm^2 for all leaves. Stomatal casts were observed using a compound microscope and magnification of 400X. A total of 15 pairs of plants and 1,845 stomates were observed for the 2 ppm systemic and control treatments and a total of 15 pairs of plants and 2,316 stomates for the 30 ppm foliar and control treatments. Stomata were categorized as fully open, partially open, or fully closed. Total frequencies were analyzed using Chi-Squared goodness of fit tests to determine if the insecticide treatment had effects on the status of the stomata. The stomate counts were adjusted to equalize the totals for each treatment and corresponding control group. This procedure makes statistical tests slightly conservative; therefore significant differences were rendered more reliable.

Imidacloprid on the Leaf Surface. Three groups of 25 plants were produced for GC/MS quantification of imidacloprid residues. As detailed above, each set was treated with 2 ppm Admire® in systemic uptake, or dipped in 2 ppm Provado® or 30 ppm Provado® for residual treatments. Following a 24 h period of darkness, extractions were done by dipping all the leaves of a set into a single beaker of 250 ml of Nanopure water for 10 s per leaf. The resultant solutions were then hydrolyzed in a basic medium (NaOH), yielding 1-(6-chloro-3-pyridylmethyl)-imidazolidin-2-one. The hydrolyzed product was neutralized with HCl and extracted with chloroform. Following dehydration with anhydrous sodium sulfate, the solution was condensed to 1000 µl and treated with 20 µl of [$^{2}H_{10}$]anthracene internal standard solution, which provided the retention time for gas chromatography. The procedure for hydrolysis of imidacloprid is described by Vilchez *et al.* (1996). Alternative degradation products for GC/MS analysis have been described (Macdonlad & Meyer, 1998), as well as other methods for determination of imidacloprid (Baskaran *et al.,* 1997; Fernandez-Alba *et al.,* 1996).

A Varian system consisting of a 3400CX gas chromatograph fitted with a Varian 8200CX Auto Sampler, a splitless injector for the DB-5 column (30 m x 0.25 mm I.D. x 0.25 µm film thickness) and a Varian 2000 mass spectrometer, a pentium computer and the proprietary software was used. The carrier gas was helium (purity 99.999%).

The parameters for the GC/MS analysis are shown in Table 1. The product of imidacloprid hydrolysis was identified via the relevant peaks in the mass spectrum as defined by Vilchez *et al.* (1996) (m/z): 211, 126, 99, and for [$^{2}H_{10}$]anthracene at (m/z) 188.

Table 1. Parameters for GC/MS analysis.

Gas Chromotography	
Detector Temperature	260°C
Column Pressure (EPC Program)	22.0 psig, 1.0 psig/min, 28.0 (1.0 min), 0.5 psig/min, 29.0 (6.0 min)
Injected Volume	2 microliters
Flow Rate	2.97 ml/min
Oven Program	150 °C (1.00 min), 18 °C/min, 270 °C (2.00 min), 10 °C/min, 290 °C (6.00 min)
Mass Spectrometry	
Emission Time	10 microamps
Scan Time	0.620 s
Segment Low Mass	97 m/z
Segment High Mass	215 m/z
Ion Preparation Technique	SIS
Ionization Mode	E1 AGC
RF Dump Value	650.0 m/z
SIS Ion Preparation	97-101, 124-128, 209-213 m/z

To insure that the hydrolysis reaction was successful and that the expected product was produced, an IR (infrared spectrum) was taken (Perkin 1720-X Infrared Fourier). The IR spectrum, in accordance with Vilchez *et al.* (1996), yielded a stretching band at 1567 cm^{-1} resulting from the C=N group. The hydrolyzed product demonstrated a new band at 1690 cm^{-1}, which is attributed to the C=O group, but did not have the C=N band. Hydrolyzed imidacloprid is the form detectable using GC/MS analysis Vlichez *et al.*, 1996).

