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9 **TYLCV Infection Alters *Bemisia tabaci* MED (Hemiptera: Aleyrodidae) Vulnerability to**
10 **Flupyradifurone**
11

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23 Abstract

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

40 Key Words

41 Insecticide, Sivanto, tolerance, *Bemisia*, TYLCV

42 **Introduction**

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

51 Although *Bemisia* feeding can itself reduce plant growth, its primary threat to agriculture
52 occurs via its ability to transmit a wide variety of plant viruses. MED is particularly effective at
53 transmitting such viruses, and its invasion is often associated with plant disease outbreaks (Ning
54 et al. 2015). The *Tomato yellow leaf curl virus* (TYLCV) is a particularly damaging pathogen
55 that has caused significant damage worldwide (Jones 2003). TYLCV relies on *B. tabaci* as a
56 vector to spread among plants (Feres and Moreno 2009). As a result, *Bemisia*- plant-TYLCV
57 interactions have been the subject of intense interest and researchers have confirmed the
58 mutualistic relationship between *B. tabaci* and the virus. It is now known, for instance, that
59 TYLCV can increase *Bemisia* fitness via its suppression of plant defense (Zhang et al. 2012,
60 Luan et al. 2013) and that MED benefits from feeding on TYLCV-infected hosts (Pan et al.
61 2013a, Shi et al. 2019).

62 Insecticides play an important role in an integrated pest management approach to
63 controlling *Bemisia* and viral outbreaks in agricultural systems. Because the whitefly can rapidly
64 develop insecticide resistance, the continued development and deployment of novel compounds

65 is essential for effective pest control. One such compound is flupyradifurone, the first
66 commercially available systemic control agent derived from the butenolide class of insecticides
67 (Nauen et al. 2015). This compound, an agonist on insect nicotinic acetylcholine receptors,
68 differs structurally from other chemicals that target these receptors. As a result, it is effective
69 against neonicotinoid- and pymetrozine-resistant *Bemisia* populations (Nauen et al. 2015).

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

82 We report the results of two experiments exploring how TYLCV infection affected MED
83 susceptibility to flupyradifurone (trade name Sivanto). We first determined the LC₁₅ and LC₅₀ for
84 control and viruliferous MED feeding on plants treated with either Sivanto or distilled water. We
85 calculated both LC₁₅ and LC₅₀ because chemical degradation and dilution gradually reduce
86 insecticide concentrations following application (e.g., Roditakis et al. 2017). We next measured
87 several demographic parameters of control and viruliferous MED exposed to either insecticide-

88 or water-treated plants. Although TYLCV infection has little direct effect on MED fitness (Pan et
89 al. 2013a, Su et al. 2015), recent work found a net downregulation of detoxification enzymes in
90 TYLCV-infected MED (Ding et al. 2019); we hypothesized that viruliferous MED would be
91 more sensitive to Sivanto than uninfected individuals.

92 **Materials and Methods**

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

100 Insects: The whitefly *Bemisia tabaci* MED (Q) was first collected in 2009 from
101 poinsettia, *Euphorbia pulcherrima* Wild. (ex Klotz.), in Beijing, China. It was reared on
102 poinsettia. In 2015, a portion of the population (~300 adults) was transferred to the Tianjin
103 Institute of Plant Protection and reared on cotton plants (*Gossypium herbaceum* L., cv DP99B) in
104 80 mesh nylon insect cages (45×45×60 cm) under $26\pm 2^{\circ}\text{C}$, $60\pm 10\%$ RH, 14L:10D photoperiod.
105 A viruliferous MED population was produced by transferring ~300 whiteflies into a cage with
106 four TYLCV-infected tomato plants; a parallel uninfected MED population was produced by
107 transferring >300 whiteflies into a cage with four healthy tomato plants. Both viruliferous and
108 uninfected MED were reared for two generations on their respective plants before being used for
109 experiments. Colony purity was monitored every 2-3 generations using a DNA marker (Khasdan
110 et al. 2005), and TYLCV infection was confirmed via PCR validation (Ghanim et al. 2007).

111 Flupyradifurone bioassay of viruliferous and uninfected MED: Sivanto 200SL (17.09%
112 flupyradifurone) was provided by Bayer Crop Science (China) Company Ltd. and diluted with
113 distilled water to five different concentrations: 200 mg[AI]kg⁻¹, 100 mg[AI]kg⁻¹, 50 mg[AI]kg⁻¹,
114 25 mg[AI]kg⁻¹, and 12.5 mg[AI]kg⁻¹. For each of the five concentrations and an additional
115 distilled water control (a total of six treatments), 200 mL was added to a 500 mL plastic spray
116 bottle. For each concentration, one spray bottle was used to spray four tomato plants that were
117 each at the 6-7 true leaf stage; plants were sprayed until drip-off. One day after spraying, 100
118 newly-emerged (within 24 hours) adult MED per plant were placed in clip cages attached to the
119 abaxial side of both the third and fourth leaves of each sprayed plant. Clip cages were kept on for
120 two days; the number of living and dead MED were then counted. This work was conducted in a
121 climate-controlled chamber at 26±1 °C and 60±10% RH with 14L:10D photoperiod.

