Rapid Detection of Insecticide Resistance in Diaphorina citri (Hemiptera: Liviidae) Populations, using a Bottle Bioassay

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Rapid detection of insecticide resistance in *Diaphorina citri* (Hemiptera: Liviidae) populations, using a bottle bioassay

*Xue Dong Chen and Lukasz L. Stelinski**

Abstract

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is a major pest of citrus crops worldwide. A large number of insecticides have been used to manage *D. citri* in Florida. Therefore, insecticide resistance could become an important problem facing citrus production. Monitoring insecticide susceptibility in populations of *D. citri* and providing a technique to use as an early warning is needed so citrus producers can modify chemical control strategies for this pest in Florida. The objective of this study was to develop a simple and fast tool to determine insecticide resistance in *D. citri* and apply it to commercial citrus production in Florida. LC50 and LC95 estimates were determined for 8 commonly used insecticides on a susceptible laboratory population of *D. citri* 24 h after treatment in a residual contact bottle assay. Five to 7 concentrations of each insecticide were tested. The LC50 values (and 95% fiducial limits) ranged from 0.06 (0.02–0.26) to 0.80 (0.26–2.46) ng/µL for each insecticide tested. Exposure time–mortality indices were determined for 0, 10, 100, 1,000, and 10,000 ng/µL concentrations of each insecticide in a laboratory susceptible strain. Knockdown was assessed after 15, 30, 45, 60, 75, 90, 105, and 120 min. Complete knockdown (100.0%) occurred within 60 min for dimethoate, fenpropathrin, imidacloprid, bifenthrin, and flupyradifurone at the 10,000 ng/µL concentration. For spinetoram, 86.7% knockdown occurred within 120 min at 10,000 ng/µL. For sulfoxaflor and cyantraniliprole, 44.0 and 42.6% knockdown, respectively, occurred within 120 min at 1,000 ng/µL. We also developed a bottle bioassay to survey field populations of *D. citri* for insecticide resistance in central Florida. Exposure time–mortality indices developed in the laboratory were used to assess susceptibility of 1 laboratory and 4 field populations of *D. citri* after 15, 30, 50, 75, 90, 105, and 120 min of exposure at the 10,000 ng/µL concentration of various insecticides. Little to no evidence of resistance was detected for bifenthrin, dimethoate, imidacloprid, and fenpropathrin in central Florida. Our investigation demonstrated that a bottle bioassay is suitable for assaying insecticide resistance in *D. citri* adults under laboratory and field conditions. It should be a flexible tool for rapid testing of insecticide resistance in possible cases of insecticide failure. Its simplicity should allow trained professionals to rapidly monitor for insecticide resistance in commercial settings where "hot spots" of *D. citri* populations may occur.

Key Words: insecticide monitoring; exposure time–concentration mortality; knockdown; Asian citrus psyllid

Resumen

El psílido cítrico asiático, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), es una plaga importante de los cítricos en todo el mundo. Se ha utilizado un gran número de insecticidas para manejar *D. citri* en la Florida. Por lo tanto, la resistencia a los insecticidas podría convertirse en un problema importante para la producción de cítricos. Se necesita monitorear la susceptibilidad a insecticidas en poblaciones de *D. citri* y proveer una técnica para usar como alerta temprana para que los productores de cítricos puedan modificar las estrategias de control químico de esta plaga en la Florida. El objetivo de este estudio fue desarrollar una herramienta simple y rápida para determinar la resistencia a los insecticidas por *D. citri* y aplicarla a la producción comercial de cítricos en la Florida. Se determinaron las estimaciones CL50 y CL95 para 8 insecticidas de uso general en una población de laboratorio susceptible de *D. citri* 24 horas después del tratamiento en un ensayo de botella de contacto residual. Se ensayaron de cinco a siete concentraciones de cada insecticida. Los valores de CL50 y los límites fiduciales del 95% oscilaron entre 0,06 (0,02 - 0,26) y 0,80 (0,26 - 2,46) ng/µl para cada insecticida ensayado. Se determinaron los índices de tiempo-mortalidad para las concentraciones de 0, 10, 100, 1.000 y 10.000 ng/µl de cada insecticida en una cepa susceptible en el laboratorio. Se evaluó el efecto de noqueo después de 15, 30, 45, 60, 75, 90, 105 y 120 minutos. El 100% del noqueo sucedio en 1 hora para el dimetoato, fenpropatrina, imidacloprid, bifentrina y flupiradifurona a la concentración de 10.000 ng/µL. Para el espinetoram, el 86.7% de noqueo ocurrió dentro de 120 minutos a 10.000 ng/µL. El efect de noqueo para sulfoxaflor y ciantraniliprole fue 44,0 y 42,6%, respectivamente, dentro de 120 minutos a 1.000 ng/µL. También desarrollamos un bioensayo de botella para examinar poblaciones de campo de *D. citri* para la resistencia a insecticidas en el centro de la Florida. Se utilizaron los índices de tiempo-mortalidad desarrollados en laboratorio para evaluar la susceptibilidad de las poblaciones de laboratorio y de 4 campos de *D. citri* después de 15, 30, 50, 75, 90, 105 y 120 minutos de exposición a la concentración de 10.000 ng/insecticida. Se detectó poco o ningún señal de resistencia para bifentrina, dimetoato, imidacloprid y fenpropatrina en el centro de la Florida. Nuestra investigación demostró que un bioensayo de botella es adecuado para ensayar la resistencia a insecticidas en adultos *D. citri* bajo condiciones de laboratorio y de campo. Debe ser una herramienta flexible para la prueba rápida de la resistencia a insecticidas en casos posibles de falla de insecticida. Su sencillez debería permitir a los profesionales capacitados vigilar rápidamente la resistencia a los insecticidas en entornos comerciales donde pueden producirse "puntos calientes" de las poblaciones de *D. citri*.

Palabras Clave: monitoreo de insecticidas; tiempo-concentración de diagnóstico; derrumbamiento; psílido asiáticos de los cítricos

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The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is one of the most important pests of citrus as it is the vector of the bacteria causing citrus greening, also called huanglongbing (Halbert & Manjunath 2004; Halbert & Núñez 2004; Bové 2006; Pelz-Stelinski et al. 2010; Boina & Bloomquist 2015). *Diaphorina citri* was first described in Taiwan in 1907 (Kuwayama 1908; Grafton-Cardwell et al. 2013), and the infectious nature of huanglongbing was described in China (Lin 1956). *Diaphorina citri* was first reported in Brazil in the 1940s (da Costa Lima 1942), Florida in 1998 (Tsai & Liu 2000), and currently, *D. citri* can be found in most citrus-producing regions of the United States (French et al. 2001; Halbert et al. 2010; Hummel & Ferrin 2010).

