# Dynamic Insecticide Susceptibility Changes in Florida Populations of *Diaphorina citri* (Hemiptera: Psyllidae)

SIDDHARTH TIWARI, NABIL KILLINY, AND LUKASZ L. STELINSKI[1]

Entomology and Nematology Department, Citrus Research and Education Center, University of Florida, 700 Experiment Station Road, Lake Alfred, FL 33850

J. Econ. Entomol. 106(1): 393–399 (2013); DOI: http://dx.doi.org/10.1603/EC12281

**ABSTRACT** Five field populations of *Diaphorina citri* Kuwayama from various regions of Florida were evaluated in 2011 for resistance to commonly used insecticides. Three diagnostic doses ($LD_{50}$, $LD_{75}$, and $LD_{95}$), developed in 2009 using a laboratory susceptible population, were used to measure changes in susceptibility levels of field-collected populations as compared with a susceptible laboratory population. Further reductions in the susceptibility levels of *D. citri* to chlorpyriphos and fenpropathrin were determined, compared with results obtained in 2010. Mean percent mortality obtained from all five locations was significantly lower than observed with the laboratory susceptible population for all insecticides tested. Previously, expression of five *CYP4* genes was implicated in contributing to insecticide metabolism in *D. citri*. In the current study, we compared the relative expression of these five *CYP4* genes and their associated levels of protein expression among field-collected and laboratory susceptible populations. Expression of all *CYP4* genes investigated was higher in field-collected populations when normalized against the laboratory susceptible population. There was an increased signal of a band corresponding to a 45 kDa protein in four of the five field populations as measured by the Western blot assay, which suggests increased production of cytochrome P450 enzymes. The current results indicate that insecticide resistance continues to increase in Florida populations of *D. citri*, particularly to chlorpyriphos and fenpropathrin. However, there was no further decrease in susceptibility of Florida populations of *D. citri* to neonicotinoid insecticides in 2011 as compared with previous years.

**KEY WORDS** *CYP4*, cytochrome P450 monooxygenase, *Diaphorina citri*, gene expression, protein expression

Development of insecticide resistance is a major obstacle to successful management of insect pests. The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), is one of the most important pests of citrus, because it transmits the causal agent of huanglongbing (HLB) or citrus greening. *D. citri* populations in Florida have developed varying levels of resistance to several insecticide chemistries (Tiwari et al. 2011a). Current management of *D. citri* involves aggressive use of insecticides. There are a limited number of modes of action available for management of *D. citri* and repeated, sequential use of the same insecticide or mode of action may occur in Florida. Increased expression of various enzymes has been correlated with decreased susceptibility of *D. citri* to insecticides (Tiwari et al. 2011a, 2012a). In addition, five *CYP4* genes have been identified that were inducible when *D. citri* were exposed to sublethal dosages of imidacloprid (Tiwari et al. 2011b). These investigations have elucidated certain basic mechanisms of insecticide resistance development in *D. citri*.

Baseline susceptibility data for both adult and immature *D. citri* to commonly used insecticides were previously documented for five populations of *D. citri* spanning Florida during 2009 and 2010 (Tiwari et al. 2011a). We present here recent results that illustrate further development of insecticide resistance in populations of *D. citri* throughout Florida. In addition, we describe expression of five *CYP4* genes and associated cytochrome P450 proteins in field populations of *D. citri* involved in insecticide metabolism.

## Materials and Methods

**Asian Citrus Psyllid Culture.** A laboratory susceptible culture (LS) of *D. citri* was maintained at the Citrus Research and Education Center (CREC), University of Florida, Lake Alfred, FL. The culture was established in 2000 using field populations collected in Polk Co., FL (28.0′ N, 81.9′ W) before the discovery of HLB in the state. The culture is maintained on 'sour orange' (*Citrus aurantium* L.) seedlings without ex-

[1] Corresponding author, e-mail: stelinski@ufl.edu.

**Table 1. Quantitative real-time PCR primer details**

| Gene | Accession no. | Forward primer | Reverse primer |
|---|---|---|---|
| *CYP4 Genes* | | | |
| *CYP4C67* | JF934716 | 5′-TGGAACGTGTCATCAAGGAG-3′ | 5′-CCGGATTGAAACTGTTAGGC-3′ |
| *CYP4DA1* | JF934718 | 5′-AGTGGTGTCGGAAATTGAGG-3′ | 5′-GTTCGAGCCACCTGGAGATA-3′ |
| *CYP4C68* | JF934717 | 5′-CTAGCCTGGACCCTCTTCCT-3′ | 5′-ACCCTCCCTATGAACGGAAC-3′ |
| *CYP4G70* | JF934720 | 5′-GCCGGAAGTTCTTTCTTCCT-3′ | 5′-TAACGGGTACTGGTGGGAAC-3′ |
| *CYP4DB1* | JF934719 | 5′-CTGTACGCTCTGGGACATCA-3′ | 5′-TTGAGCGGTGCATAGAGTTG-3′ |
| *Actin* | DQ675553 | 5′-CCCTGGACTTTGAACAGGAA-3′ | 5′-CTCGTGGATACCGCAAGATT-3′ |

