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Available at: https://doi.org/10.1093/jee/toaa118

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TYLCV Infection Alters *Bemisia tabaci* MED (Hemiptera: Aleyrodidae) Vulnerability to Flupyradifurone

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†These authors contributed equally to this work.
Abstract

The whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae) is a major phloem-feeding pest of agricultural crops that is also an important vector of many plant diseases. The *B. tabaci* Mediterranean (‘MED’) biotype is a particularly effective vector of *Tomato yellow leaf curl virus* (TYLCV), a devastating plant pathogen. While insecticides play an important role in the control of MED and TYLCV, little is known about how TYLCV infection affects MED susceptibility to insecticides. We conducted research addressing how MED susceptibility to flupyradifurone, the first commercially available systemic control agent derived from the butenolide class of insecticides, was affected by TYLCV infection. We first conducted bioassays determining the LC$_{15}$ and LC$_{50}$ for control and viruliferous MED feeding on either water- or insecticide-treated plants. We next measured several demographic parameters of control and viruliferous MED exposed to either insecticide- or water-treated plants. TYLCV infection increased MED tolerance of flupyradifurone: the LC$_{15}$ and LC$_{50}$ of viruliferous MED were double that of uninfected MED. Viral infection also altered MED demographic responses to flupyradifurone, but in an inconsistent manner. While the ability of TYLCV and other persistently-transmitted viruses to benefit *Bemisia* via manipulation of host plant defense is well-known, this appears to be the first example of virally-mediated changes in vector susceptibility to an insecticide.

Key Words

Insecticide, Sivanto, tolerance, *Bemisia*, TYLCV
The whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) is a major phloem-feeding pest of both field and greenhouse crops worldwide (Stansly and Naranjo 2010). Its management is complicated by the fact that *B. tabaci* contains over 30 phenotypically identical but genetically distinct cryptic species (Liu et al. 2012, Hadjistylli et al. 2016) that vary widely in traits such as insecticide resistance (Chen et al. 2016, Xie et al. 2017). *Bemisia tabaci* Mediterranean (MED) poses a particular threat to agriculture due to its invasiveness. Since its arrival in China in 2003 (Chu et al. 2006), it has displaced both native and invasive *B. tabaci* throughout the country (Teng et al. 2010).

Although *Bemisia* feeding can itself reduce plant growth, its primary threat to agriculture occurs via its ability to transmit a wide variety of plant viruses. MED is particularly effective at transmitting such viruses, and its invasion is often associated with plant disease outbreaks (Ning et al. 2015). The *Tomato yellow leaf curl virus* (TYLCV) is a particularly damaging pathogen that has caused significant damage worldwide (Jones 2003). TYLCV relies on *B. tabaci* as a vector to spread among plants (Fereres and Moreno 2009). As a result, *Bemisia*- plant-TYLCV interactions have been the subject of intense interest and researchers have confirmed the mutualistic relationship between *B. tabaci* and the virus. It is now known, for instance, that TYLCV can increase *Bemisia* fitness via its suppression of plant defense (Zhang et al. 2012, Luan et al. 2013) and that MED benefits from feeding on TYLCV-infected hosts (Pan et al. 2013a, Shi et al. 2019).

Insecticides play an important role in an integrated pest management approach to controlling *Bemisia* and viral outbreaks in agricultural systems. Because the whitefly can rapidly develop insecticide resistance, the continued development and deployment of novel compounds
is essential for effective pest control. One such compound is flupyradifurone, the first commercially available systemic control agent derived from the butenolide class of insecticides (Nauen et al. 2015). This compound, an agonist on insect nicotinic acetylcholine receptors, differs structurally from other chemicals that target these receptors. As a result, it is effective against neonicotinoid- and pymetrozine-resistant *Bemisia* populations (Nauen et al. 2015).