Huanglongbing is one of the most economically important diseases of citrus throughout the world (Halbert & Manjunath 2004; Manjunath et al. 2008). Huanglongbing previously occurred in Asia and Africa (Gottwald 2010). It was first found in the western hemisphere in Brazil in 2004 (Texeira et al. 2005; Grafton-Cardwell et al. 2013), and Florida in 2005 (Halbert 2005), and has since spread to Central America and most citrus production areas in the United States. Citrus trees infected by this disease produce misshapen, small, discolored, and reduced-quality fruit over a shortened lifespan (Bové 2006; Gottwald et al. 2007; Grafton-Cardwell et al. 2013). In Florida, about 10.8 out of 60 million orange trees have been infected with huanglongbing (Quarles 2014; Boina & Bloomquist 2015). It is estimated that over the last 5 yr in Florida, huanglongbing has caused over US$1.3 billion in lost revenue to the citrus industry and a loss of over US$3.6 billion in total economic activity (Hodges & Spreen 2012). The 2014 citrus harvest may have been the lowest in approximately 50 yr without the impact of a serious weather event. Prevention of disease transmission has proven difficult worldwide (Aubert et al. 1996). There is currently no method of curing diseased trees (Morris et al. 2009; Tiwari et al. 2010, 2011). Currently, the most common practice for managing huanglongbing relies on chemical insecticides for vector suppression (Tiwari et al. 2011).

Insecticides are presently a critical component of *D. citri* management in Florida, and 8 to 12 treatments are commonly applied per year. Under such intensive pressure, declines of susceptibility among *D. citri* populations to neonicotinoid, organophosphate and pyrethroid insecticides have been observed (Tiwari et al. 2011; Grafton-Cardwell et al. 2013; Kanga et al. 2016). Insecticide resistance is one of the most important problems facing citrus production. A recent study from Mexico showed that *D. citri* populations had become 100-fold and 4,000-fold resistant to organophosphates and neonicotinoids, respectively (Vazquez Garcia et al. 2013).

Monitoring for insecticide susceptibility in *D. citri* populations is a proactive approach to detect changes in insecticide performance, and could provide an early warning to modify chemical control strategies. Solving this problem requires the development and validation of a reliable, rapid, and inexpensive bioassay to detect insecticide resistance in *D. citri* populations, which would assist growers, consultants, and extension personnel in making informed decisions on adequate control measures. Currently, novel biochemical or immunological methods hold considerable promise for resistance management, but inheritance of resistance may be complex or there may be multiple mechanisms for the resistance, which may preclude early detection in field populations (Robertson et al. 1984; Preisler 1988; ffrench Constant & Roush 1990; Brogdon & McAllister 1998; Tiwari et al. 2012a).

The bottle bioassay is an efficient tool for determining insecticide susceptibility in the laboratory and has been successfully used to evaluate susceptibility of various pest species such as *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse) (both Diptera: Culicidae) (Dunford et al. 2015), *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (Sivasupramaniam et al. 1997), *Diuraphis noxia* (Kurdjumov) (Hemiptera: Aphididae) (Bayoun et al. 1995), *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) (Pietrantonio et al. 2007), *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) (Nielsen et al. 2008), *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) (Snodgrass 1996), *Nezara viridula* (L.) (Hemiptera: Pentatomidae) (Snodgrass et al. 2005), *Pseudatomoscelis seriatus* (Reuter) (Hemiptera: Miridae) (Lòpez et al. 2008), *Tetranychus urticae* Koch (Acari: Tetranychidae) (Latheef & Hoffmann 2014), and *D. citri* (Kanga et al. 2016). However, the baseline and diagnostic exposure time–mortality data for insecticides used against *D. citri* obtained with a bottle bioassay technique are currently unavailable.

In this investigation, we developed a simplified bottle bioassay technique to test the exposure time–mortality relationship for multiple insecticide concentrations and determined the LC50 and LC95 (24 h mortality) values for 8 insecticides. The objectives of this study were to evaluate this bioassay technique and determine baseline susceptibility of *D. citri* field populations to pyrethroid, organophosphate, neonicotinoid, diamide, sulfoximine, butenolide, and spinosyn insecticides that are commonly used in Florida citrus production. Our investigation provides exposure time–mortality data for future surveys of insecticide susceptibility of *D. citri* populations with this bottle assay.

Materials and Methods

INSECTS

A susceptible laboratory population of *D. citri* was reared in a greenhouse at the Citrus Research and Education Center, University of Florida, Lake Alfred, Florida. It originated from adults collected in 2000 from citrus in Polk County, Florida, with no history of insecticide exposure. This strain has been reared without exposure to insecticides or subsequent input of field-collected *D. citri*. The culture was maintained on sweet orange (*Citrus sinensis* [L.] Osbeck; Rutaceae) in a greenhouse at 27 to 28 °C, with 60 to 65% relative humidity, and a 14:10 h L:D photoperiod. Collections of *D. citri* adults from commercial citrus groves in Florida were conducted in 2016 in central Florida (locations and methods described below) to measure insecticide susceptibility in field populations. In all cases, mixed-sex groups were used and the age of the adults (laboratory and field) was not determined.

INSECTICIDES

Tested insecticides were analytical grade and included bifenthrin (99.8%), dimethoate (99.8%), fenpropathrin (99.1%), imidacloprid (99.9%), cyantraniliprole (98.0%), sulfoxaflor (99.5%), flupyradifurone (99.5%), and spinetoram (a mixture of 76.2% spinetoram J and 21.0% spintoram L) representing several insecticide classes (Table 1). Dimethoate, fenpropathrin, and imidacloprid were obtained from Sigma-Aldrich (St. Louis, Missouri). Bifenthrin, cyantraniliprole, sulfoxaflor, flupyradifurone, and spinetoram were obtained from Chem Service, Inc. (West Chester, Pennsylvania). Each insecticide was stored according to the manufacturer's recommendation. Serial dilutions of technical insecticide in acetone (ACS grade, 99.5%, Fisher Scientific, Hampton, New Hampshire) were made to generate test concentrations ranging from 0.0001 to 10,000 ng/µL. All insecticide solutions were kept at −20 °C for a maximum of 30 d and new solutions were prepared thereafter.