posure to insecticides in a greenhouse at 27–28°C, 60–65% relative humidity (RH), and a photoperiod of 14:10 (L: D) h. Field populations of *D. citri* adults were collected from five commercial citrus groves in Florida during 2011 (see map in Fig. 1 of Tiwari et al. 2011a). Adults were collected using sweep-nets and aspirators, transferred to the laboratory, and released onto citrus plants in Plexiglas cages ($40 \times 40 \times 40$ cm) until use in bioassays.

**Insecticides.** All bioassays were conducted with analytical grade insecticides. The susceptibility of adult *D. citri* was tested against six insecticides, belonging to various chemistry classes and modes of action. The insecticides evaluated were carbaryl (99.5%) (ChemService, West Chester, PA), chlorpyriphos (99.5%) (Sigma–Aldrich, St Louis, MO), fenpropathrin (99.5%) (ChemService), imidacloprid (99.5%) (ChemService), spinetoram (%) (Dow AgroSciences LLC, Indianapolis, IN; and ChemService), and thiamethoxam (99.7%) (Sigma–Aldrich).

**Adult Topical Application Bioassay.** All data were collected from adult populations of *D. citri* during the summer (May–August) of 2011. Susceptibility was evaluated using three diagnostic doses (previously established $LD_{50}$, $LD_{75}$, and $LD_{95}$, respectively) of each insecticide obtained from bioassays conducted on the adults from the LS culture (Table 2 in Tiwari et al. 2011a). *D. citri* adults of mixed gender were anesthetized under $CO_2$ and a 0.2 $\mu$l droplet of technical grade insecticide in analytical grade acetone was administered to the dorsal part of the thorax using a 10-$\mu$l Hamilton syringe attached to a microapplicator (PAX 100–3 Automatic Micro-Dispensing System, Burkard Agronomic Instruments, Uxbridge, United Kingdom). The same amount of acetone alone was applied as a control. Diagnostic doses for each insecticide were replicated three times per population tested, using 20–30 adults of mixed gender per replicate. Treated insects were placed into 35-mm plastic disposable petri dishes with 35-mm citrus leaf disks placed over agar beds as a source of food. Petri dishes with insects were kept at 25 ± 1°C and 50 ± 5% RH under a photoperiod of 14:10 (L:D) h in a growth chamber for 24 h. After 24 h, mortality was assessed. Adults that did not move upon prodding were considered dead. Mortality data were corrected for the control treatment using Abbott's formula (Abbott 1925). Percent mortality data were analyzed by analysis of variance (ANOVA) using a general linear model (PROC GLM) (SAS Institute 2004) for each diagnostic dose and insecticide, followed by Fishers protected least significant difference (LSD) tests.

**Quantitative Real-Time Polymerase Chain Reaction (qPCR) for Quantifying *CYP4* Gene Expression in Field-Collected and Laboratory Susceptible Populations of *D. citri*.** Field-collected *D. citri* and LS adults were subjected to RNA isolation and cDNA synthesis. RNA isolations were performed using the SV total RNA isolation kit (Promega, Madison, WI). RNA isolation was performed on groups of 40–50 adults per population. Quality and quantity of RNA from each sample was measured on a NanoDrop 1000 Spectrophotometer using $A_{260}$ and $A_{260}/A_{280}$ ratio, respectively (Scharf et al. 2008). Thereafter, RNA samples were used to synthesize cDNA using the iScript cDNA synthesis kit (Bio–Rad, Hercules, CA).

Relative expression of five *CYP4* genes in each population was detected by qPCR using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) in an ABI 7500 Real-Time PCR System (Applied Biosystems). Primers for the five *CYP4* genes and the reference gene, *Actin*, are reported in Table 1, as described by Tiwari et al. (2011b). The production of gene-specific products and absence of 'primer-dimers' was verified by 1% agarose electrophoresis in Tris-acetate EDTA (TAE) buffer with ethidium bromide staining. qPCR was performed in a 20-$\mu$l reaction volume containing 10-$\mu$l of SYBR green PCR master mix, 1-$\mu$l of cDNA, 1-$\mu$l of each forward and reverse primers, and 7-$\mu$l of nuclease-free water. Amplification cycles consisted of an initial denaturing step at 95°C for 10 min, followed by 45 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, an extension step at 72°C for 10 min, and the final melting-curve step (90 cycles of temperature reduction from 90 to 50°C at a rate of 0.5°C/10 s). Three biological replicates were performed for each gene.