A recent assessment of MED survival and TYLCV transmission found that while flupyradifurone rapidly killed MED and reduced TYLCV transmission by 85%, treatment with the neonicotinoid thiomethoxam only reduced viral transmission by 25% (Roditakis et al. 2017). Other work confirming the general efficacy of flupyradifurone against *B. tabaci* Middle East-Asia Minor 1 (MEAM1) nonetheless found a few field populations with high levels of flupyradifurone tolerance (Smith et al. 2016). While increasing insecticide tolerance in its vector would clearly benefit TYLCV and similar viruses, there is no published research assessing whether viruses can provide such benefits. Alternately, TYLCV could affect MED in a manner similar to Rickettsia, which is correlated with increased *Bemisia* sensitivity to a range of different insecticides (Kontsedalov et al. 2008, but see Pan et al. 2013b). Understanding how TYLCV affects the flupyradifurone tolerance of its vector is important to maximize the effective use of this important insecticide.

We report the results of two experiments exploring how TYLCV infection affected MED susceptibility to flupyradifurone (trade name Sivanto). We first determined the LC$_{15}$ and LC$_{50}$ for control and viruliferous MED feeding on plants treated with either Sivanto or distilled water. We calculated both LC$_{15}$ and LC$_{50}$ because chemical degradation and dilution gradually reduce insecticide concentrations following application (e.g., Roditakis et al. 2017).

We next measured several demographic parameters of control and viruliferous MED exposed to either insecticide-
or water-treated plants. Although TYLCV infection has little direct effect on MED fitness (Pan et al. 2013a, Su et al. 2015), recent work found a net downregulation of detoxification enzymes in TYLCV-infected MED (Ding et al. 2019); we hypothesized that viruliferous MED would be more sensitive to Sivanto than uninfected individuals.

**Materials and Methods**

**Plants:** Tomato plants (*Solanum lycopersicum* L., cv. Zhongza 9) were grown individually in two-liter pots in a greenhouse with natural lighting and controlled temperature (26±2°C). All plants were grown in a 10:5:1 (by volume) mixture of peat moss, vermiculite, and organic 8-8-8 fertilizer. TYLCV-infected plants were produced with injection of *Agrobacterium tumefaciens*-mediated TYLCV clones (Shanghai isolate) at the 3-4 true leaf stage (Zhang et al. 2009). The plants were grown for four weeks post-injection to give them time to display infection-associated pathological symptoms.

**Insects:** The whitefly *Bemisia tabaci* MED (Q) was first collected in 2009 from poinsettia, *Euphorbia pulcherrima* Wild. (ex Klotz.), in Beijing, China. It was reared on poinsettia. In 2015, a portion of the population (~300 adults) was transferred to the Tianjin Institute of Plant Protection and reared on cotton plants (*Gossypium herbaceum* L., cv DP99B) in 80 mesh nylon insect cages (45×45×60 cm) under 26±2°C, 60±10% RH, 14L:10D photoperiod. A viruliferous MED population was produced by transferring ~300 whiteflies into a cage with four TYLCV-infected tomato plants; a parallel uninfected MED population was produced by transferring >300 whiteflies into a cage with four healthy tomato plants. Both viruliferous and uninfected MED were reared for two generations on their respective plants before being used for experiments. Colony purity was monitored every 2-3 generations using a DNA marker (Khasdan et al. 2005), and TYLCV infection was confirmed via PCR validation (Ghanim et al. 2007).
Flupyradifurone bioassay of viruliferous and uninfected MED: Sivanto 200SL (17.09% flupyradifurone) was provided by Bayer Crop Science (China) Company Ltd. and diluted with distilled water to five different concentrations: 200 mg[Al]kg⁻¹, 100 mg[Al]kg⁻¹, 50 mg[Al]kg⁻¹, 25 mg[Al]kg⁻¹, and 12.5 mg[Al]kg⁻¹. For each of the five concentrations and an additional distilled water control (a total of six treatments), 200 mL was added to a 500 mL plastic spray bottle. For each concentration, one spray bottle was used to spray four tomato plants that were each at the 6-7 true leaf stage; plants were sprayed until drip-off. One day after spraying, 100 newly-emerged (within 24 hours) adult MED per plant were placed in clip cages attached to the abaxial side of both the third and fourth leaves of each sprayed plant. Clip cages were kept on for two days; the number of living and dead MED were then counted. This work was conducted in a climate-controlled chamber at 26±1 °C and 60±10% RH with 14L:10D photoperiod.