SUSCEPTIBILITY BOTTLE BIOASSAY

The susceptibility bottle bioassay methods described by Snodgrass & Scott (2000), Miller et al. (2010), Liu et al. (2012), and Tiwari & Stelinski (2013) were used to measure direct toxicity of insecticides to *D. citri*. In the bioassay, 20 mL glass scintillation vials (Wheaton Industries

Table 1. Insecticides tested on *Diaphorina citri.*

Insecticide	IRAC Group[a]	Chemical Class	Biochemical target	Manufacturer
Dimethoate	1B	Organophosphate	Acetylcholinesterase Inhibitor	Sigma-Aldrich
Fenpropathrin	3A	Pyrethroid	Sodium channel modulators	Sigma-Aldrich
Imidacloprid	4A	Neonicotinoid	Nicotinic acetylcholine receptor	Sigma-Aldrich
Bifenthrin	3A	Pyrethroid	Sodium channel modulators	Chem Service
Cyantraniliprole	28	Diamides	Ryanodine receptor modulator	Chem Service
Sulfoxaflor	4C	Sulfoximines	Nicotinic acetylcholine receptor	Chem Service
Flupyradifurone	4D	Butenolides	Nicotinic acetylcholine	Chem Service
Spinetoram	5	Spinosyn	Nicotinic acetylcholine receptor allosteric modulators	Chem Service

[a]Insecticide Resistance Action Committee mode of action group number.

Inc., Millville, New Jersey) with a total inner surface of 46.71 cm^2 measuring 4.7 cm in height and 2.5 cm in diameter were coated with 150 µL of an insecticide–acetone dilution or acetone alone (as a control). The vials were then rotated on a mechanical roller for 30 min to achieve a uniform coat of insecticide on the inside of the vial at which point the acetone had evaporated. Ten 2- to 10-d-old *D. citri* adults were placed into each vial and the cap was secured loosely. Five to 7 concentrations of each insecticide were tested. Each concentration was replicated 5 times and each experiment was repeated 3 times. The vials were held upright in a growth chamber at 25 ± 1 °C and 60 ± 1% RH with a 14:10 h L:D photoperiod. Mortality of *D. citri* was assessed 24 h after transfer into the growth chamber. Insects were considered dead when found on their sides or back and unable to move when probed with a camel-hair brush.

DIAGNOSTIC EXPOSURE TIME AND CONCENTRATION FOR KNOCKDOWN OF *D. CITRI*

The appropriate concentration of each insecticide tested for treating vials to achieve 100% knockdown at 1 h was determined for the laboratory susceptible strain of *D. citri*. Knockdown in this case was defined as occurring after 1 h whereas the above-described mortality assessments were made after 24 h. For this experiment, vials were prepared with 150 µL of insecticide–acetone dilution per vial or acetone alone as a control. The concentrations used were 0, 10, 100, 1,000, and 10,000 ng/µL of insecticide. The rate of knockdown was assessed after 15, 30, 45, 60, 75, 90, 105, and 120 min. There were 5 replicates and the entire experiment was conducted 3 times. The minimum concentration that registered 100% knockdown of *D. citri* within 45 to 60 min of exposure was considered as the concentration for discriminating between susceptible and resistant *D. citri* populations.

SURVEILLANCE OF *D. CITRI* FIELD POPULATIONS WITH THE BOTTLE BIOASSAY

Field testing of *D. citri* populations from citrus groves in central Florida was conducted during 2016 by using the bottle bioassay described above. Collections were made from Winter Garden, Frostproof, and 2 locations in Lake Alfred (Table 2). Adults of *D. citri* were collected by vacuum (D-Vac, Rincon-Vitova Insectaries, Ventura, California) as described in Coy et al. (2016) with permission from grove managers. Based on sensitivity ratios, an earlier investigation showed no difference in insecticide susceptibility between mouth-aspirated and vacuum-collected psyllids (Coy et al. 2016).

Field-collected adults were transported to the laboratory in coolers and released into 40 × 40 × 40 cm Plexiglas cages, and were provided with four 25- to 35-cm-tall citrange (*Poncirus trifoliate* (L.) Raf × *C. sinensis* 'Kuharski Carrizo' saplings. Approximately 500 to 700 *D. citri* adults were kept under controlled conditions in the laboratory (25 °C, with 60 to 65% relative humidity, and a 14:10 h L:D photoperiod) and allowed to acclimate for 24 h prior to use in the bioassay. All assays were completed within 3 d of psyllid collection. For testing of the insecticide-susceptible population, *D. citri* adults were moved from the main laboratory colony into Plexiglas cages and maintained under the same conditions as the field-collected psyllids prior to testing. All newly emerged adults and those appearing damaged from our field collections were not used in subsequent bioassays. Adults were mouth aspirated from plants and tested with the bottle bioassay. The 4 insecticides that had produced 100% knockdown in the knockdown experiments were tested. Mixed-sex groups of adults were exposed to the concentrations of insecticides (10, 100, 1,000, and 10,000 ng/µL). Knockdown was recorded after 15, 30, 45, and 60 min of initial exposure as described for the knockdown experiments. Over 100 adults were tested at each tested time interval for each insecticide, including an acetone-only control treatment. The bioassay was repeated twice for each insecticide. Each insecticide was replicated 5 times for each bioassay.

Table 2. Site and dates of collection of *Diaphorina citri* during spring and summer of 2016 in central Florida.

Site	Location in Florida	County	Collection date	Geographic coordinates
1	Lake Alfred 1	Polk	5 May 2016	28.3525°N 81.9583°W
2	Frostproof	Polk	17 Jun 2016	27.8422°N 81.6642°W
3	Winter Garden	Orange	13 Jul 2016	28.5156°N 81.8383°W
4	Lake Alfred 2	Polk	3 Aug 2016	28.2314°N 81.7339°W

STATISTICAL ANALYSES

Mortality data were analyzed by probit analysis (Finney 1971) and were computed using PROC Probit in SAS® software version 9.4 (SAS 2013). A likelihood ratio was conducted to test the hypothesis that all *P* values were equal. The mortality in the control treatment never exceeded 3% and Abbott's formula was used to adjust for mortality of the control when it occurred (Abbott 1925). Statistical differences between LC50 values were determined using the presence or absence of overlap in the 95% fiducial limits. For each chemical, the LC50 was tested for significance according to Robertson & Preisler (1992) and Wheeler et al. (2006) to determine differences at $P \leq 0.05$.