Relative expression of each gene among populations was compared using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). First normalization was performed using *Actin* (reference gene) gene expression, followed by normalization to the treatment giving the lowest gene expression. Separate one-way ANOVAs were performed to compare the relative expression of each gene among various populations, followed by Fisher protected LSD tests for mean separation (PROC GLM) (SAS Institute 2004).

**Western Blot of Field-Collected and Laboratory Susceptible Populations of *D. citri*.** Adults from field-collected populations and the LS culture were subjected to extractions and quantifications of subcellular

**Table 2. Mean percent (±SEM) mortality of laboratory susceptible and field populations of adult *Diaphorina citri* as a result of an $LD_{50}$ diagnostic dose for various insecticides in 2011**

| Insecticide[a] | Laboratory susceptible | Fort Pierce | Lake Alfred | Vero Beach | La Belle | Lake Alfred-2 |
|---|---|---|---|---|---|---|
| Carbaryl | 48.07 ± 3.86a[b,c] | 47.22 ± 6.41a | 48.70 ± 6.37a | — | 42.58 ± 1.29a | 46.11 ± 6.55a |
| Chlorpyriphos | 48.77 ± 1.56a | 22.99 ± 9.99b | 46.67 ± 4.41a | — | 48.32 ± 2.03a | 44.52 ± 3.47a |
| Fenpropathrin | 52.67 ± 1.76a | 38.31 ± 4.22a | 49.63 ± 5.19a | — | 49.70 ± 5.51a | 48.34 ± 3.39a |
| Imidacloprid | 52.69 ± 1.08a | 48.75 ± 4.73ab | 38.07 ± 5.05c | 38.06 ± 3.14c | 40.33 ± 2.60bc | 46.02 ± 0.81abc |
| Spinetoram | 52.84 ± 0.62a | 46.11 ± 13.06a | 48.25 ± 3.16a | 37.82 ± 2.43a | 41.96 ± 9.69a | 50.72 ± 2.03a |
| Thiamethoxam | 51.21 ± 3.00a | 38.89 ± 2.00bc | 37.52 ± 3.11bc | 35.67 ± 1.17c | 40.84 ± 2.17bc | 43.49 ± 2.22b |

[a] $LD_{50}$ as reported in Table 2 from Tiwari et al. (2011a) was selected as the diagnostic dose for each insecticide.
[b] Mean percent mortalities followed by different letters within each insecticide (row) are significantly different ($P < 0.05$).
[c] Mean percent mortality was calculated using 60–90 adults for each insecticide and pop.

protein fractions. Protein concentration was determined by the Bradford procedure (Bradford 1976) using a protein assay kit (Bio–Rad Laboratories) with ovalbumin as a standard. Subcellular protein fractions were extracted using methods described by Wheeler et al. (2010). Each protein fraction dissolved in potassium phosphate buffer was loaded on a 10% polyacrylamide gel separately and subjected to electrophoresis using methods described by Mouchés et al. (1979). Protein fractions were visualized by staining with colloidal Coomassie blue G250. Microsomal proteins were subsequently used for the Western blot. Microsomal proteins from each population were transferred to a PVDF membrane for 60 min at 10 V using a semidry transfer apparatus (Bio–Rad). For renaturation of proteins and blocking of the unoccupied spaces, the membrane was incubated overnight at 4°C in TBS (100 mM Tris-HCl, 150 mM NaCl), pH 7.4, containing 6% nonfat dry milk (Sigma–Aldrich). After blocking, the membrane was used immediately for the Western blot assays. The membrane from the blocking solution was transferred to the primary antibody solution (polyclonal rabbit antibody, Anti-Cytochrome P450 19A1, Sigma–Aldrich, with the concentration of 1:1,000 in TBS), placed on a shaker for 1 h, rinsed with TBS-Tween-20 three times and then transferred to a secondary antibody solution (Anti-Rabbit IgGs-Alkaline phosphatase with the concentration of 1:10,000 in TBS) and placed on a shaker for 1 h. After three rinses with TBS-Tween-20 buffer, the membrane was placed into the BCIP/NBT solution. After developing color, the membrane was transferred to fresh nanopure water, placed on clean filter paper, and photographed as soon as possible.

## Results

**Adult Topical Application Bioassay.** Three diagnostic doses corresponding to 50, 75, and 95% mortalities as determined from the LS culture (Table 2 in Tiwari et al. 2011a) were chosen to assess the susceptibility of *D. citri* field populations in 2011. Susceptibilities of field populations were compared with the LS culture (Tables 2–4). At the $LD_{50}$ diagnostic dose, mortality of *D. citri* from the Fort Pierce population was significantly lower to chlorpyriphos; Lake Alfred, Vero Beach, and La Belle populations to imidacloprid; and all five populations to thiamethoxam, than from the LS culture (Table 2). Likewise, at the $LD_{75}$ diagnostic dose, mean percent mortality was lower for the Fort Pierce and Lake Alfred populations to chlorpyriphos; Fort Pierce, Lake Alfred, LA Belle, and Lake Alfred-2 populations to fenpropathrin; Fort Pierce, Lake Alfred, Vero Beach, and La Belle populations to imidacloprid; and Lake Alfred, Vero Beach, and La Belle populations to thiamethoxam, than for the LS culture (Table 3). At the $LD_{95}$ diagnostic dose, mean percent mortality of various field populations was lower to carbaryl, chlorpyriphos, fenpropathrin, imidacloprid, spinetoram, and thiamethoxam, than for the LS culture (Table 4).