Demographic responses of viruliferous and uninfected MED to flupyradifurone (LC₁₅): Data from the above-mentioned experiment was used to calculate the LC₁₅ for viruliferous MED. A solution of this concentration was sprayed on healthy tomato plants at the 6-7 true leaf stage until drip-off. Another group of healthy tomato plants was sprayed to drip-off with distilled water. After 24 hours, approximately 100 newly emerged (within one day) viruliferous or uninfected MED were attached in separate clip cages to the abaxial side of the third and fourth leaves of either a Sivanto-treated or control plant. This produced four treatments: TYLCV (uninfected, viruliferous) crossed with insecticide (dH₂O, Sivanto). This work were conducted in a climate-controlled chamber at 26±1 °C and 60±10% RH with 14L:10D photoperiod. Clip cages were removed after two days and the living and dead adult MED collected and counted.

Female adult longevity and first-week fecundity: Thirty female MED from each of the four treatments (=120 total) were placed individually in clip cages. Each cage was then clipped
on the abaxial side of a middle leaf of an unsprayed healthy tomato plant (6-7 true leaf stage). A total of two MED were clipped onto each plant, one per leaf, and both MED on a given plant had the same infection status (i.e., they were both either uninfected or viruliferous). The clip cages were checked each day for MED mortality; after one week, all surviving adults were individually transferred to new unsprayed healthy tomato plants and the number of eggs laid during the first week counted.

Egg-to-adult survival and developmental time: Five pairs of newly-emerged MED (within one day; 1:1 sex ratio) from a given treatment were placed into a single clip cage and clipped onto the abaxial side of a middle leaf of an unsprayed healthy tomato plant (6-7 true leaf stage). Only one clip cage was attached to each plant. This was replicated 10 times in each of the four treatments, for a total of 40 replicates. After one day, each clip cage was opened and the adults were removed, leaving only the eggs and nymphs. Each clip cage was then inspected daily and the number of nymphs and adults recorded. Daily inspections continued until the last nymph had either entered adulthood or died.

Statistical analysis: Probit parameter estimation of the concentration-mortality response for viruliferous and uninfected MED in the six concentrations were calculated using POLO-PC (Russell et al. 1977, LeOra 1987). These parameters included LC$_{15}$ and LC$_{50}$ values expressed in mg[Al]kg$^{-1}$ and their corresponding 95% confidence limit (CL) along with the slopes of the probit regressions. Between-treatment differences in the mortality of viruliferous and uninfected MED were calculated using 95% CLs; LC$_{15}$ or LC$_{50}$ values for viruliferous and uninfected MED were considered significantly different if their corresponding 95% CLs did not overlap.

Data on each of the demographic responses was analyzed using two-way ANOVA to assess the main effects of TYLCV (uninfected, viruliferous) and insecticide (dH2O, Sivanto) as
well as their interaction. When one or more main effects or their interaction was significant at $p = 0.05$, Tukeys’ HSD was used for means separation tests. Data on adult longevity and survival was sqrt transformed before analysis. All analyses were conducted using JMP 9.0.0 (SAS 2010).

**Results**

Viruliferous MED were more tolerant of Sivanto than uninfected MED (Table 1). The $LC_{15}$ of viruliferous MED was more than twice that of uninfected MED (11.8 versus 5.8, respectively), and the $LC_{50}$ of viruliferous MED was almost twice as high (31.3 versus 17.3). The 95% CLs of viruliferous and uninfected MED did not overlap, meaning that the two groups differed significantly in both their $LC_{15}$ and $LC_{50}$ values (Table 1).

Exposure to Sivanto (at $LC_{15}$ concentration determined for viruliferous MED) marginally increased adult female longevity (Fig. 1A), increased first-week fecundity (Fig. 1B) and decreased egg-adult development time (Fig. 1C) in both MED groups (Table 2). In contrast, the only significant main effect of TYLCV was a 28% decrease in first-week fecundity (Fig. 1B).

The TYLCV*Sivanto interaction was marginally significant ($P = 0.067 – 0.085$) for three of the four variables: Sivanto had a greater impact on the first-week fecundity and egg-adult development time of uninfected MED than viruliferous MED (Fig. 1B, 1C), but increased the adult female lifespan of viruliferous MED more than for uninfected MED (Fig. 1A). Survival from egg to adult (Fig. 1D) was not affected by either main effect or their interaction (Table 2).