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

132 Female adult longevity and first-week fecundity: Thirty female MED from each of the
133 four treatments (=120 total) were placed individually in clip cages. Each cage was then clipped

134 on the abaxial side of a middle leaf of an unsprayed healthy tomato plant (6-7 true leaf stage). A
135 total of two MED were clipped onto each plant, one per leaf, and both MED on a given plant had
136 the same infection status (i.e., they were both either uninfected or viruliferous). The clip cages
137 were checked each day for MED mortality; after one week, all surviving adults were individually
138 transferred to new unsprayed healthy tomato plants and the number of eggs laid during the first
139 week counted.

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

148 Statistical analysis: Probit parameter estimation of the concentration-mortality response
149 for viruliferous and uninfected MED in the six concentrations were calculated using POLO-PC
150 (Russell et al. 1977, LeOra 1987). These parameters included LC_{15} and LC_{50} values expressed in
151 $mg[AI]kg^{-1}$ and their corresponding 95% confidence limit (CL) along with the slopes of the
152 probit regressions. Between-treatment differences in the mortality of viruliferous and uninfected
153 MED were calculated using 95% CLs; LC_{15} or LC_{50} values for viruliferous and uninfected MED
154 were considered significantly different if their corresponding 95% CLs did not overlap.

155 Data on each of the demographic responses was analyzed using two-way ANOVA to
156 assess the main effects of TYLCV (uninfected, viruliferous) and insecticide (dH₂O, Sivanto) as

157 well as their interaction. When one or more main effects or their interaction was significant at p
158 = 0.05, Tukeys' HSD was used for means separation tests. Data on adult longevity and survival
159 was sqrt transformed before analysis. All analyses were conducted using JMP 9.0.0 (SAS 2010).

160 **Results**

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

166 Exposure to Sivanto (at LC_{15} concentration determined for viruliferous MED) marginally
167 increased adult female longevity (Fig. 1A), increased first-week fecundity (Fig. 1B) and
168 decreased egg-adult development time (Fig. 1C) in both MED groups (Table 2). In contrast, the
169 only significant main effect of TYLCV was a 28% decrease in first-week fecundity (Fig. 1B).
170 The TYLCV*Sivanto interaction was marginally significant ($P = 0.067 - 0.085$) for three of the
171 four variables: Sivanto had a greater impact on the first-week fecundity and egg-adult
172 development time of uninfected MED than viruliferous MED (Fig. 1B, 1C), but increased the
173 adult female lifespan of viruliferous MED more than for uninfected MED (Fig. 1A). Survival
174 from egg to adult (Fig. 1D) was not affected by either main effect or their interaction (Table 2).

175 **Discussion**

176 Contrary to expectations, we found that TYLCV did not increase MED vulnerability to
177 flupyradifurone. Instead, both the LC_{15} and LC_{50} values for viruliferous MED were significantly
178 higher than those of uninfected MED (Table 1). In three of the four demographic variables, there
179 was also a marginally significant interaction between Sivanto and TYLCV: Sivanto tended to

180 increase adult longevity only in viruliferous MED and first-week fecundity only in uninfected
181 MED, and tended to decrease egg-adult development time only in uninfected MED (Fig.
182 1A,B,C). While the ability of TYLCV and other persistently-transmitted viruses to benefit
183 *Bemisia* via manipulation of host plant defense is well-known, this appears to be the first
184 example of virally-mediated changes in vector susceptibility to an insecticide.

185 While our results were surprising, there have been other reports of microorganism-
186 mediated changes in insecticide susceptibility (Pietri and Liang 2018). Gut symbionts in both the
187 cigarette beetle *Lasioderma serricornis* (Shen and Dowd 1991) and the apple fly *Rhagoletis*
188 *pomonella* (Lauzon et al. 2003) are involved with the detoxification of natural and synthetic
189 toxins. In contrast, the symbiotic microorganism *Rickettsia* increased *Bemisia* sensitivity to a
190 range of different insecticides (Kontsedalov et al. 2008, but see Pan et al. 2013b); later research
191 linked increases in *Bemisia* symbiont diversity and density to greater insecticide susceptibility
192 (Ghanim and Kontsedalov 2009). Similar results have been reported in the psyllid *Diaphorina*
193 *citri*, where infection with *Candidatus Liberibacter asiaticus* increased its vulnerability to several
194 insecticides (Tiwari et al. 2011). A recent review (Pietri and Liang 2018) suggested these
195 variable results may partially reflect symbiont-specific effects on both host detoxification
196 enzymes and their immune/stress response. A transcriptomic analysis of gene regulation in
197 TYLCV-infected MED found that while TYLCV generally downregulated detoxification
198 enzymes, genes involved in both stress and immune responses were upregulated (Ding et al.
199 2019). It seems likely that some of these upregulated genes alter MED susceptibility to
200 flupyradifurone.