Susceptibility data of the laboratory versus field populations were analyzed using 2-way mixed model analysis of variance (strain × time). First order interactions were removed from the analytical model if they were not significant (Sokal & Rohlf 1995). If first order interactions between the 2 factors were significant, the data were tested with a Bonferroni test in each time period ($P = 0.05$) (Sokal & Rohlf 1995). Percentage data were arcsine transformed before analysis.

Table 3. Lethal concentration values (ng/µL) for 24 h response of an insecticide-susceptible *Diaphorina citri* strain to selected insecticides using a bottle bioassay.

Insecticide	*n*[a]	χ^2	Intercept ± SE	LC50[b] (95% FL)[c]	LC95 (95% FL)[c]
Dimethoate	1,210	17.73	0.90 ± 0.21	0.06 (0.02–0.26) a	9.48 (2.03–540.76)
Fenpropathrin	1,051	1.34	0.13 ± 0.11	0.60 (0.17–2.26) b	390.24 (50.11–20,194.00)
Imidacloprid	1,201	3.52	0.39 ± 0.21	0.20 (0.02—2.27) b	191.50 (10.09–522,264.00)
Bifenthrin	1,050	41.76	0.66 ± 0.10	0.13 (0.06–0.28) b	21.08 (6.45–128.88)
Cyantraniliprole	1,051	4.39	0.35 ± 0.17	0.31 (0.06–1.62) b	78.47 (9.06–12,660.00)
Sulfoxaflor	1,200	0.29	0.05 ± 0.10	0.80 (0.26–2.46) c	797.77 (130.13–16,474.00)
Spinetoram	1,200	57.03	0.40 ± 0.05	0.19 (0.13–0.28) b	185.94 (87.95–454.45)
Flupyradifurone	1,060	19.14	0.21 ± 0.05	0.32 (0.20–0.53) b	2,341.00 (755.21–9,925.00)

[a]Number of adults tested per insecticide.
[b]LC50 values within a column follow by the same letter are not significantly different (Roberston & Preisler 1992; $P > 0.05$).
[c]FL = Fiducial limits.

Table 4. Percentage of knockdown of an insecticide-susceptible *Diaphorina citri* strain in a bottle assay treated with dilutions of standard grade insecticides (ng/µL)[a].

Insecticide	Concentration (ng/µL)	Amount (mg per bottle)	Time (min)	Knockdown rate (% ± SE)[b]
Dimethoate	10,000	1.5000	45	100.0 ± 0.0
	1,000	0.1500	45	76.7 ± 1.1
	100	0.0150	45	22.0 ± 1.3
	10	0.0015	45	17.3 ± 0.7
Fenpropathrin	10,000	1.5000	45	100.0 ± 0.0
	1,000	0.1500	45	87.3 ± 3.4
	100	0.0150	45	91.3 ± 1.3
	10	0.0015	45	80.0 ± 3.1
Imidacloprid	10,000	1.5000	60	100.0 ± 0.0
	1,000	0.1500	60	99.3 ± 1.5
	100	0.1500	60	84.7 ± 3.8
	10	0.0015	60	31.3 ± 2.3
Bifenthrin	10,000	1.5000	45	100.0 ± 0.0
	1,000	0.1500	45	82.7 ± 1.9
	100	0.0150	45	69.3 ± 1.3
	10	0.0015	45	30.7 ± 1.2
Cyantraniliprole	10,000	-[c]	-	-
	1,000	0.1500	120	42.6 ± 0.7
	100	0.0150	120	40.7 ± 3.8
	10	0.0015	120	17.3 ± 1.6
Sulfoxaflor	10,000	—	—	—
	1,000	0.1500	120	44.0 ± 3.7
	100	0.0150	120	38.0 ± 2.3
	10	0.0015	120	22.7 ± 1.6
Spinetoram	10,000	1.5000	120	86.7 ± 3.3
	1,000	0.1500	120	70.0 ± 2.8
	100	0.0150	120	70.7 ± 2.5
	10	0.0015	120	67.3 ± 2.5
Flupyradifurone	10,000	1.5000	60	100.0 ± 0.0
	1,000	0.1500	60	85.3 ± 1.3
	100	0.0150	60	68.7 ± 1.3
	10	0.0015	60	47.3 ± 2.7

[a]Each concentration of each insecticide replicated 5 times in 3 separate experiments; no knockdown recorded in controls.
[b]Ten adults per bottle per concentration.
[c]A dash (-) appearing in a column indicates that that concentration was not tested.