Results

Whitefly Susceptibility to Systemic versus Residual Exposure with Imidacloprid. Toxicity of imidacloprid to whiteflies exposed 48 h to residual or systemic residues of imidacloprid was not significantly different (likelihood

ratio test $X^2 = 0.954$, $df = 2$, $P = 0.621$) for concentrations ranging from 0.1 ug/ml to 3.2 ug/ml (Figure 1). Similarly, LC_{50} estimates derived from the full range of concentrations tested (0.1 to 100 ppm) did not differ statistically and were 1.46 ppm (95% FL 0.877 to 2.80) and 1.62 ppm (95% FL 0.605 to 6.54) for residual and systemic bioassays, respectively. However, mortality diverged between methods at the higher range of concentrations (Figure 1). Tests with 10, 32, and 100 ppm, residual contact resulted in significantly higher mortality than did systemic uptake. Probit analysis of all concentrations tested resulted in rejection of hypotheses that systemic and residual bioassay lines had the same slopes and intercepts (likelihood ratio test $X^2 = 41.0$, $df = 2$, $P < 0.001$) or that lines were parallel (likelihood ratio test $X^2 = 28.1$, $df = 1$, $P < 0.001$). Though the LC90s for residual uptake (87.2 ppm) and systemic uptake (199 ppm) differed by over 2-fold, their, 95% confidence intervals overlapped.

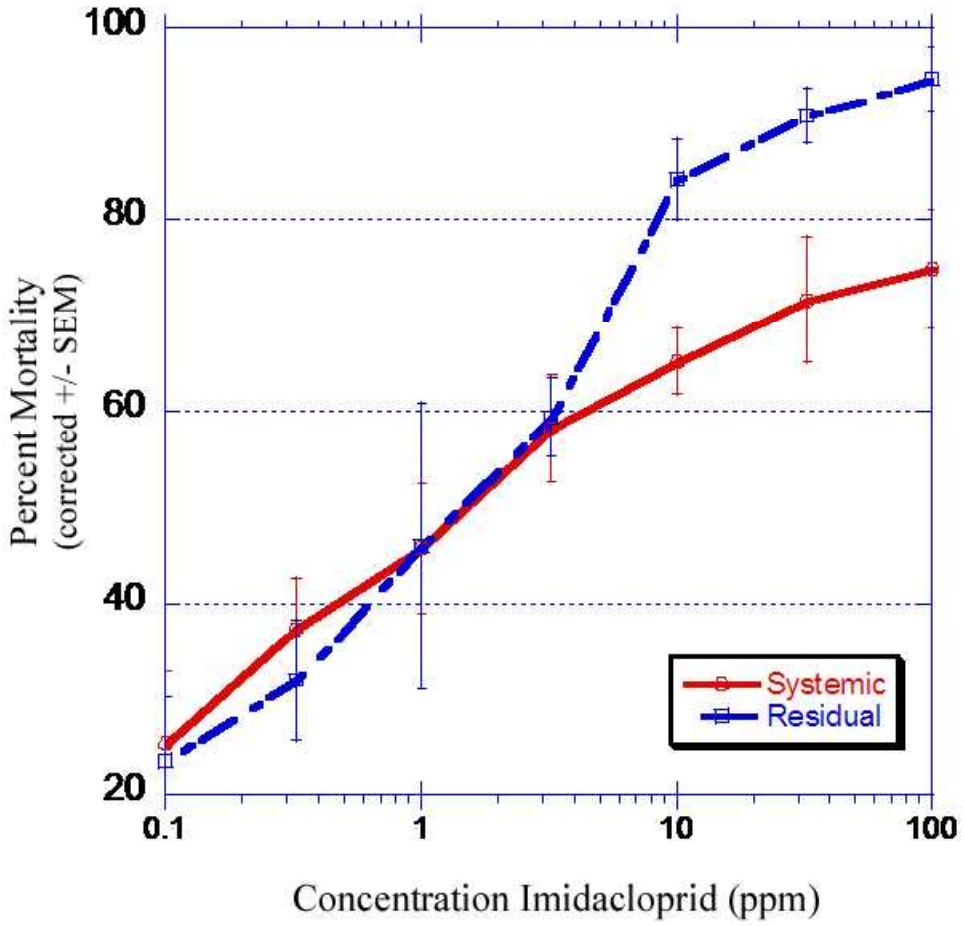

Figure 1. Mortality of whiteflies in residual and systemic leaf disk bioassays using cantaloupe plants. Prior to placing whiteflies on excised leaf disks, plants were either dipped in imidacloprid solutions (residual) or allowed to take up imidacloprid through the root system (systemic).