**Discussion**

Contrary to expectations, we found that TYLCV did not increase MED vulnerability to flupyradifurone. Instead, both the $LC_{15}$ and $LC_{50}$ values for viruliferous MED were significantly higher than those of uninfected MED (Table 1). In three of the four demographic variables, there was also a marginally significant interaction between Sivanto and TYLCV: Sivanto tended to
increase adult longevity only in viruliferous MED and first-week fecundity only in uninfected MED, and tended to decrease egg-adult development time only in uninfected MED (Fig. 1A,B,C). While the ability of TYLCV and other persistently-transmitted viruses to benefit *Bemisia* via manipulation of host plant defense is well-known, this appears to be the first example of virally-mediated changes in vector susceptibility to an insecticide.

While our results were surprising, there have been other reports of microorganism-mediated changes in insecticide susceptibility (Pietri and Liang 2018). Gut symbionts in both the cigarette beetle *Lasioderna serricorne* (Shen and Dowd 1991) and the apple fly *Rhagoletis pomonella* (Lauzon et al. 2003) are involved with the detoxification of natural and synthetic toxins. In contrast, the symbiotic microorganism *Rickettsia* increased *Bemisia* sensitivity to a range of different insecticides (Kontsedalov et al. 2008, but see Pan et al. 2013b); later research linked increases in *Bemisia* symbiont diversity and density to greater insecticide susceptibility (Ghanim and Kontsedalov 2009). Similar results have been reported in the psyllid *Diaphorina citri*, where infection with *Candidatus Liberibacter asiaticus* increased its vulnerability to several insecticides (Tiwari et al. 2011). A recent review (Pietri and Liang 2018) suggested these variable results may partially reflect symbiont-specific effects on both host detoxification enzymes and their immune/stress response. A transcriptomic analysis of gene regulation in TYLCV-infected MED found that while TYLCV generally downregulated detoxification enzymes, genes involved in both stress and immune responses were upregulated (Ding et al. 2019). It seems likely that some of these upregulated genes alter MED susceptibility to flupyradifurone.

The negative impact of flupyradifurone revealed in the LC$_{15}$ and LC$_{50}$ bioassays appears at odds with its equivocal effect on various aspects of MED demography. MED that survived one
day of flupyradifurone exposure had slightly higher female longevity, higher first-week fecundity, and a shorter egg-adult development time than MED in the control treatment. These ‘benefits’ of flupyradifurone are almost certainly an experiment artifact: a day of insecticide exposure removed the weakest and/or most susceptible MED from the population that was subsequently used for our demographic work. They may also reflect hormesis, a phenomenon in which sublethal dosages of insecticide improve fecundity or provide other benefits to the targeted insects (Cutler 2012). It is also worth noting that both uninfected and viruliferous MED were exposed to flupyradifurone at the LC15 concentration determined for viruliferous MED. Because the LC15 value for uninfected MED was lower than for viruliferous MED, this flupyradifurone concentration was more lethal to the uninfected population than to the viruliferous one. Higher rates of exposure-related mortality in our uninfected group may have had the unintended effect of minimizing differences between the uninfected and viruliferous groups. It should also be noted that the recommended label rate of flupyradifurone, 150 mg/l, was substantially higher than the concentrations we used; we chose to work with lower concentrations in order to assess MED that survive initial exposure. The effect of TYLCV on MED insecticide tolerance may be reduced or eliminated at these higher concentrations.

Pesticides can indirectly control insect-vectored plant diseases via their impact on vector density. This control may be lessened, however, if vectors feeding on pesticide-sprayed plants survive long enough to transmit TYLCV and other viruses. Viruliferous Bemisia efficiently transmit TYLCV to uninfected plants. Less than two minutes of Bemisia salivation is necessary to infect a healthy tomato plant (Jiang et al. 2000). As a result, thiamethoxam and other insecticides that do not quickly kill Bemisia may prove inefficient at decreasing TYLCV
transmission (Roditakis et al. 2017). Flupyradifurone has a higher knockdown rate than thiamethoxam and is more effective at reducing TYLCV transmission (Roditakis et al. 2017).