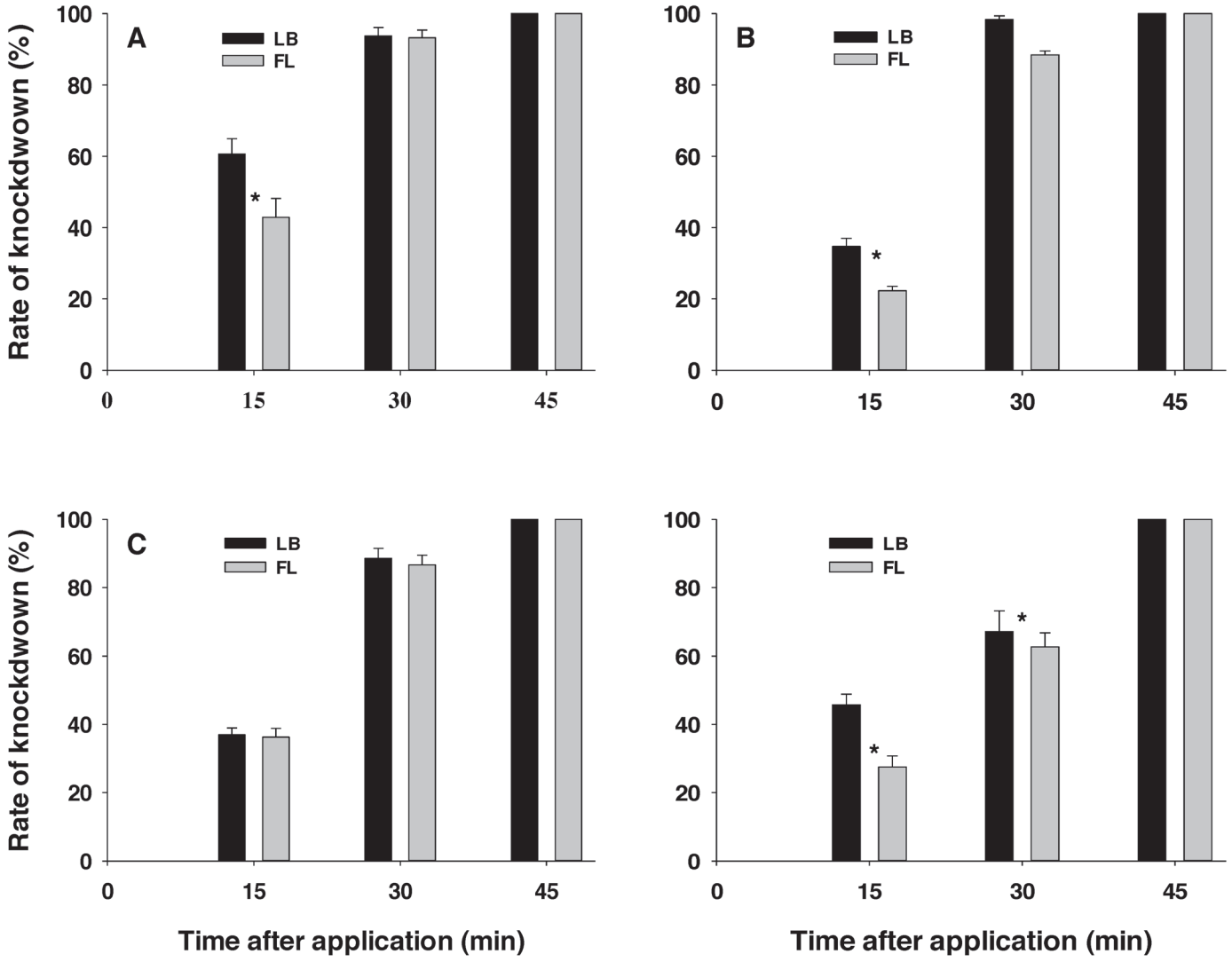

Fig. 1. Susceptibility of laboratory and field-collected populations of *Diaphorina citri* of bifenthrin tested at the diagnostic exposure time–concentration combination (A: Lake Alfred 1; B: Winter Garden; C: Lake Alfred 2; D: Frostproof; LB: laboratory strain, FL: Florida field population). Each bar represents mean ± SE. An asterisk (*) indicates significant difference between laboratory and field population at a time period based on a Bonferroni test ($P \leq 0.05$).

Results

DETERMINATION OF SUSCEPTIBILITY VALUES

The susceptibility bottle bioassay results for dimethoate, fenpropathrin, imidacloprid, bifenthrin, cyantraniliprole, sulfoxaflor, spinetoram, and flupyradifurone against *D. citri* adults are listed in Table 3. Dimethoate was the most toxic of the insecticides tested with an LC50 of 0.06 (95% fiducial limits: 0.02–0.26) ng/µL. Susceptibility to dimethoate was significantly higher than to the other insecticides tested. Sulfoxaflor was the least toxic with an LC50 of 0.80 (0.26–2.46) ng/µL. Susceptibility to sulfoxaflor was significantly lower than to the other insecticides tested. Of the 2 pyrethroids tested, bifenthrin was the most toxic with an LC50 of 0.13 (0.06–0.28) ng/µL, but the toxicity was not statistically different from fenpropathrin with an LC50 of 0.60 (0.17–2.26) ng/µL. The LC50 for imidacloprid was 0.20 (0.02–2.27) ng/µL, which was lower than for dimethoate and fenpropathrin. The diamide insecticide cyantraniliprole had an LC50 of 0.31 (0.06–1.62) ng/µL. Spinetoram had an LC50 of 0.19 (0.13–0.28) ng/µL, whereas flupyradifurone had an LC50 of 0.32 (0.20–0.53) ng/µL. These last 2 insecticides were more toxic than sulfoxaflor. In summary, the toxicities of the 8 insecticides in order from highest to lowest were dimethoate > fenpropathrin = imidacloprid = bifenthrin = cyantraniliprole = spinetoram = flupyradifurone > sulfoxaflor (Table 3).

DIAGNOSTIC INSECTICIDE CONCENTRATIONS AND EXPOSURE TIMES

The responses of the laboratory susceptible population of *D. citri* to each insecticide were used to select the diagnostic exposure times listed in Table 4. We determined the intervals at which 100% knockdown of *D. citri* occurred after exposure to an insecticide at concentrations ranging from 10 to 10,000 ng/µL. The time required for 100% knockdown of *D. citri* for dimethoate, fenpropathrin, and bifenthrin was 45 min at the 10,000 ng/µL concentration. The time required for imidacloprid and flupyradifurone was 60 min at the 10,000 ng/µL concentration.

SURVEILLANCE OF *D. CITRI* FIELD POPULATIONS WITH THE BOTTLE BIOASSAY

Using the established diagnostic exposure time and concentration, we investigated for possible insecticide resistance of field populations of *D. citri* during the spring and summer of 2016 in central Florida. Four