**qPCR for Quantifying *CYP4* Gene Expression in Field-Collected and Laboratory Susceptible Populations of *D. citri*.** There were differences in expression of five *CYP4* genes among the various field-collected populations of *D. citri* (Fig. 1). Expression of *CYP4C67* (One-way ANOVA results: $F = 10.94$; df = 5, 12; $P = 0.0004$), *CYP4DA1* ($F = 9.87$; df = 5, 12; $P = 0.0006$), *CYP4C68* ($F = 41.54$; df = 5, 12; $P < 0.0001$), *CYP4G70*

**Table 3. Mean percent (±SEM) mortality of laboratory susceptible and field populations of adult *Diaphorina citri* as a result of an $LD_{75}$ diagnostic dose for various insecticides in 2011**

| Insecticide[a] | Laboratory susceptible | Fort Pierce | Lake Alfred | Vero Beach | La Belle | Lake Alfred-2 |
|---|---|---|---|---|---|---|
| Carbaryl | 77.70 ± 4.60a[b,c] | 42.78 ± 21.65a | 52.83 ± 8.02a | — | 70.50 ± 5.17a | 49.90 ± 6.66a |
| Chlorpyriphos | 77.92 ± 1.63a | 29.44 ± 16.00c | 48.17 ± 11.15bc | — | 67.19 ± 5.76ab | 67.42 ± 3.42ab |
| Fenpropathrin | 78.86 ± 1.82a | 50.37 ± 4.86c | 59.84 ± 5.36bc | — | 58.71 ± 4.10bc | 63.06 ± 2.69b |
| Imidacloprid | 77.67 ± 0.90a | 59.44 ± 5.30bc | 62.48 ± 3.11bc | 52.15 ± 3.55c | 53.58 ± 6.26bc | 65.63 ± 2.36ab |
| Spinetoram | 76.19 ± 1.19a | 62.42 ± 4.40a | 60.04 ± 3.38a | 60.81 ± 3.48a | 70.70 ± 9.10a | 67.59 ± 5.68a |
| Thiamethoxam | 75.19 ± 1.90a | 66.62 ± 4.81ab | 49.22 ± 5.98c | 50.58 ± 9.06c | 57.05 ± 2.65bc | 62.02 ± 2.13abc |

[a] $LD_{75}$ as reported in Table 2 from Tiwari et al. (2011a) was selected as the diagnostic dose for each insecticide.
[b] Mean percent mortalities followed by different letters within each insecticide (row) are significantly different ($P < 0.05$).
[c] Mean percent mortality was calculated using 60–90 adults for each insecticide and pop.

**Table 4. Mean percent (±SEM) mortality of laboratory susceptible and field populations of adult *Diaphorina citri* as a result of an $LD_{95}$ diagnostic dose for various insecticides in 2011**

| Insecticide[a] | Laboratory susceptible | Fort Pierce | Lake Alfred | Vero Beach | La Belle | Lake Alfred-2 |
|---|---|---|---|---|---|---|
| Carbaryl | 94.29 ± 2.97a[b,c] | 74.26 ± 2.28bc | 82.22 ± 4.94b | — | 82.59 ± 0.44b | 71.31 ± 2.47c |
| Chlorpyriphos | 95.40 ± 1.15a | 29.74 ± 6.52d | 48.33 ± 0.84c | — | 88.70 ± 2.44ab | 76.17 ± 9.17b |
| Fenpropathrin | 95.63 ± 1.15a | 44.21 ± 5.70c | 41.92 ± 8.38c | — | 84.65 ± 5.88ab | 72.07 ± 3.78b |
| Imidacloprid | 95.52 ± 1.09a | 90.79 ± 4.61a | 87.72 ± 4.64a | 62.04 ± 5.35b | 81.30 ± 11.20a | 85.32 ± 2.62a |
| Spinetoram | 95.03 ± 1.52a | 70.56 ± 4.59c | 61.89 ± 2.56c | 68.37 ± 0.86c | 86.35 ± 8.92ab | 73.45 ± 1.42bc |
| Thiamethoxam | 94.09 ± 1.23a | 96.25 ± 1.91a | 83.80 ± 5.07b | 78.33 ± 2.52b | 81.25 ± 1.53b | 76.19 ± 2.75b |

[a] $LD_{95}$ as reported in Table 2 from Tiwari et al. (2011a) was selected as the diagnostic dose for each insecticide.
[b] Mean percent mortalities followed by different letters within each insecticide (row) are significantly different ($P < 0.05$).
[c] Mean percent mortality was calculated using 60–90 adults for each insecticide and pop.

