Intraorchard Variation of Resistance to Imidacloprid in *Diaphorina citri* (Hemiptera: Liviidae) Adults[1]

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Abstract *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) is the most severe pest of citrus worldwide, and it has a high capacity to develop insecticide resistance. We estimated the intraplot variation of resistance to imidacloprid in adults collected from an orchard (8 ha) of Persian lemon, *Citrus latifolia* Tan., in Martínez de la Torre, Veracruz, Mexico. We divided the orchard into eight sections of similar size. Adults were sampled from each section to assess their response in the F_1. We conducted two field samplings: November 2020 and May 2021. The relative response (RR_{50}) at the median lethal mortality (LC_{50}) level in adults collected in the first sampling varied from 518× to 16,701×. Adults collected from Sections 2 and 5 exhibited the highest LC_{50} values. In the second sampling, adults with the highest LC_{50} values were collected from Sections 3, 5, and 6. The range of intraorchard variation at the LC_{50} level (RR_{50}) ranged from 635× to 6,626×. The RR_{95} values could be estimated in two sections of the first sampling: 7,421× (Section 7) and 58,958× (Section 8). For the remainder of the intra-orchard sections in both samplings, the maximum concentration of imidacloprid that could be prepared was 100,000 mg/L, which caused a level of mortality that reached $\leq$87.9%. The range of variation at the LC_{50} among sections (FRR_{50}) was low: 1 to 32.17× in the first sampling and 1 to 10.43× in the second. The resistance detected to imidacloprid is the highest recorded worldwide for *D. citri*.

Key Words Asian citrus psyllid, *Candidatus,* Liberibacter, insect vector, insecticide resistance

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is a vector of the bacteria *Candidatus* Liberibacter var. *asiaticus* and *Candidatus* Liberibacter *americanus*, causal agents of the Huanglongbing disease (Bové 2006, Halbert and Manjunath 2004). This disease has caused losses estimated at US$1 billion per year in Florida (da Costa et al. 2021, Li et al. 2020). In Mexico, it has

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economically affected more than 67,000 citrus producers, who harvest more than seven million tons, with an approximate value of US$500 million (SENASICA 2018). To mitigate its dispersion, actions have been taken to prevent the trade of infested plant material (Qureshi and Stansly 2007, SENASICA 2018). However, these efforts have focused mainly on conventional insecticides. In most cases, growers do not have rational management programs for these products (Boina et al. 2009, Chen and Stelinski 2017, García-Méndez et al. 2019). Confronted with biological efficacy problems, growers usually increase the dose and frequency of insecticide applications. This scenario raises the probability and number of incidences of insecticide resistance (Hawkins et al. 2018) and the risks to environmental and human health (Damalas and Eleftherohorinos 2011). To lessen these adverse effects, a resistance monitoring program was initiated in 2008 to estimate the response to insecticides in *D. citri* in Florida (Boina et al. 2009). In 2009, this insect vector expressed 35× resistance, under field conditions, to imidacloprid (Tiwari et al. 2011). Resistance to chlorpyrifos (124×), imidacloprid (759×), and bifenthrin (107×) has been detected in Pakistan (Naeem et al. 2016). In 2018, in the Mexican state of Michoacán, high levels of resistance to insecticides were detected: 2,435× to chlorpyrifos and 4,265× to imidacloprid (Pardo et al. 2018, Vásquez-García et al. 2013). These studies demonstrated the ability of this species to survive and reproduce in habitats treated with commercial doses of the products used to combat it.

The response to a toxicant in field pest populations does not have the same intensity in time and space. These differences have been attributed to variations in the use of insecticides (García-Méndez et al. 2019) and to the complex patterns of insect dispersal (Collins and Schlipalius 2018, Pasteur and Raymond 1996), among others. For example, Castle and Prabhaker (2013) estimated the susceptibility of *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) to four neonicotinoid insecticides: thiamethoxam, dinotefuran, imidacloprid, and thiamethoxam. They found variation in resistance in spring, summer, and autumn. During the 3 yr of study, they observed that resistance in the summer season was reduced due to insect dispersal. Chevillone et al. (1995) studied the gene flow of resistance to chlorpyrifos in populations of *Culex pipiens* L. (Diptera: Culicidae) in different geographical areas in southern France and northern Spain. They considered that variation in response was attributed to the mobility of this mosquito species, insecticide use, and a possible differential effect of resistance genes on fitness.