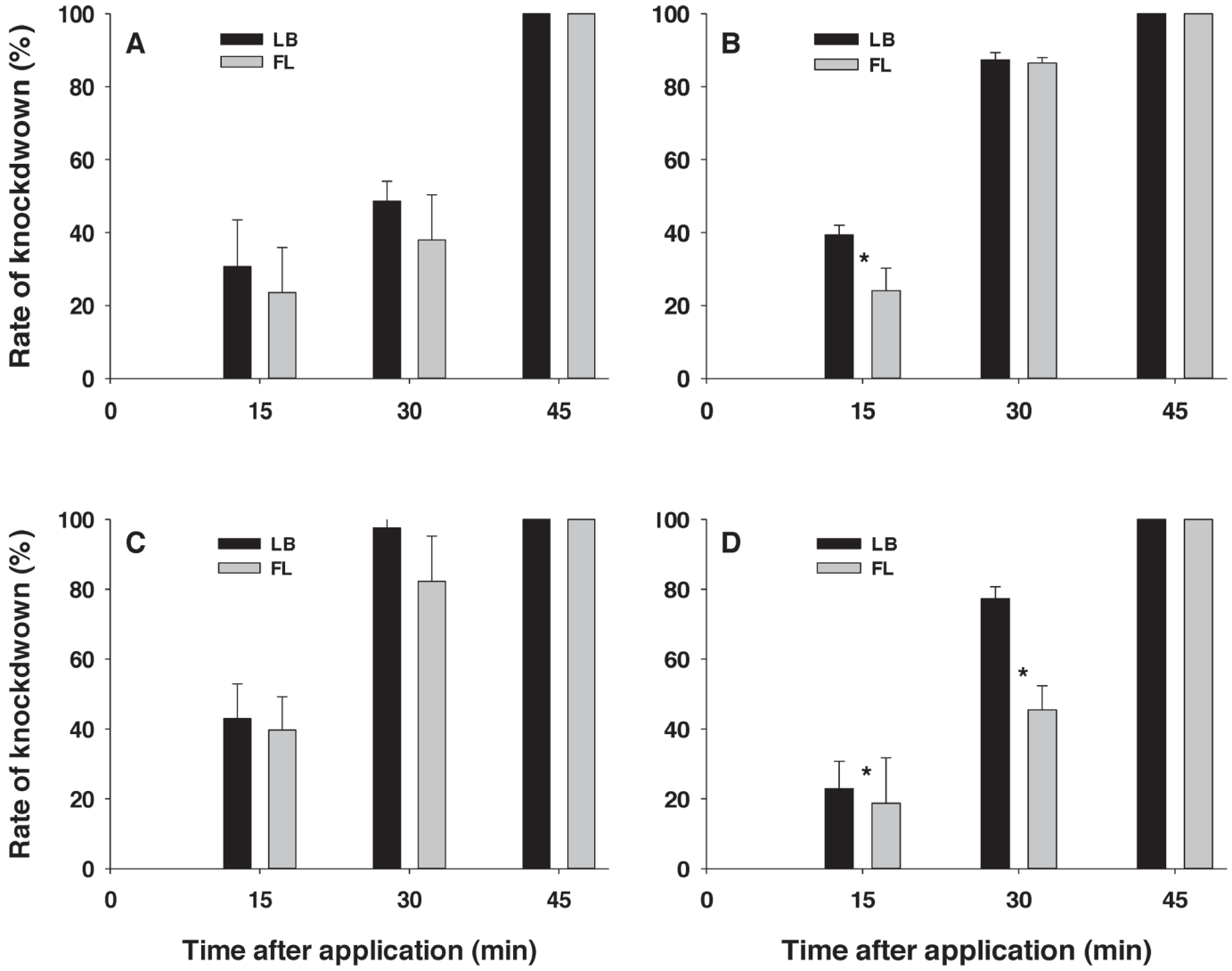

Fig. 2. Susceptibility of laboratory and field-collected populations of *Diaphornia citri* to dimethoate tested at the diagnostic exposure time–concentration combination (A: Lake Alfred 1; B: Winter Garden; C: Lake Alfred 2; D: Frostproof; LB: laboratory strain, FL: Florida field population). Each bar represents mean ± SE. An asterisk (*****) indicates significant difference between laboratory and field population based on a Bonferroni test ($P \leq 0.05$).

insecticides were evaluated using the bottle bioassay on *D. citri* adults of mixed sex and unknown ages collected from 4 locations (i.e., Lake Alfred 1, Lake Alfred 2, Winter Garden, and Frostproof). For binfenthrin, there were no significant strain × time interactions for Lake Alfred 2 (F = 1.04; df = 3; P = 0.3808) (Fig. 1C). However, the strain × time interaction was significant for Lake Alfred 1 (F = 5.82; df = 3; P = 0.0013) (Fig. 1A), Winter Garden (F = 25.94; df = 3; P < 0.0001) (Fig. 1B), and Frostpoof (F = 12.54; df = 3; P < 0.0001) (Fig. 1D). At the 15 min time point for Lake Alfred 1, and the 15 min and 30 min time points for Winter Garden and Frostpoof, there were significant differences between the field population and the laboratory population ($P \leq 0.05$). At the other time points, there were no significant differences between the laboratory and field population for each site (Fig. 1A–D; $P > 0.05$).

For dimethoate, the strain × time interaction was not significant for Lake Alfred 1 (F = 1.31; df = 3; P = 0.2794) and Lake Alfred 2 (F = 0.56; df = 3; P = 0.6438) (Fig. 2 A–C). However, there were significant differences for Winter Garden (F = 4.70; df = 3; P = 0.0047) and Frostpoof (F = 4.27; df = 3; P = 0.0078). At the 15 min time point for Winter Garden and at the 15 min and 30 min time points for Frostpoof, there were significant differences between the field populations and laboratory population ($P \leq 0.05$) (Fig. 2B and D). At the other time points, there were no significant differences between the laboratory and field population for each site (Fig. 2A–D; $P > 0.05$).

For imidacloprid, the strain × time interaction was not significant for Lake Alfred 1 (F = 0.14; df = 3; P = 0.9368) or Frostpoof (F = 1.55; df = 3; P = 0.2082) (Fig. 3A, and D). However, the strain × time interaction was significant for Winter Garden (F = 3.17; df = 3; P = 0.0293) and Lake Alfred 2 (F = 4.80; df = 3; P = 0.0042). There were significant differences between the field and laboratory populations at the 15 min and 30 min time points in Winter Garden ($P \leq 0.05$) (Fig. 3B) and at 15 min at Lake Alfred 2 ($P < 0.05$) (Fig. 3C). At the other time points, there were no significant differences between the laboratory and field population for each site (Fig. 3A–D; $P > 0.05$).

For fenpropathrin, the strain × time interaction was not significant for Winter Garden (F = 1.51; df = 3; P = 0.2200) or Lake Alfred 2 (F = 0.23; df = 3; P = 0.8781) (Fig. 4B and C). However, the strain × time interaction was significant for Lake Alfred 1 (F = 0.31; df = 3; P = 0.0318) and for Frostpoof (F = 21.92; df = 3; P < 0.0001) (Fig. 4A and D). At the 1, 15, and 30 min time points for Lake Alfred, there were significant differences between the field and laboratory populations ($P \leq 0.05$) (Fig. 4D).