($F = 15.42$; df = 5, 12; $P < 0.0001$), and *CYP4DB1* ($F = 14.35$; df = 5, 12; $P = 0.0001$) was significantly greater in the field-collected populations than the LS culture. Expression of *CYP4C67* was 108- to 2,075-fold higher in field-collected than LS *D. citri*. Likewise, expression of *CYP4DA1*, *CYP4C68*, *CYP4G70*, and *CYP4DB1* was 1.8–25.2, 2.0–40.3, 3.3–12.9, and 1.2–6.8-fold higher in field-collected than LS *D. citri*, respectively. Expression of *CYP4DA1*, *CYP4C68*, *CYP4G70*, and *CYP4DB1* was lower in one field population than the *D. citri* from the LS culture (Fig. 1).

**Western Blot of Field-Collected and Laboratory Susceptible Populations of *D. citri*.** The SDS page of the LS and five field-collected populations of *D. citri* showed various differences in the protein profiles as depicted by black arrows in Fig. 2A. The SDS page was conducted using total protein fractions from each population. The Western blot analysis was conducted using 25 $\mu$g of proteins prepared from the microsomal fraction of *D. citri* to confirm differential expression of cytochrome P450 proteins among the six populations of *D. citri*. The highest signal of a band corresponding to a protein of 45 kDa molecular mass was observed in the Fort Pierce population (Fig. 2B), followed by Vero Beach, Lake Alfred, and Lake Alfred two populations. This band corresponded to the protein that cross-reacted with the anti-cytochrome P450 19A1 antibody. The lowest signal was observed in the LS and La Belle populations.

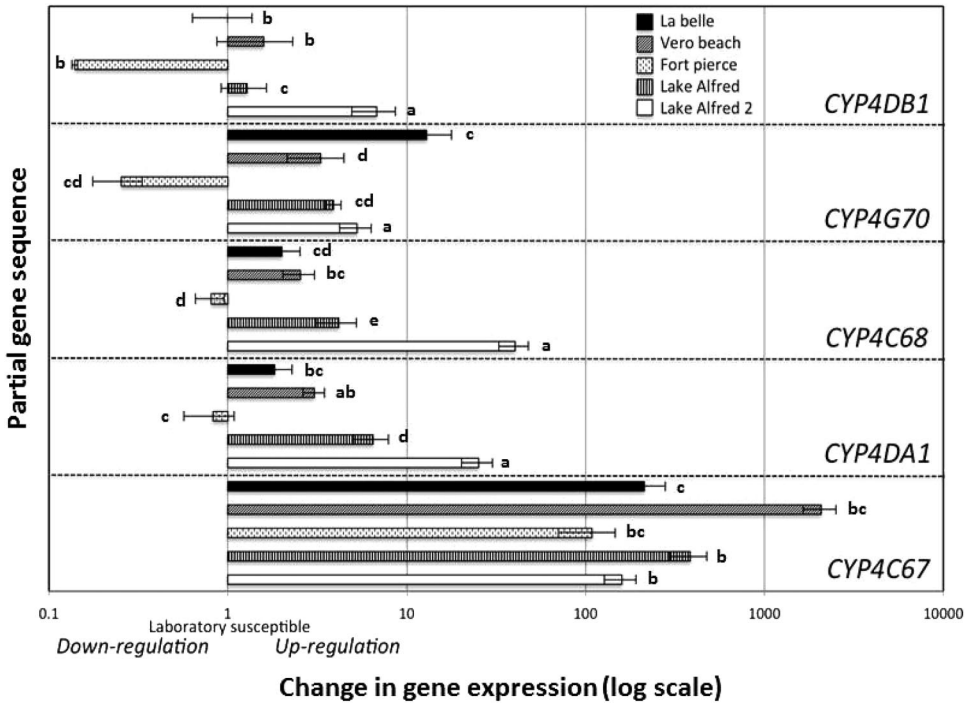


**Fig. 1.** Expression levels of five *CYP4* genes in five field-collected *Diaphorina citri* populations. Results were normalized to the treatment giving the lowest gene expression (laboratory susceptible population) using the $2^{-\Delta\Delta CT}$ method. Values within each *CYP4* gene sharing the same letter are not significantly different ($P \geq 0.05$; Fisher protected LSD). ANOVA and mean separation tests were performed on the ΔCT values. The reference gene was *Actin*. qPCR primers and sequence accession numbers are reported in Table 1.