In monitoring the response to insecticides in *D. citri*, variations in resistance levels have been observed and are attributed to differences in the management of this species (García-Méndez et al. 2019, Naeem et al. 2016). Short and long-distance movement patterns are also assumed to influence the variation in insecticide response. The adult of this species can fly up to 150 m within an orchard (Hall and Hentz 2011, Martini et al. 2014, Stelinski 2019) and may contribute to differences in response at the intraorchard level. This knowledge constitutes an essential input for making decisions on the rational use of insecticides. In addition, it will be possible to infer the possible orchard system's contribution of resistant individuals to other crops that share the same pest species. Consequently, the dispersion of these alleles in the agroecosystem could have a positive or negative contribution to chemical pest management. If those alleles express susceptibility to insecticides, they will contribute to reversing the existing resistance levels. Otherwise, they will represent a threat to the rational use of insecticides.

Bioassays are generally conducted with samples of individuals from a population, and inferences are made at the agroecosystem level. However, researchers usually ignore the variation that could exist at the orchard level, where specific chemical combat decisions are made. Therefore, the objectives of this study were to estimate the level of resistance to imidacloprid in *D. citri* adults and its variation at the intraorchard level.

Materials and Methods

Insects. Individuals from two populations of *D. citri* were used: one susceptible to insecticides and one from the field. The susceptible population was field collected in 2009 in Martínez de la Torre, Veracruz, Mexico, and has since been maintained on lemongrass plants, *Murraya paniculata* (L.) Jack, under greenhouse conditions and without exposure to pesticides.

The field population was collected in Martínez de la Torre's citrus zone, Mexico (20°05′28.09″N 97°04′2.70″W), from an 8-ha orchard with 5-yr-old Persian lime trees, *Citrus latifolia* Tan. This orchard was divided into eight sections of similar size, and 12 trees were randomly chosen from each. About 500 adults of *D. citri* were collected from each section and placed separately in entomological cages (60 × 45 × 60 cm) covered with organza fabric (Grupo Parisina S.A de C.V., Mexico City, Mexico) and appropriately labeled. For the feeding and oviposition of this vector, each cage contained eight plants (2 yr old) of budding *M. paniculata*. Then, the individuals of *D. citri* from each section were reproduced separately to obtain enough adults in the F_1 generation to conduct the bioassays. The infested plants were kept under greenhouse conditions at a temperature of 25 ± 5°C and 60 ± 5% relative humidity (RH). Two field collections were made: November 2020 and May 2021. The response to imidacloprid was estimated separately for each section and sampling date.

Insecticides. For the bioassays, the Confidor® commercial formulation was used (imidacloprid, concentrated suspension, 35%, 35 g active ingredient/L, Bayer CropScience, Mexico). We used distilled water with 1 ml/L of the adjuvant INEX® (20.2% ethoxylated fatty alcohol and 1% polydimethylsiloxane, aqueous solution, Cosmocel®, Mexico) to prepare dilutions.

Bioassays. The immersion method proposed by the Insecticide Resistance Action Committee (IRAC 2019) was used with slight modifications. From the middle stratum of 4-yr-old orange trees, *Citrus sinensis* (L.) Osbeck var. Valencia, not exposed to pesticides, we collected leaves to obtain discs (4.0-cm diameter) that we dipped for 30 s in the respective insecticide concentration. Subsequently, the discs were allowed to air dry for 1 h at room temperature and then placed adaxial surface down in a petri dish containing a 3-mm layer of agar-agar (Merck®, Darmstadt, Germany).

Initially, the concentrations at which 0 and 100% mortality (biological window) was estimated. To obtain this range of mortality, one or two repetitions were made, with 24-h exposure to the toxicant. Concentrations of 0.01, 0.001, 0.0001, 0.00001% were used for the susceptible population, and 10, 3, 1, 0.1, 0.01, 0.001% for insects collected from the different intraorchard sections. If the evaluated concentrations did not cover the range from 0 to 100%, additional repetitions were tested, increasing or decreasing the concentrations of imidacloprid as needed. Subsequently, at least eight intermediate concentrations within that range were introduced. Each repetition consisted of all insecticide concentrations within 0 to 100% mortality plus an untreated control to which only distilled water was applied. At least five repetitions were performed on different days. For statistical analysis, these concentrations of imidacloprid were expressed in milligrams per liter.