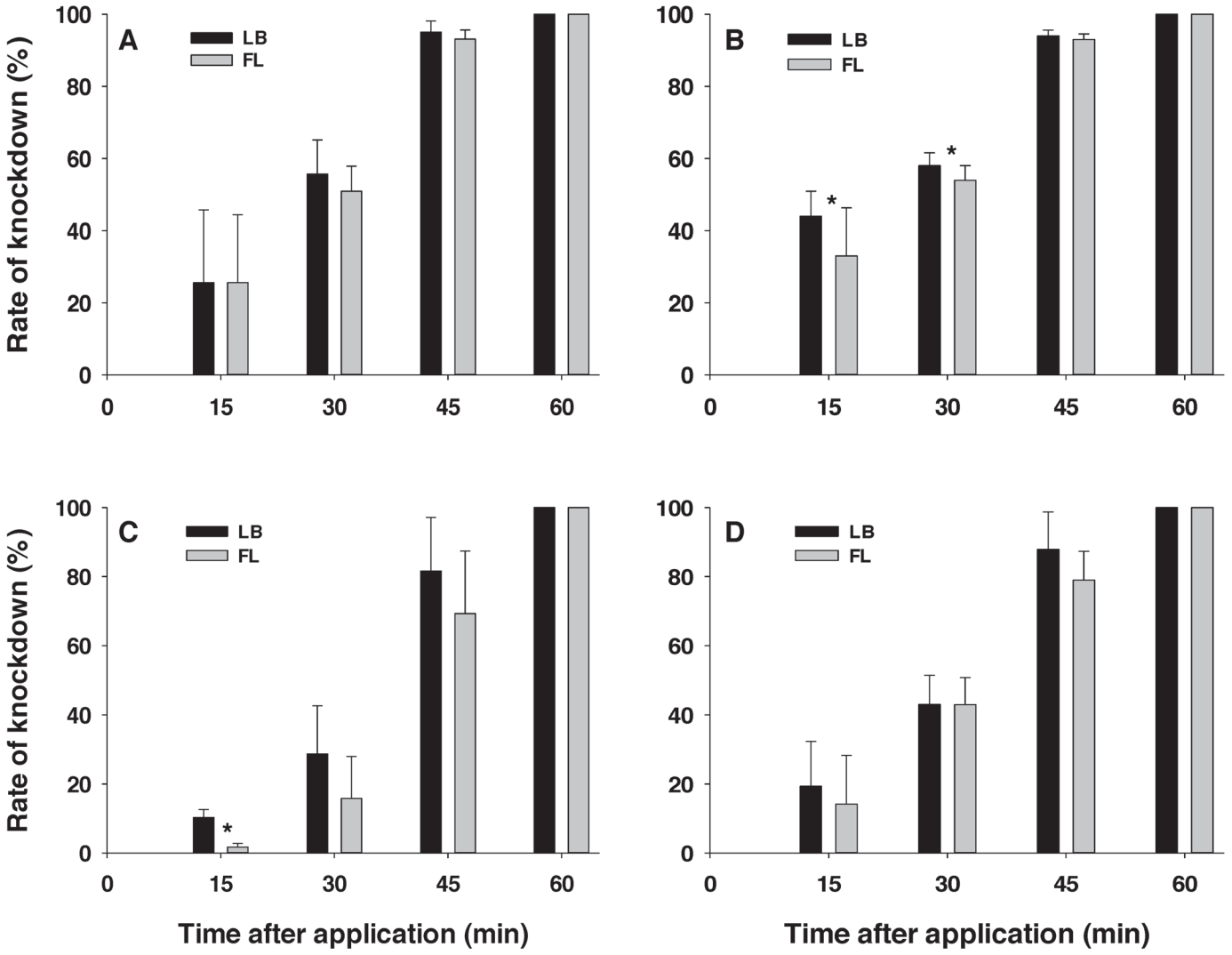

Fig. 3. Susceptibility of laboratory and field-collected populations of *Diaphorina citri* to imidacloprid tested at the diagnostic exposure time–concentration combination (A: Lake Alfred 1; B: Winter Garden; C: Lake Alfred 2; D: Frostproof; LB: laboratory strain, FL: Florida field population). Each bar represents mean ± SE. An asterisk (*) indicates significant difference between laboratory and field population at a time period based on a Bonferroni test ($P \leq 0.05$).

At the other time points, there were no significant differences between the laboratory and field population for each site (Fig. 4A–D; $P > 0.05$).

Discussion

We developed a simple bottle bioassay for measuring insecticide susceptibility in *D. citri* adults and used it to investigate susceptibility to 8 recommended insecticides. Using a laboratory susceptible population, we established diagnostic exposure times for a bottle bioassay at which 100% knockdown of *D. citri* occurred reliably for each insecticide tested. Whereas 100% knockdown occurred with dimethoate, bifenthrin, and fenpropathrin within 45 min at the 10,000 ng/µL concentration, 60 min at 10,000 ng/µL was required for flupyradifurone and imidacloprid (Table 4). If resistance is suspected in the field, the bottle assay should reveal less than 100% mortality at these exposure time–concentration diagnostic cut-offs.

The currently described bioassay is based on previously published methods (Sivasupramaniam 1997; Zamora Perea et al. 2009; Miller et al. 2010; Elamathi et al. 2014; Latheef & Hoffmann 2014). A useful bottle assay was recently developed and field tested for *D. citri* and evidence of reduced insecticide susceptibility was found in certain populations of *D. citri* in southern Florida (Kanga et al. 2016). Our bioassay specifically provides a discriminating exposure time–concentration combination that kills all exposed adult insects within approximately 1 h. The fixed concentration and exposure time represents a predetermined discriminating dose based upon data collected using a susceptible population of the test species for subsequent use against exposed field populations. Therefore, it can be used by anyone to obtain repeatable data based on a known susceptible laboratory culture that has existed since 2000 and that has been used to monitor insecticide resistance in Florida since 2008 when the first resistance monitoring program for *D. citri* was initiated (Tiwari et al. 2011).

Our method indicates that exposure time may have quantitative value, and the interaction of time and increasing concentration can be used to determine exposure time–concentration diagnostics required for 100% knockdown occurring within approximately 1 h. This potentially simplifies monitoring for possible indications of resistance in the field for pests such as *D. citri*, which occur as many populations across many thousands of hectares of crop. The cost of such a monitoring