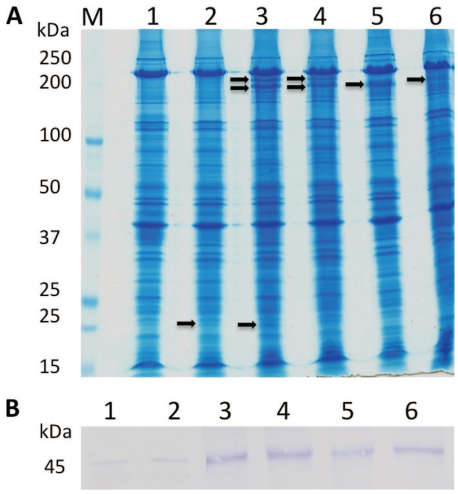


**Fig. 2.** (A) Protein fractions of the six populations of *Diaphorina citri* analyzed by SDS-PAGE using 10% polyacrylamide gel stained with colloidal Coomassie blue G250. Twenty-five micrograms of protein was loaded in each lane. M refers to the molecular mass marker. The six *D. citri* populations used in the assay and numbered 1–6 are laboratory susceptible, LA Belle, Vero Beach, Fort Pierce, Lake Alfred, and Lake Alfred 2, respectively. (B) Western blot of *D. citri* microsomal proteins probed with anti-cytochrome P450 19A1. Alkaline phosphatase-conjugated goat anti-rapid IgGs were used as secondary antibodies and detection was performed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT). Twenty-five micrograms of protein was loaded for each sample. The six *D. citri* populations used in the assay and numbered 1–6 are laboratory susceptible, LA Belle, Vero Beach, Fort Pierce, Lake Alfred, and Lake Alfred 2, respectively. (Online figure in color.)

## Discussion

The present investigation provides the most current insecticide susceptibility data of field populations of *D. citri* in Florida. The results revealed that insecticide susceptibility levels to certain modes of action continue to change rapidly, which is likely the result of intense insecticide input for management of this pest. Significant reduction in insecticide susceptibility of field-collected *D. citri* was observed for all six insecticides when compared with the LS culture. Collectively, mean percent mortality from all field-collected populations was significantly lower to the insecticides tested in 2011 than in 2010, with the exception of imidacloprid and thiamethoxam. This comparison is based on observed mortalities of field-collected *D. citri* populations during the 2 yr using the diagnostic dose of $LD_{95}$ (dose causing 95% mortality) (Tiwari et al. 2011a). In 2009, we measured reduced susceptibility of *D. citri* populations only to fenpropathrin, imidacloprid, and thiamethoxam; however, by 2010, *D. citri* adults were less susceptible to 13 insecticides tested as compared with the LS culture for one or more of the diagnostic doses tested (Tiwari et al. 2011a). Likewise, in 2011, field-collected *D. citri* adults exhibited lower susceptibility to chlorpyriphos, fenpropathrin, imidacloprid, and thiamethoxam at the $LD_{50}$ and $LD_{75}$ diagnostic doses, when compared with the LS culture. However, at the highest dose tested ($LD_{95}$), field-collected *D. citri* adults exhibited lower susceptibility to all six insecticides, when compared with the LS culture. In 2011, we were forced to reduce the number of insecticides and sites for evaluation because *D. citri* populations were significantly lower throughout the state than in previous years. This may have been because of the intensified insecticide use by growers on an area-wide scale through Citrus Health Management Areas (CHMA) (National Research Council [NRC] 2010).

There were significant reductions in the susceptibility of *D. citri* to certain insecticides in 2011 as compared with the LS culture. Susceptibility to carbaryl was not significantly different between field-collected populations and LS culture at $LD_{50}$ and $LD_{75}$ doses; however, susceptibility at the $LD_{95}$ dose was significantly lower in all field-collected populations than for the LS culture. In 2011, *D. citri* collected from populations in Lake Alfred and La Belle were less susceptible to carbaryl, whereas those from Fort Pierce were more susceptible than psyllids collected from these same areas in 2010. These changes in susceptibility levels within a year suggest that selection pressure may have risen because of increased product use.

Susceptibility to chlorpyrifos differed between field-collected populations and the LS culture at all three doses tested in 2011. In Fort Pierce, *D. citri* were up to 2.1- to 3.2-fold less susceptible to chlorpyriphos as compared with the LS culture, depending on the diagnostic dose used. In 2011, *D. citri* populations collected from Fort Pierce, Lake Alfred, and La Belle were 2.4-, 1.7-, and 1.1-fold less susceptible to chlorpyrifos than psyllids collected from the same areas in 2010. This rate of reduction in susceptibility levels within a year suggests that use of chlorpyrifos and other organophosphates should be judicious and appropriately rotated with other modes of action.

Susceptibility to fenpropathrin was not different between field-collected populations and the LS culture at the $LD_{50}$ diagnostic dose in 2011; however, it was lower at the $LD_{75}$ and $LD_{95}$ doses. Susceptibility to fenpropathrin was lower in 2011 for psyllids collected from Fort Pierce, Lake Alfred, and La Belle than for those locations in 2010 at the $LD_{95}$ diagnostic dose. This may be another example of suboptimal rotation or intensified use of a chemistry that may require more judicious use.