Status of stomatal opening in leaves systemically treated with 2 ppm imidacloprid. A significantly greater number of stomata were closed on the leaves systemically treated with 2 ppm imidacloprid than on untreated leaves. The total number of stomata in the open position for control and imidacloprid-treated groups was 17 and 0, respectively (X^2=17; p<<0.05). Total stomata partially open for control and treated groups was 213 and 155, respectively (X^2=7.17; p<<0.05). Total stomata fully closed for control and treated was 631 and 829, respectively (X^2=6.57; p<<0.05) (Figure 1).

Status of stomatal opening in leaves dipped in 30 ppm of imidacloprid. There was a statistically significant difference in the number of open stomata between control and treated leaves dipped in 30 ppm imidacloprid (X^2=7.56, p<<0.05) but the actual number of stomates involved was very small. The greatest numerical difference between control and treated groups was the number of closed stomates for control and treated leaves which numbered 907 and 868, respectively. However, this difference was not statistically significant (X^2=0.073, p>>0.05). Total stomata partially open for control and treated were also not statistically significant (X^2=0.027, p>>0.05) (Figure 2).

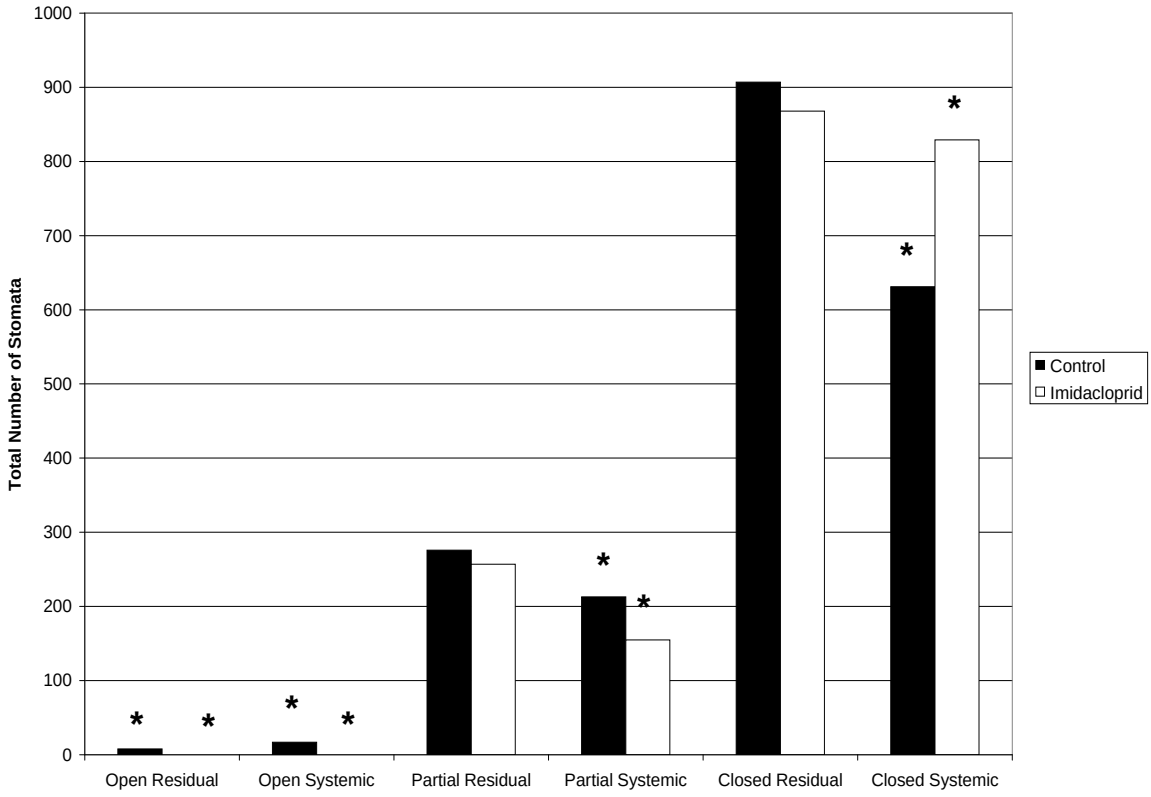

Figure 2. Total number of stomata in fully open, partially open, and fully closed positions for control vs. 2 ppm systemically and control vs. 30 ppm residually treated leaves. The asterisked bars indicate pairs of treatment groups that differ from one another statistically.

