

Effect of Cyantraniliprole, a Novel Insecticide, on the Inoculation of *Candidatus Liberibacter Asiaticus* Associated with Citrus Huanglongbing by the Asian Citrus Psyllid (Hemiptera: Liviidae)

EL-DESOUKY AMMAR,¹ DAVID G. HALL,^{1,2} AND JUAN M. ALVAREZ³

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ABSTRACT The Asian citrus psyllid (*Diaphorina citri* Kuwayama) is the principal vector of ‘*Candidatus Liberibacter asiaticus*’ (CLAs) associated with huanglongbing (HLB), the most serious citrus disease worldwide. New control measures including pesticides are urgently needed to combat HLB, especially to protect young or newly planted citrus trees from CLAs-inoculation by vector psyllids. Here, we tested CLAs-inoculation by *D. citri* adults (CLAs-exposed, reared on infected plants) by feeding them for 7 d on excised healthy citrus leaves with dry residues of cyantraniliprole (Exirel), a novel insecticide, in comparison with fenpropathrin (Danitol 2.4EC), an insecticide commonly used against *D. citri*. Fewer adults settled (putatively feeding or probing) on leaves treated with cyantraniliprole than those treated with fenpropathrin or water controls. Also, psyllid adults died at a slower rate on leaves treated with cyantraniliprole than those treated with fenpropathrin, although the final cumulative mortality did not differ between the two treatments. In quantitative real-time polymerase chain reaction tests, 59.0–65.3% of the CLAs-exposed psyllid adults were proven to be CLAs-positive. Inoculation rates of CLAs (using 10 adults per leaf) into untreated healthy citrus leaves (47.5–85%) were significantly higher than rates into leaves treated with cyantraniliprole or fenpropathrin (2.5–12.5%). Reduced inoculation rates to leaves treated with cyantraniliprole probably occurred as a result of reduced feeding or probing by *D. citri*. The excised leaf assay method, which took only a few weeks compared with up to a year or longer using whole plants, can be an effective tool for testing the effect of new pesticides or other treatments in reducing CLAs inoculation or transmission by psyllid vectors.

KEY WORDS *Diaphorina citri*, Exirel, Cyazypyr, fenpropathrin, citrus greening

Introduction

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is an invasive citrus pest that transmits the phloem-limited bacterium ‘*Candidatus Liberibacter asiaticus*’ (CLAs) implicated as the causative agent of huanglongbing (HLB, or citrus greening), the most serious disease of citrus worldwide (Halbert and Manjunath 2004, Bové 2006, Gottwald 2010, Hall et al. 2013a). *Diaphorina citri* and HLB apparently originated in Asia, but this psyllid species has invaded many countries in Central, South, and North America including the Caribbean Basin starting in the 1990s. HLB has subsequently been detected in southern United States (notably Florida), Brazil, Mexico, Belize, and several other countries in Central and South America. In Brazil and Florida, HLB is spreading rapidly throughout commercial and residential citrus plantings, causing considerable losses to these citrus industries.

HLB infection impacts tree size and vigor, fruit quality and fruit production; yield reduction can reach 30 to 100% (Gottwald 2010).

Following the discovery of HLB in Florida during 2005, a three-component management program against HLB was advocated: intensive chemical control of *D. citri*, removal of HLB-symptomatic trees, and planting disease-free nursery stock (Hall et al. 2013a). However, even repetitive applications of an array of insecticides registered for use against *D. citri* in citrus have been proven to be ineffective in preventing the spread of HLB into new citrus plantings, (Gottwald 2010, Hall et al. 2013b). Novel or improved control measures to combat *D. citri* and HLB are urgently needed to maintain a viable citrus industry in Florida, Brazil, and other areas where the HLB situation is now critical. Protecting young and newly planted citrus trees from CLAs-inoculation by infected psyllids is critical in this endeavor (Gottwald 2010, Hall et al. 2013b). However, one of the great handicaps in both applied and fundamental research on HLB is the length of time needed to test the ability of *D. citri* to inoculate or transmit CLAs into citrus plants, which can take from several months to a year to confirm when using

¹ ARS-USDA, Fort Pierce, FL 34945.

² Corresponding author, e-mail: david.hall@ars.usda.gov.

³ DuPont Crop Protection, 1090 Elkton Road, Newark, DE 19711.

whole citrus plants for inoculation (Gottwald 2010, Pelz-Stelinski et al. 2010). The rapid excised leaf “inoculativity” assay method, which has been recently developed, proved to be as efficient as and much faster than using whole citrus plants for CLAs inoculation by psyllids (Ammar et al. 2013).