Susceptibility to imidacloprid and thiamethoxam was higher in field-collected populations than the LS culture at all three doses tested in 2011. For example, at the diagnostic dose of $LD_{95}$, susceptibility to imidacloprid was higher in 2011 for psyllids collected from Fort Pierce, Lake Alfred, Vero Beach, and La Belle than for psyllids collected from these same locations in 2010. At the diagnostic dose of $LD_{95}$, susceptibility to thiamethoxam was higher in 2011 for pysllids collected from Fort Pierce and La Belle, whereas lower for those collected from Vero Beach than susceptibility levels measured for the same populations in 2010. Susceptibility to spinetoram was

lower in 2011 for psyllids collected from Fort Pierce, while higher for those from Lake Alfred and Vero Beach than observed for psyllids collected from these same locations in the 2010 at the $LD_{95}$ diagnostic dose.

The overall mortality data from all locations at the $LD_{95}$ diagnostic dose indicates that psyllids collected from various populations in 2011 were 4.3, 31.4, 23.1, and 1.2% less susceptible to carbaryl, chlorpyriphos, fenpropathrin, and spinetoram, respectively, when compared with those collected from the same locations in 2010. However, at the same dose, psyllids collected in 2011 were 11.2 and 9.3% more susceptible to imidacloprid and thiamethoxam, respectively, than those collected in 2010 for the locations surveyed in this ongoing investigation. Imidacloprid yielded the highest resistance ratio (ratio of $LD_{50}$ of field-collected populations and LS culture) of 35 in 2009, with a similar trend in 2010 (Tiwari et al. 2011a). Therefore, higher susceptibility levels to imidacloprid and thiamethoxam in field-collected populations of *D. citri* in 2011 than in 2010 suggest that selection pressure for evolution of resistance to this class of insecticides has plateaued in Florida or possibly decreased as compared with previous years.

Varying levels of detoxifying enzymes, and specifically cytochrome P450 monooxygenases were found in *D. citri* adults and nymphs from various populations collected in 2010 (Tiwari et al. 2011a). Nymphs collected from one site, which exhibited the highest cytochrome P450 monooxygenase levels, as well as other detoxifying enzymes, also were relatively more resistant to carbaryl, imidacloprid, and spinetoram as compared with the LS culture (Tiwari et al. 2011a). This suggested that the one of the mechanisms conferring resistance in field-collected *D. citri* is enzyme based and may involve cytochrome P450 monooxygenases. In addition, we have previously reported that five cytochrome P450 genes (*CYP4C67*, *CYP4DA1*, *CYP4C68*, *CYP4DB1*, and *CYP4G70*) from *D. citri* were overexpressed in insecticide tolerant *D. citri* as compared with susceptible counterparts (Tiwari et al. 2011b,c). Expression of all five genes was induced by imidacloprid treatment (Tiwari et al. 2011b). These findings suggested potential involvement of five *CYP4* genes associated with insecticide metabolism in *D. citri*, and therefore warranted an investigation of their expression levels among various field populations of *D. citri.* In the current study, all five *CYP4* genes were generally upregulated in the field populations tested as compared with the LS culture, with one exception. In the Fort Pierce population, four of the *CYP4* genes were downregulated, suggesting that the resistance level in psyllids from this population may not be mediated by the above mentioned *CYP4* genes. However, the Western blot revealed the strongest signal for a 45 kDa cytochrome P450 monooxygenase protein in this population, which suggests that the resistance level might be related to other cytochrome P450 proteins. Therefore, further investigations are needed to quantify *CYP* genes belonging to families other than four or designing an antibody (antibodies) specific to the *CYP4* sequences used in the current study. In addition, populations of *D. citri* from Lake Alfred, Lake Alfred 2, and Vero Beach were characterized by stronger expression of a 45 kDa cytochrome P450 monooxygenase protein than those from the LS culture and La Belle population.

Based on the present and previous results, there appear to be varying levels of selection pressure for resistance development among the insecticide chemistries used for *D. citri* management. For example, resistance to carbaryl, chlorpyriphos, fenpropathrin, and spinetoram further increased from 2010 to 2011, whereas it appeared to stabilize or reverse for imidacloprid and thiamethoxam. These results suggest a possible strategy for rotation of modes of action. For example, it may be useful to apply neonicotinoids or IGRs before carbamates or organophosphates or pyrethroids. This is mainly because resistance to organophosphate/carbamate/pyrethroid insecticides has increased at a higher rate than that for neonicotinoids. Therefore, rotation of organophosphate or carbamate insecticides with a pyrethroid may be less effective than rotation of these modes of action with neonicotinoids. Development of optimal rotation modules will require further investigations. Application of carbamates or organophosphates during the winter dormant period will also reduce the impact on biological control agents, which are less abundant at this time of the year (Qureshi and Stansly 2009, 2010). IGRs are effective against *D. citri* immature stages (Boina et al. 2010, Tiwari et al. 2012b) and may also be included in insecticide rotation modules for this pest. The current results also emphasize the need for insecticide alternatives or non-neurotoxic tools as supplements for management of *D. citri.* In addition, area-wide management programs called "Citrus Health Management Areas or CHMAs" tailored for minimizing resistance development of *D. citri* should be extended to other citrus growing regions of the United States.