201 The negative impact of flupyradifurone revealed in the LC₁₅ and LC₅₀ bioassays appears
202 at odds with its equivocal effect on various aspects of MED demography. MED that survived one

203 day of flupyradifurone exposure had slightly higher female longevity, higher first-week
204 fecundity, and a shorter egg-adult development time than MED in the control treatment. These
205 ‘benefits’ of flupyradifurone are almost certainly an experiment artifact: a day of insecticide
206 exposure removed the weakest and/or most susceptible MED from the population that was
207 subsequently used for our demographic work. They may also reflect hormesis, a phenomenon in
208 which sublethal dosages of insecticide improve fecundity or provide other benefits to the
209 targeted insects (Cutler 2012). It is also worth noting that both uninfected and viruliferous MED
210 were exposed to flupyradifurone at the LC_{15} concentration determined for viruliferous MED.
211 Because the LC_{15} value for uninfected MED was lower than for viruliferous MED, this
212 flupyradifurone concentration was more lethal to the uninfected population than to the
213 viruliferous one. Higher rates of exposure-related mortality in our uninfected group may have
214 had the unintended effect of minimizing differences between the uninfected and viruliferous
215 groups. It should also be noted that the recommended label rate of flupyradifurone, 150 mg/l,
216 was substantially higher than the concentrations we used; we chose to work with lower
217 concentrations in order to assess MED that survive initial exposure. The effect of TYLCV on
218 MED insecticide tolerance may be reduced or eliminated at these higher concentrations.

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

225 transmission (Roditakis et al. 2017). Flupyradifurone has a higher knockdown rate than
226 thiamethoxam and is more effective at reducing TYLCV transmission (Roditakis et al. 2017).

227 Research assessing the impact of flupyradifurone on *Bemisia* feeding behavior is
228 necessary to understand the mechanism(s) underlying its effect on viral transmission. Aphids
229 feeding on thiamethoxam-treated plants, for example, spend less time in the sieve element phase
230 required for viral transmission to an uninfected plant (Cho et al. 2011, Stamm et al. 2013).

231 Although TYLCV increased MED tolerance to flupyradifurone (Table 1), it might still change
232 MED feeding behavior in ways that make this pesticide effective at reducing or eliminating viral
233 transmission. Alternately, TYLCV-linked increases in flupyradifurone tolerance may provide
234 viruliferous MED an advantage over uninfected individuals in pesticide-treated fields. If so,
235 insecticide application could, under some conditions, favor viral outbreaks in agricultural
236 systems (Pan et al. 2015).

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

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- 345

346 **Table 1:** Median lethal concentration (LC₁₅ and LC₅₀) of flupyradifurone (Sivanto) to uninfected
 347 and viruliferous MED. LC₁₅ and LC₅₀ followed by different upper-case letters indicate that
 348 uninfected and viruliferous MED are significantly different based on overlap of 95% CLs.

Treatment	N	Slope ± SE	LC ₁₅ (mg[AI]kg ⁻¹) (95% CL)	LC ₅₀ (mg[AI]kg ⁻¹) (95% CL)	X ² (df)	P value
Uninfected	478	3.64 ± 0.39	5.78 (3.72-7.82) A	17.33 (14.08-20.48) A	1.61 (3)	0.66
Viruliferous	476	4.07 ± 0.36	11.75 (8.91-14.44) B	31.33 (27.22-35.82) B	2.4 (3)	0.49

349

350

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

Treatment	Female longevity (d)			First week fecundity (# eggs)			Egg-adult developmental time (d)			Egg-adult survival (%)		
	F	df	P	F	df	P	F	df	P	F	df	P
TYLCV [†]	0.43	1,101	0.513	15.71	1,101	<0.001	0.86	1,18	0.364	1.03	1,18	0.324
Sivanto	3.55	1,101	0.063	10.24	1,101	0.002	7.26	1,18	0.015	0.31	1,18	0.586
TYLCV*Sivanto	3.43	1,101	0.067	3.18	1,101	0.078	3.34	1,18	0.085	0.97	1,18	0.338

[†]Tomato yellow leaf curl virus

353

354 **Figure Legends**

355 Figure 1. *Bemisia tabaci* MED feeding on *Lycopersicon esculentum*. Mean \pm SE values for the
356 demographic variables A) Female longevity (days); B) Eggs per female over one week; C) Egg-
357 adult development time (days); and D) Egg-adult survival (%). Light gray bars: uninfected MED;
358 dark gray bars: viruliferous MED. Unstriped bars (S-): plants sprayed with distilled water;
359 striped bars (S+): plants sprayed with 11.75 mg[AI]kg⁻¹ Sivanto (LC₁₅ for viruliferous MED).
360 Different upper-case letters above bars indicate significant differences (Tukeys' HSD with $\alpha =$
361 0.05); in figure 1D, there were no significant between-treatment differences.

Figure 1.