Using this new assay method, we compared two insecticides in their ability to prevent or reduce inoculation or transmission of CLAs by infected psyllids: a novel insecticide cyantraniliprole (Exirel, 100 SE Cyazypyr, DuPont Crop Protection, Wilmington, DE) and fenpropathrin (Danitol 2.4EC, Valent USA Corporation, Walnut Creek, CA), a standard pesticide popular with growers for controlling *D. citri*. The Insecticide Resistance Action Committee classifies cyantraniliprole and fenpropathrin as having completely different modes of action (groups 28 and 3, respectively, <http://www.irac-online.org/eClassification/>). Fenpropathrin is a sodium channel modulator, while cyantraniliprole is a novel cross-spectrum anthranilic diamide insecticide that selectively activates the ryanodine receptors in insect muscles (Sattelle et al. 2008). Cyantraniliprole has been shown to reduce transmission of some plant viral and bacterial pathogens that are transmitted by thrips and an array of Hemipteran pests including whiteflies, aphids, and psyllids (Castle et al. 2009; Stansly and Kostyk 2010; Jacobson and Kennedy 2011, 2013a,b; Govindappa et al. 2013; Cameron et al. 2014). The investigation presented here was thus conducted to study the effect of cyantraniliprole and fenpropathrin on *D. citri* survival and settling (presumed feeding and probing), as well as CLAs inoculation into citrus leaves by infected psyllids.

Materials and Methods

Psyllids, Plants, and CLAs Bacterium. We used “CLAs-exposed” and “non-exposed” *D. citri* adults in this experiment. The “CLAs-exposed” adults were from a laboratory colony that has been maintained for several generations (eggs, nymphs and adults) on HLB-symptomatic, CLAs-infected (PCR-positive) citrus plants (rough lemon, *Citrus jambhiri* Lushington). Nonexposed (CLAs-free) *D. citri* adults were taken from a laboratory colony maintained since 2000, originally on healthy orange jasmine (*Murraya exotica* L.) trees, and more recently on healthy citrus (*Citrus macrophylla* Wester) in a greenhouse (Hall and Richardson 2013). No wild or new *D. citri* are introduced into this colony, and individuals from the colony are assayed with quantitative real-time polymerase chain reaction (qPCR) every 3 mo to ensure that the colony remains CLAs-free.

Mortality, Settling, and Inoculativity Tests. The excised leaf assay method described by Ammar et al. (2013) was used to test the effects of cyantraniliprole and fenpropathrin on *D. citri* survival, settling behavior and inoculation of CLAs into healthy citrus leaves. Medium-size mature leaves were excised from healthy (non-CLAs infected) young sweet orange plants [*Citrus sinensis* (L.) Osbeck ‘Ridge Pineapple’] grown in the

greenhouse. These leaves were washed twice with deionized (DI) water, air-dried, dipped briefly into solutions of cyantraniliprole or fenpropathrin (see below) or DI water control, and air-dried in a fume hood for 24 h. For each leaf, a 0.6-ml microcentrifuge tube was filled with DI water and sealed tightly with Parafilm “M” laboratory film (American National Can, Chicago, IL), a small hole was created through the center of the Parafilm membrane, and the petiole of a leaf was slipped through this hole down into the water. The microcentrifuge tube with the leaf was then placed into a 50-ml BD Falcon Conical Tube (BD Biosciences, San Jose, CA). Psyllid adults (10 per leaf) were added to each tube and the tube was covered with a nylon mesh screen under the cap (with the flip-top removed) for ventilation. These tubes were placed in a tube-rack on a bench top in the laboratory under ambient conditions ($23.7 \pm 1.5^\circ\text{C}$ and a photoperiod of 14:10 [L:D] h) for 7 d. All tubes were observed with naked eye and/or under a stereomicroscope at 1, 3.5, 5, 24, 29, 48, 72, 144, and 168 h after the psyllids were introduced into the tubes. At each time point, the number of *D. citri* adults in the following categories was recorded: live adults that settled on the leaf (mostly in their normal feeding or probing position, with their body angled at 45° with the leaf); live adults but not on the leaf; dead adults (completely motionless); or moribund adults (not actively moving but twitching). Dead or moribund psyllid adults were collected daily and stored at -80° for qPCR assays.