## Acknowledgments

This project was supported by a grant from the Citrus Research and Development Foundation to L.L.S. We acknowledge H. Anderson, R. Blanco, P. Clayson, Y. Cruz–Plemons, D. Diaz, M. Flores, B. Holladay, S. Holladay, A. Hoyte, I. Jackson, E. H. Kuhns, and W. Meyer for *D. citri* collections and technical assistance.

## References Cited

**Abbott, W. S. 1925.** A method of computing the effectiveness of an insecticide. J. Econ. Entomol. 18: 265–267.

**Boina, D. R., M. E. Rogers, N. Wang, and L. L. Stelinski. 2010.** Effect of pyriproxyfen, a juvenile hormone mimic, on egg hatch, nymph development, adult emergence and reproduction of the Asian citrus psyllid, *Diaphorina citri* Kuwayama. Pest Manag. Sci. 66: 349–357.

**Bradford, M. M. 1976.** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

**Livak, K. J., and T. D. Schmittgen. 2001.** Analysis of relative gene expression data using real-time quantitative PCR and the 2-DDCT method. Methods 25: 402–408.

**Mouchés, C., J. C. Vignault, J. G. Tully, R. F. Whitcomb, and J. M. Bové. 1979.** Characterization of spiroplasmas by one- and two-dimensional protein analysis on polyacrylamide slab gels. Curr. Microbiol. 2: 69–74.

**(NRC) National Research Council. 2010.** Strategic planning for the Florida citrus industry: addressing citrus greening disease. The National Academies Press, Washington, DC.

**Qureshi, J. A., and P. A. Stansly. 2009.** Exclusion techniques reveal significant biotic mortality suffered by Asian citrus psyllid *Diaphorina citri* (Hemiptera: Psyllidae) populations in Florida citrus. Biol. Control 50: 129–136.

**Qureshi, J. A., and P. A. Stansly. 2010.** Dormant season foliar sprays of broad-spectrum insecticides: an effective component of integrated management for *Diaphorina citri* (Hemiptera: Psyllidae) in citrus orchards. Crop Prot. 29: 860–866.

**SAS Institute. 2004.** SAS users guide. SAS Institute, Cary, NC.

**Scharf, M. E., X. Zhou, and M. A. Schwinghammer. 2008.** Application of RNA interference in functional genomics studies of a social insect, pp. 205–229. *In* S. Barik (ed.), Methods in Molecular Biology (Vol. 442): Sirna, Shrna and Mirna Protocols. Humana Press, Totowa, NJ.

**Tiwari, S., R. S. Mann, M. E. Rogers, and L. L. Stelinski. 2011a.** Insecticide resistance in field populations of Asian citrus psyllid in Florida. Pest Manag. Sci. 67: 1258–1268.

**Tiwari, S., A. Gondhalekar, R. S. Mann, M. E. Scharf, and L. L. Stelinski. 2011b.** Characterization of five *CYP4* genes of Asian citrus psyllid and their expression levels in *Candidatus Liberibacter asiaticus* infected and uninfected adults. Insect Mol. Biol. 20: 733–744.

**Tiwari, S., K. Pelz–Stelinski, and L. L. Stelinski. 2011c.** Effect of *Candidatus Liberibacter asiaticus* infection on susceptibility of Asian citrus psyllid, *Diaphorina citri*, to selected insecticides. Pest Manag. Sci. 67: 94–99.

**Tiwari, S., L. L. Stelinski, and M. E. Rogers. 2012a.** Biochemical basis of organophosphate and carbamate resistance in Asian citrus psyllid. J. Econ. Entomol. 105: 540–548.

**Tiwari, S., P. J. Clayson, E. H. Kuhns, and L. L. Stelinski. 2012b.** Effects of buprofezin and diflubenzuron on various developmental stages of Asian citrus psyllid, *Diaphorina citri.* Pest Manag. Sci. (doi:10.1002/ps.3323).

**Wheeler, M. M., M. R. Tarver, M. R. Coy, and M. E. Scharf. 2010.** Characterization of four esterase genes and esterase activity from the gut of the termite *Reticulitermes flavipes.* Arch. Insect Biochem. Physiol. 73: 30–48.

*Received 18 July 2012; accepted 20 November 2012.*