Determination of imidacloprid on the surface of the leaves. Extraction of the 30 ppm residual treatment yielded a peak of the hydrolyzed product with a retention time ranging between 7.00-7.25 min. The mass spectrum yielded the relevant peaks. Neither residual or systemic 2 ppm treatments yielded peaks at the corresponding retention time for the hydrolyzed product (Figure 3).

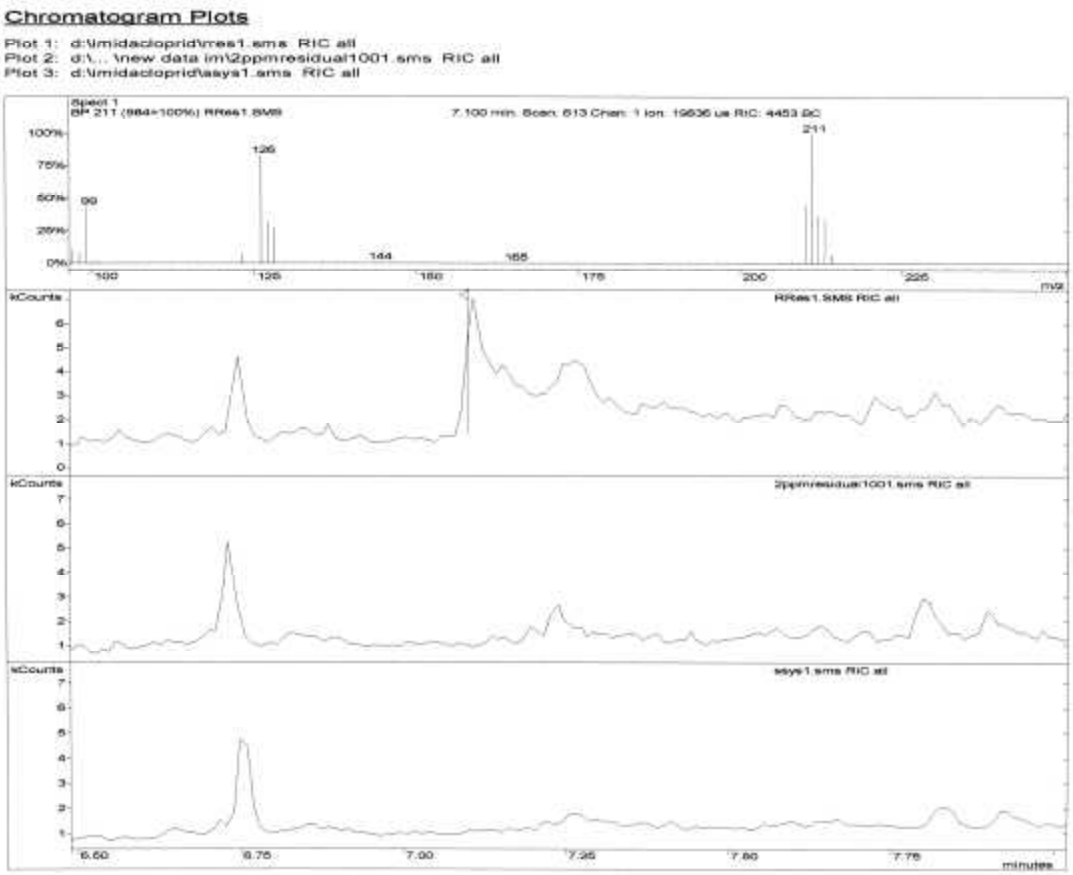

Figure 3.
Top: Mass spectrum at 30 ppm with relevant peaks of the hydrolyzed product at 211, 126 and 99 m/z.
Second: Gas Chromatogram of 30 ppm residual treatment. The peak occurring between 7.00 and 7.25 minutes is the hydrolyzed product.
Third: Gas Chromatogram of 2 ppm residual treatment. No hydrolyzed product was detected.
Bottom: Gas Chromatogram of 2 ppm systemic treatment. No hydrolyzed product was detected.