Four treatments were tested: 1) CLAs-exposed psyllids on cyantraniliprole-treated leaves; 2) CLAs-exposed psyllids on fenpropathrin-treated leaves; 3) positive controls (CLAs-exposed psyllids on leaves treated with DI water); and 4) negative controls (non-CLAs-exposed psyllids on DI water-treated leaves). Cyantraniliprole was used as a 125 ppm ai solution (184 mg of product per 150 ml of water, equivalent to 1.18 liter of product or 117 g ai in 946 liter of water per Ha), whereas fenpropathrin was used as a 450 ppm ai solution (218.5 mg of product per 150 ml of water, equivalent to 1.48 liter of product or 425 g ai in 946 liter of water per Ha). The cyantraniliprole rate was intermediate with respect to labeled rates (0.99–1.49 liter per Ha); the fenpropathrin rate was near the upper limit of the labeled rate (1.17–1.56 liter per Ha). Twenty replicates were tested per treatment (10 adults per leaf per tube), and the experiment was repeated twice: the first on 10–17 September 2013 and the second on 22–29 October 2013.

Quantitative Real-time Polymerase Chain Reaction. Following the 1-wk exposure time on healthy excised leaves, live psyllids were removed from each tube, and each leaf was placed individually into a zipped plastic bag and incubated for 7 d ($25.7 \pm 0.4^\circ\text{C}$ and a photoperiod of 14:10 [L:D] h). The leaves were then washed thoroughly with DI water (to remove honeydew or eggs possibly laid on the leaves by *D. citri* adults), stored at -80°C , and later processed for DNA extraction and qPCR using two CLAs primer sets separately: HLBasper primers (Li et al. 2006) and the more sensitive LJ900 primers (Morgan et al. 2012). Almost

all psyllids (live and dead) from the cyantraniliprole and fenpropathrin treatments (193–198 of the original 200 psyllids per replication of each treatment), 100 positive control (CLAs-exposed) psyllids per replication, and 46–50 negative control (nonexposed) psyllids per replication were also stored at -80°C and later processed for qPCR testing using only the HLBasper primers. DNA extraction and qPCR procedures for psyllids and leaves were as described earlier (Ammar et al. 2013), and each leaf or psyllid DNA sample was tested in two to three replicates (wells) in the qPCR plates. Psyllid or leaf samples were considered CLAs-positive when at least two replicates had cycle quantification (Cq) values <40 , and in such a case, an average Cq value was calculated for each positive psyllid or leaf.

Statistical Analyses. The following variables were subjected to analyses of variance (PROC ANOVA or PROC GLM, SAS Institute 2010), and mean separations were investigated using the Ryan–Einot–Gabriel–Welsch multiple range test ($P=0.05$): percentages of psyllids that settled on leaves over time; final percentage of psyllid mortality under each treatment; percentages of psyllids positive for CLAs and titers of CLAs within these psyllids; and for each primer set, percentages of CLAs-positive leaves and titers (Cq values) of CLAs within these leaves. Main effects of the analysis on percentages of psyllids settling on leaves were treatment, replication, and observation hour. For all other analyses, means per treatment per replication were computed and then subjected to an analysis of variance with main effects treatment and replication. All percentage data were arcsine-transformed for the statistical analyses (Gomez and Gomez 1984).

Results

Settling and Mortality of *D. citri* Adults on Citrus Leaves. *Diaphorina citri* adults exposed to leaves treated with cyantraniliprole died much slower than those in the fenpropathrin treatment. Within 1 h of confinements on treated leaves, $>60\%$ of adults exposed to leaves treated with fenpropathrin were either dead or showed signs of poisoning (twitching), while much fewer adults exposed to leaves treated with cyantraniliprole died or showed such signs (Fig. 1A). However, by the end of the 7-d observation period, mortality was not significantly different between psyllid adults exposed to fenpropathrin (86.7%) or cyantraniliprole (90.8%; Fig. 1B; Table 1). Among live adults, significantly fewer adults were observed to settle on leaves (putatively feeding or probing) treated with cyantraniliprole than on leaves treated with fenpropathrin or on the positive or negative control leaves (Fig. 1C; Table 2). Mortality in the positive and negative controls was very low and not significantly different (Table 1).