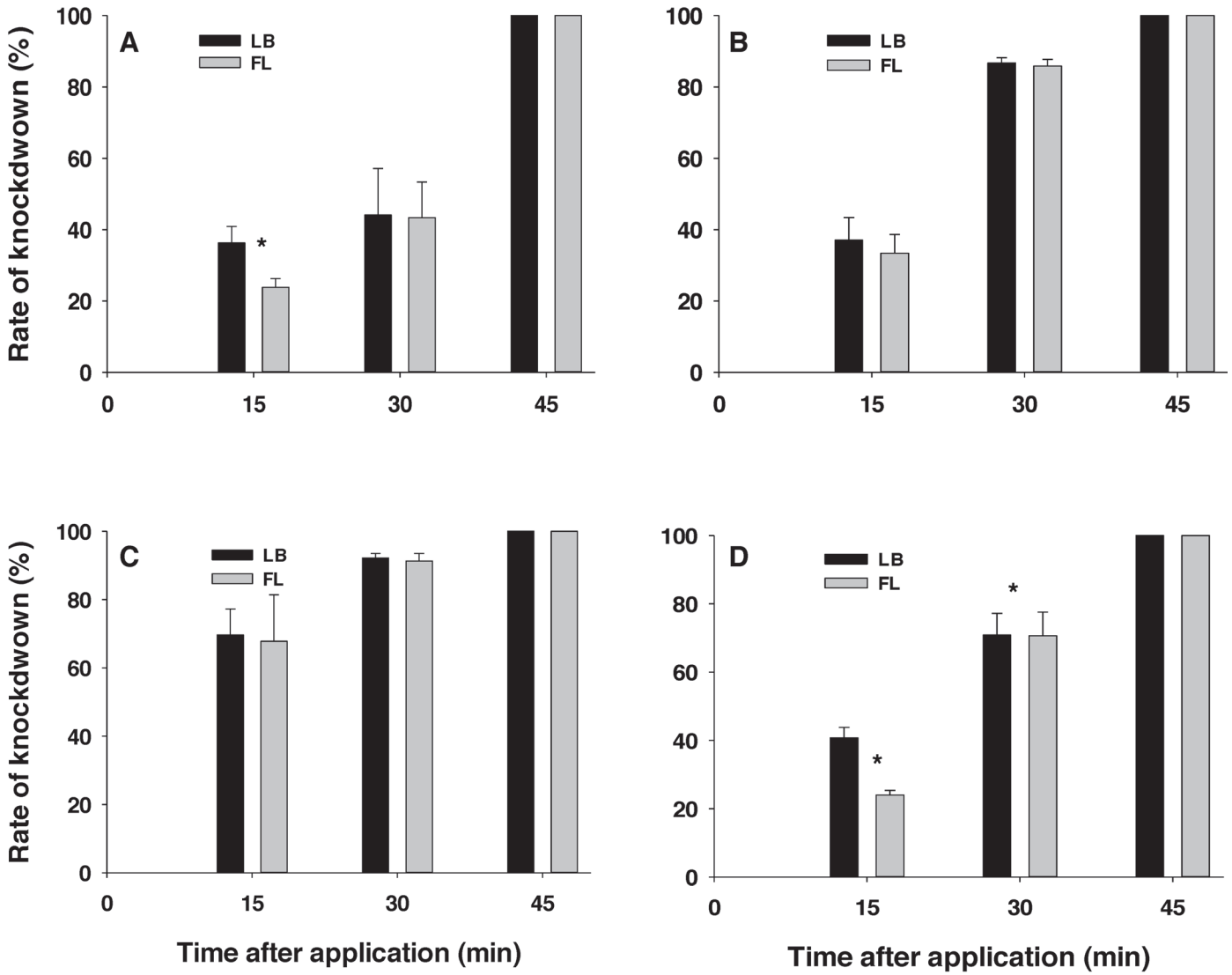

Fig. 4. Susceptibility of laboratory and field-collected populations of *Diaphorina citri* to fenpropathrin tested at the diagnostic exposure time–concentration combination (A: Lake Alfred 1; B: Winter Garden; C: Lake Alfred 2; Frostproof; LB: Laboratory strain, FL: Florida Field strain). Each bar represents mean ± SE. An asterisk (*) indicates significant difference between laboratory and field population at a time period based on a Bonferroni test ($P \leq 0.05$).

program based on this assay to cover the several hundreds of thousands of hectares of citrus in Florida is to be determined, but given the reduction in labor, the cost should be reduced as compared with other techniques. However, the finding that there were no significant differences between some of the chemicals tested at the LC50 (e.g., dimethoate and sulfoxaflor, Table 3), but were observable with others (e.g., cyantraniliprole and spinetoram, Table 4), suggests that the time factor may not be adequately calibrated in this current bottle assay for all of the chemicals tested here. In future work, we will optimize the concentrations and exposure time required to cause 100% mortality to improve the sensitivity of the bioassay for these and other insecticides used in Florida citrus.

We used diagnostic exposure time–mortality monitoring to determine possible resistance to organophosphate, pyrethroid, and neonicotinoid insecticides in field populations of *D. citri* in central Florida. Our results did not suggest current occurrence of resistance to these insecticides in 2016, which is a similar trend to that recorded throughout the state in 2015 (Coy et al. 2016). However, reduced insecticide susceptibility to some of these insecticides was reported earlier in Florida (Tiwari et al. 2011, 2015) and appears to have occurred as late as 2015 in certain regions of southern Florida (Kanga et al. 2016). Therefore, continued vigilant monitoring of insecticide resistance in Florida populations of *D. citri* is needed. Also, monitoring programs in other citrus production regions where insecticides are heavily used against *D. citri*, such as in Asia and Brazil, should be initiated. A quick and simple bottle assay that eliminates the need for having or maintaining a laboratory susceptible culture of *D. citri* should simplify establishing insecticide resistance programs for *D. citri* worldwide.

The molecular and biochemical mechanisms of insecticide resistance in *D. citri* have received significant recent attention (Tiwari et al. 2011, 2013; Liu et al. 2016). A proactive approach has been taken to characterize the molecular and biochemical response of *D. citri* to insecticides for optimization of rotation schedules (Tiwari et al. 2011; Coy et al. 2016). These investigations have applied several methods of assaying mortality of *D. citri*, including leaf disc, topical application, and glass vial bioassays (Boina et al. 2009; Tiwari et al. 2011; Coy et al. 2016; Kanga et al. 2016). The currently described diagnostic bottle bioassay for use in the field is a supplement to the previous techniques

with the specific purpose of allowing rapid determination of population-level susceptibility to a suite of commonly used insecticides. Although the list of insecticides tested here is not exhaustive for *D. citri*, other insecticides can be added rapidly with minimal time investment.

We describe a simple, rapid, and inexpensive technique for determining insecticide susceptibility in populations of *D. citri* that can be used anywhere this pest occurs without the need for measuring against a laboratory susceptible strain. This method provides results within approximately 1 h and can be used with any contact insecticide class or formulation. The bioassay can be adjusted for use with lower concentrations of insecticides by simply increasing the diagnostic exposure time beyond 1 h to further reduce the needed concentrations of insecticides used. Using this bioassay, we found little to no evidence of insecticide resistance among populations of *D. citri* in central Florida in 2016, which is consistent with a trend for reversal to susceptibility observed in Florida populations of *D. citri* since 2014 (Coy et al. 2016). In our future research, we will expand field testing of *D. citri* populations with the bottle bioassay throughout Florida for regular monitoring on an area-wide scale and for monitoring cross resistance.

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