Discussion

Residual and topical routes of exposure to imidacloprid were remarkably similar in toxicity to whiteflies. Although, residual exposure to imidacloprid caused statistically greater toxicity at concentrations of 10 ppm or greater, resulting in >60% mortality, the magnitude of such differences were relatively small. Containing imidacloprid within plants systemically treated likely provides benefits of extended activity (Horowitz *et al.*, 1998), reduced exposure of field workers, and non-target organisms, as well as reduced photodegradation (Wamhoff & Schneider, 1999) of active ingredient.

In a comparison of two neonicotinoid insecticides against *B. tabaci* on cotton crops, Horowitz *et al*., (1998) found that field residual activity of foliar imidacloprid treatments lasted three days, while soil applications of imidacloprid lasted over two weeks. Boiteau *et al.* (1997) used foliar applications at intervals of seven days for control of Colorado potato beetle (Coleoptera: Chrysomelidae) and three species of potato colonizing aphids (Homoptera: Aphidae) on potatoes. Soil applications however provided control for 62-65 days after plant emergence. In systemic treatments, imidacloprid produces active metabolites, two of which demonstrate greater toxicity to whiteflies than the parent imidacloprid molecule. Hence systemic treatments protect imidacloprid from photolysis

and base hydrolysis, and they also bioactivate the molecule (Nauen *et al.*, 1998b). Foliar treatments would also result in bioactivation as the molecules moves into the plant, however, photolysis rapidly removes parent imidacloprid present.

Distinct influences of imidacloprid were observed on plant physiology. Stomatal opening on cantaloupe plants was significantly altered when imidacloprid was applied systemically with 2 ppm treatments, but not when applied as foliar 30 ppm treatments. Systemically treated plants had a greater proportion of closed stomates. We hypothesize that this phenomenon of systemic imidacloprid treatments causing stomates to close could permit whiteflies to distinguish between chemical stimuli emanating from treated versus untreated plants, and thus have contributed to the significant repellency demonstrated for cantaloupe plants undergoing a 2 ppm systemic imidacloprid treatment (Marklund *et al.*, 2003). Although imidacloprid treatments neither enhanced or negatively affected growth and yield in muskmelon (Palumbo & Sanchez, 1995), closed stomates in cotton crops grown during extended periods of high temperatures have been attributed to reduce lint yields due to poor stomatal conductance (Ulloa *et al.*, 2000).

Measurement of residues of hydrolyzed imidacloprid on leaf surfaces with Gas Chromatography/Mass Spectroscopy demonstrated benefits of reduced chances of worker exposure to residues when leaves are treated systemically. Hydrolyzed active ingredient was not detected on the surface of leaves treated with systemic or residual treatments of 2.0 ppm imidacloprid but were detected on 30 ppm residual treatments (Figure 2). Thus, rates of systemic imidacloprid that are very potent against whiteflies yielded no detectable residue that could expose non-target organisms.

Lastly, our GC/MS analysis indicated that the repellency of 2 ppm systemic imidacloprid treatments previously reported by Marklund *et al.* (2003) were unlikely to have resulted from detectable amounts of imidacloprid on the leaf surface. No detectable surface residue of hydrolyzed imidacloprid was found on any but the 30 ppm residual treatment (Figure 2). This result is consistent with the significant differences in landing detected by Marklund *et al.* (2003) in a 30 ppm residual treatment group (Marklund *et al.*, 2003). We hypothesize that the higher concentration of active ingredient in residual treatments is both detectable by whiteflies on the leaf surface, and affects landing choice of whiteflies.

Isaacs *et al.* (1999) found a significant difference in host evaluation for systemically treated leaves, but not for foliar treatments. Likewise, probing behavior of *B. tabaci* was significantly greater on systemically treated leaves, but not on foliarly treated leaves. Isaacs *et al.*'s (1999) findings were for 16 ppm systemic and foliar treatments. Our GC/MS analysis of leaf surfaces cannot speak to whether a detectable level of imidacloprid might be present on the leaf surface after a 16 ppm systemic or foliar treatment, and follow-up experiments on those concentrations would help integrate behavioral findings of Isaacs *et al.* (1999) and Marklund *et al.* (2003).

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