CLAs-Infectivity and Inoculativity of *D. citri*. In qPCR tests with HLBasper primers, a high percentage of the CLAs-exposed *D. citri* adults were

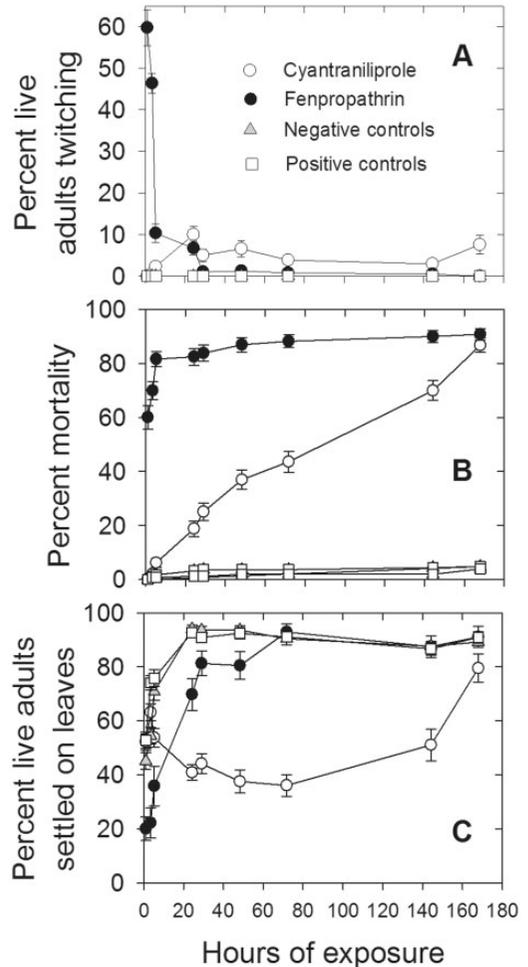


Fig. 1. Mortality, apparent paralysis (twitching), and settling activity over time of *D. citri* adults exposed to excised citrus leaves treated with cyantraniliprole, fenpropathrin, or water controls. (A) Percentage of live adults incapacitated (twitching). (B) Cumulative percent mortality of adults. (C) Percentage of live adults settled on citrus leaves (probably feeding or probing). All adults were from a colony of CLAs-exposed psyllids except for those included as negative controls, which were from a colony of nonexposed psyllids.

Table 1. Mean cumulative percentage mortality of *D. citri* adults after 7 d of exposure to leaves treated with cyantraniliprole, fenpropathrin, or water controls

Treatment	Mean (SEM) % mortality ^a
Cyantraniliprole	86.7 (7.8)a
Fenpropathrin	90.8 (6.9)a
Positive control	4.8 (3.4)b
Negative control	3.9 (0.5)b

All adults were from a colony of CLAs-exposed psyllids except for those included as negative control ($N=400$ adults per treatment)

^aMeans followed by the same letter are not significantly different according to the Ryan–Einot–Gabriel–Welsch multiple range test, $F_{3,3}=29.6$; $P=0.01$. Analysis on arcsine-transformed percentages, raw mean percentages are presented.

Table 2. Mean percentage over nine sampling times of live *D. citri* adults that settled on leaves treated with cyantraniliprole, fenpropathrin, or water controls

Treatment	Mean (SEM) % settled on leaves ^a
Cyantraniliprole	50.9 (6.2)c
Fenpropathrin	64.5 (5.5)b
Positive control	83.0 (0.9)a
Negative control	79.8 (2.2)a

All adults were from a colony of CLAs-exposed psyllids except for those included as negative control ($N = 400$ adults per treatment on start date).

^aMeans in the same column followed by the same letter are not significantly different according to the Ryan–Einot–Gabriel–Welsch multiple range test, $F_{3,59} = 17.9$; $P = 0.005$. Analysis on arcsine-transformed percentages, raw mean percentages are presented.

Table 3. Percentages of *D. citri* adults testing positive for CLAs in each treatment (using HLBasper primers in qPCR)

Treatment	Mean (SEM) % positive ^a	Mean (SEM) Cq value of Clas-positive psyllids ^b
Cyantraniliprole	65.3 (14.5)a	31.5 (0.1)a
Fenpropathrin	64.3 (11.9)a	32.8 (0.3)a
Positive controls	59.0 (20.0)a	31.5 (0.3)a
Negative controls	0.0 (0.0)b	–

All adults were from a colony of CLAs-exposed psyllids except for those included as negative control

Means in the same column followed by the same letter are not significantly different according to the Ryan–Einot–Gabriel–Welsch multiple range test, $P = 0.05$.