Research assessing the impact of flupyradifurone on *Bemisia* feeding behavior is necessary to understand the mechanism(s) underlying its effect on viral transmission. Aphids feeding on thiamethoxam-treated plants, for example, spend less time in the sieve element phase required for viral transmission to an uninfected plant (Cho et al. 2011, Stamm et al. 2013). Although TYLCV increased MED tolerance to flupyradifurone (Table 1), it might still change MED feeding behavior in ways that make this pesticide effective at reducing or eliminating viral transmission. Alternately, TYLCV-linked increases in flupyradifurone tolerance may provide viruliferous MED an advantage over uninfected individuals in pesticide-treated fields. If so, insecticide application could, under some conditions, favor viral outbreaks in agricultural systems (Pan et al. 2015).

In summary, our work found that infection with TYLCV altered the susceptibility of *Bemisia tabaci* MED to flupyradifurone. While the mechanism underlying our results is unknown, our findings suggest that viral infection may be capable of changing population-level responses to current management practices. Even for novel insecticides, such interactions highlight how work exploring pesticide impacts on each part of the vector-virus-plant interaction can contribute to the development of effective strategies to control MED and TYLCV.
Acknowledgements

This work was funded by the National Natural Science Foundation of China (31772171, 31401785), Tianjin Natural Science Foundation (17JCZDJC33700), Innovative research and experimental projects for young researchers of Tianjin Academy of Agricultural Science (201903). The authors declare that no conflict of interest exists.
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Table 1: Median lethal concentration ($LC_{15}$ and $LC_{50}$) of flupyradifurone (Sivanto) to uninfected and viruliferous MED. $LC_{15}$ and $LC_{50}$ followed by different upper-case letters indicate that uninfected and viruliferous MED are significantly different based on overlap of 95% CLs.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$N$</th>
<th>Slope ± SE</th>
<th>$LC_{15}$ (mg[AI]kg$^{-1}$) (95% CL)</th>
<th>$LC_{50}$ (mg[AI]kg$^{-1}$) (95% CL)</th>
<th>$X^2$ (df)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>478</td>
<td>3.64 ± 0.39</td>
<td>5.78 (3.72-7.82) A</td>
<td>17.33 (14.08-20.48) A</td>
<td>1.61</td>
<td>0.66</td>
</tr>
<tr>
<td>Viruliferous</td>
<td>476</td>
<td>4.07 ± 0.36</td>
<td>11.75 (8.91-14.44) B</td>
<td>31.33 (27.22-35.82) B</td>
<td>2.4 (3)</td>
<td>0.49</td>
</tr>
</tbody>
</table>

Table 2: Results of ANOVA assessing the impact of TYLCV infection, Sivanto exposure, and their interaction on MED demographic variables.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Female longevity (d)</th>
<th>First week fecundity (# eggs)</th>
<th>Egg-adult developmental time (d)</th>
<th>Egg-adult survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F$</td>
<td>$df$</td>
<td>$P$</td>
<td>$F$</td>
</tr>
<tr>
<td>TYLCV$^+$</td>
<td>0.43</td>
<td>1,101</td>
<td>0.513</td>
<td>15.71</td>
</tr>
<tr>
<td>Sivanto</td>
<td>3.55</td>
<td>1,101</td>
<td>0.063</td>
<td>10.24</td>
</tr>
<tr>
<td>TYLCV*Sivanto</td>
<td>3.43</td>
<td>1,101</td>
<td>0.067</td>
<td>3.18</td>
</tr>
</tbody>
</table>

$^+$Tomato yellow leaf curl virus
Figure 1. *Bemisia tabaci* MED feeding on *Lycopersicon esculentum*. Mean ± SE values for the demographic variables A) Female longevity (days); B) Eggs per female over one week; C) Egg-adult development time (days); and D) Egg-adult survival (%). Light gray bars: uninfected MED; dark gray bars: viruliferous MED. Unstriped bars (S-): plants sprayed with distilled water; striped bars (S+): plants sprayed with 11.75 mg[AI]kg⁻¹ Sivanto (LC₁₅ for viruliferous MED). Different upper-case letters above bars indicate significant differences (Tukeys’ HSD with α = 0.05); in figure 1D, there were no significant between-treatment differences.
Figure 1.