^a $F_{3,3} = 24.1$; $P = 0.01$. Analysis on arcsine -transformed percentages, raw mean percentages are presented.

^b $F_{2,2} = 7.5$; $P = 0.12$.

CLAs-positive (59.0–65.3%), with no significant difference between treatments (Table 3). CLAs titer in the exposed psyllids was relatively high, as indicated by low mean Cq values (31.5–32.7). None of the negative control (nonexposed) psyllid adults tested CLAs-positive in these qPCR assays.

Relatively high rates of CLAs inoculation occurred by CLAs-exposed *D. citri* (using 10 adults per leaf) when they fed on noninsecticide treated leaves: 47.5 and 85% of these leaves became CLAs-infected based on the HLBasper and LJ900 primers, respectively. Significantly lower inoculation levels were obtained when CLAs-exposed psyllids fed on leaves treated with cyantraniliprole (10.0–17.5% leaves infected) or fenpropathrin (2.5–10% leaves infected). There were no significant differences in inoculation rates by CLAs-exposed psyllids between the cyantraniliprole and fenpropathrin treatments based on either primer set ($N = 40$ per treatment). None of the negative control (nonexposed) psyllids inoculated CLAs into leaves based on HLBasper primers. However, using the more sensitive LJ900 primers, 1 of the 40 leaves exposed to non-CLAs-exposed psyllids tested positive (Table 4). CLAs titer, as indicated by the Cq values, in leaves that were considered positive for CLAs did not differ significantly among treatments (Table 5).

Table 4. Percentage of CLAs-infected leaves 1 wk after inoculation by *D. citri* adults to sweet orange leaves treated with cyantraniliprole, fenpropathrin or water controls, as detected by qPCR (10 adults/leaf, 40 leaves/treatment)

Treatment	Mean (SEM) % transmission ^a	
	LJ900 primers ^b	HLBasper primers ^c
Cyantraniliprole	17.5 (12.5)b	10.0 (5.0)b
Fenpropathrin	10.0 (5.0)b	2.5 (2.5)bc
Positive control	85.0 (15.0)a	47.5 (7.5)a
Negative control ^d	2.5 (2.5)b	0.0 (0.0)c

All adults were from a colony of CLAs-exposed psyllids except for those included as negative control

^aMeans in the same column followed by the same letter are not significantly different according to the Ryan–Einot–Gabriel–Welsch multiple range test, $P = 0.05$. Analyses on arcsine-transformed percentages, raw percentages are presented.

^b $F_{3,3} = 34.2$; $P = 0.008$.

^c $F_{3,3} = 56.9$; $P = 0.004$.

^dOne of the 40 leaves tested positive based on the LJ900 primers but not with the HLBasper primers.

Table 5. Mean Cq values for leaves that proved CLAs-positive (Cq < 40 in qPCR tests) 1 wk after inoculation by *D. citri* adults (10 adults per leaf, 40 leaves per treatment)

Treatment	Mean (SEM) Cq value ^a	
	LJ900 primers ^b	HLBasper primers ^c
Cyantraniliprole	32.0 (0.5)a	38.5 (0.6)a
Fenpropathrin	32.1 (0.6)a	39.0 (-)a
Positive control	30.6 (0.1)a	37.6 (0.5)a
Negative control ^d	–	–

All adults were from a colony of CLAs-exposed psyllids except for those included as negative control.

^aMeans in the same column followed by the same letter are not significantly different according to the Ryan–Einot–Gabriel–Welsch multiple range test, $P = 0.05$.

^b $F_{2,2} = 2.3$; $P = 0.3$.

^c $F_{2,1} = 0.7$; $P = 0.6$. One replication of the fenpropathrin treatment had no leaves testing positive.

^dOne of the 40 leaves tested positive based on the LJ900 primers (Cq = 31.5), but not with the HLBasper primers.

Discussion

Fenpropathrin is an effective insecticide for controlling *D. citri* (Rogers et al. 2012). Our research indicated that *D. citri* adults exposed to leaves treated with cyantraniliprole were much slower to die than those in the fenpropathrin treatment. However, CLAs inoculation into excised leaves by CLAs-infected *D. citri* was only 2.5 to 17.5% on leaves treated with either cyantraniliprole or fenpropathrin compared with 47.5–85% on leaves not treated with insecticide. In our previous work (Ammar et al. 2013), we showed that CLAs inoculation rates by *D. citri* using excised citrus leaves were comparable with CLAs inoculation or transmission rates by *D. citri* using whole citrus plants. Thus, both insecticides appear to reduce CLAs inoculation or transmission into citrus, despite their different modes of action and the difference in how fast death occurred when psyllids were exposed to dry residues of these insecticides.

In contrast to the quick kill of *D. citri* by fenpropathrin, mortality of *D. citri* exposed to cyantraniliprole occurred slower, with >80 h of exposure required for 50% *D. citri* mortality. This slow mortality rate may be a consequence of cyantraniliprole-induced repellency or antifeeding effect, as large proportions of *D. citri* remained alive in the assay vials for many hours but settled at the bottom or on the inside surface of the vials rather than on leaves. Relatively few adults exposed to cyantraniliprole exhibited any visible symptoms of being poisoned (e.g., twitching) before they died. It was not determined if *D. citri* exposed to cyantraniliprole died in response to toxic properties of cyantraniliprole or to starvation. Mortality rates of psyllids exposed to cyantraniliprole treatment in this study were similar to mortality rates reported for psyllids held without food (Hall and McCollum 2011), supporting the notion that these adults died of starvation or lack of feeding. In addition, Tiwari and Stelinski (2013) reported that the number of honeydew droplets produced by *D. citri* exposed to leaves treated with cyantraniliprole were significantly fewer than those on control leaves treated with water. These authors also noted a reduced settling of psyllids on cyantraniliprole-treated trees and consequently speculated that cyantraniliprole might reduce CLAs transmission (Tiwari and Stelinski 2013), as indirectly demonstrated in our study.

Our observations of reduced feeding by *D. citri* on leaves treated with cyantraniliprole is consistent with reports that cyantraniliprole reduces feeding by other sap-sucking insects including *Bemisia tabaci* (Gennadius) (Cameron et al. 2013, 2014; Civolani et al. 2014), thrips (Jacobson and Kennedy 2011, 2013a), and aphids (Jacobson and Kennedy 2013b). Significant reductions occurred in inoculation rates of CLAs by *D. citri* to leaves treated with cyantraniliprole, which was probably a result of adults avoiding to feed on these leaves. Among leaves which *D. citri* did inoculate, CLAs titers were similar among leaves treated with fenpropathrin, cyantraniliprole, or water. Thus, the two insecticides reduced inoculation occurrence, but apparently CLAs titer was not affected once the bacteria was inoculated into the leaf. In the negative control group, 1 leaf (of the 40) tested CLAs-positive by qPCR using the LJ900 primers, but as none of the psyllids from the non-CLAs-exposed colony tested positive for the pathogen, we attributed this to possible sample contamination or a false positive result known to occasionally occur with these more sensitive primers (Morgan et al. 2012).

Managing *D. citri* populations is considered one of the three principle control strategies for HLB, and chemical control with insecticides is currently considered the only option in conventional citrus. In orchards infected by HLB, the ultimate goal of an insecticide program against *D. citri* is to reduce the spread of CLAs. Our results confirm that residues of contact insecticides like the acutely toxic fenpropathrin can significantly reduce inoculation or transmission of CLAs through rapid *D. citri* knockdown and death. Our results also show that fresh, dry residues of insecticides with antifeeding activity or repellency such as

cyantraniliprole can be effective in reducing CLAs inoculation or transmission, even if the materials have low acute toxicity to *D. citri*. Such materials would likely be less toxic to other insects including beneficials, and in fact, cyantraniliprole has been shown to be more compatible than other insecticides with some natural enemies including coccinellid predators, staphylinid beetles, carabid beetles, *Trichogramma* parasitoids, and Phytoseiid predatory mites (Mandal 2012, Misra and Mukherjee 2012, Misra 2013, Rebelles et al. 2014). Notably, fresh dry residues of cyantraniliprole were recently reported to be 296 times more toxic to *D. citri* than to one of its parasitoids, *Tamarixia radiata* (Waterson) (Tiwari and Stelinski 2013). Dry residues of fenpropathrin and other conventional insecticides have been shown to be highly toxic to *T. radiata* (Hall and Nguyen 2010).

A logical next research step would be to evaluate cyantraniliprole under field conditions for efficacy in reducing transmission of CLAs. Of interest would be the residual period of protection against transmission in the field, which might be as long as a month, based on the residual activity of cyantraniliprole reported against population levels of *D. citri* (Tiwari and Stelinski 2013).

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