With the help of a manual aspirator, unsexed adults (5–7 d old) of healthy appearance were collected and placed in resealable plastic bags (Ziploc hermetic bags [16.5 $\times$ 14.9 cm] with double closure, S.C. Johnson, Racine, WI) and then anesthetized with CO_2 at a pressure of 70 kg/cm^2 for 1 min. Subsequently, they were placed in groups of 20 on the underside of the leaf discs. A lid with a hole (2.0 cm diameter) covered with organza fabric was placed on these boxes. After 10 min, the adults were visually inspected to discard those that had suffered any damage from handling. Subsequently, the position of the petri dishes was inverted so that the insects remained in a normal position, as they are on the leaves of the plants under field conditions.

The treated individuals were kept in bioclimatic chambers at 25 $\pm$ 5°C, 60 $\pm$ 5% RH, and a photoperiod of 12:12-h light:darkness. Mortality was recorded 24 h after exposure to the discs. Adults that did not react to being stimulated with the bristles of a brush were considered dead, as suggested by Naeem et al. (2016). In the untreated control, maximum mortality of 5% was accepted, and this variable was corrected with Abbott's formula (Abbott 1925).

Statistical analysis. Using SAS software (SAS 9.0), mortality data were subjected to probit analysis (Finney 1971). This analysis allowed us to estimate the values of the slope, median lethal concentration (LC_{50}), the concentration that causes 95% mortality (LC_{95}), 95% confidence limits, and the goodness-of-fit test to a straight line ($Pr > \chi^2$). The relative response values (RR) at the LC_{50} (RR_{50}) and LC_{95} (RR_{95}) were obtained by dividing the $LC_{50(95)}$ of the individuals of each section of the orchard by the $LC_{50(95)}$ of the susceptible population. To estimate the variation in response among samples from each orchard's sections, we used the relative field response variable at LC_{50} (FRR_{50}) and LC_{95} (FRR_{95}). These values were obtained by dividing the lowest value of $LC_{50(95)}$ by the $LC_{50(95)}$ observed in each of the evaluated sections. At both LC_{50} and LC_{95}, the response to imidacloprid in the orchard sections was considered different if their respective fiducial limits did not overlap, as suggested by Robertson and Preisler (1992).

Results

In the bioassays with field-collected insects on both sampling dates, we observed significant variation in the response with LC_{50} values (Tables 1, 2) among the sections. In the first sampling (November 2020), the LC_{50} of the intraorchard sections ranged from 690.5 mg/L (Section 7) to 22,212 mg/L (Section 5), which corresponded to a range in variation at the LC_{50} (RR_{50}) between 518$\times$ and 16,701$\times$, respectively (Table 1). The highest LC_{50} values were observed in

Table 1. Variation in resistance to imidacloprid in adult *Diaphorina citri* from eight sections of an 8-ha orchard of Persian lemon, *Citrus latifolia* Tan. Martínez de la Torre, Veracruz, Mexico. First field collection: November 2020.

Orchard Section	n*	df	Slope ($b \pm$ SE)	LC_{50}** 95%FL (mg/L)	LC_{95}** 95%FL (mg/L)	Pr $>\chi 2$§	RR_{50}/RR_{95}§§	FRR_{50}/FRR_{95}§§§
1	1,194	7	0.4 ± 0.06	2,142 (906.80–5,590)	>100,000 (77.39)***	0.002	1,611	3.10
2	1,184	7	0.5 ± 0.03	9,721 (6,641–15,012)	>100,000 (66.38)	0.69	7,309	14.07
3	1,154	7	0.5 ± 0.03	7,744 (5,322–11,830)	>100,000 (78.07)	0.27	5,823	11.22
4	1,181	7	0.5 ± 0.03	1,114 (784.55–1,585)	>100,000 (87.9)	0.57	838	1.61
5	1,229	7	0.5 ± 0.04	22,212 (14,860–35,696)	>100,000 (64.5)	0.73	16,701	32.17
6	763	7	0.6 ± 0.04	1,451 (995.11–2,125)	>100,000 (76.68)	0.82	1,091	2.10
7	776	7	0.8 ± 0.05	690.5 (507.86–934.20)	56,032 (32,372–110,750)	0.16	518/7,421	1.0/1.0
8	1198	7	0.6 ± 0.06	918.5 (445.46–1,876)	445,130 (108,956–4,415,040)	<0.0001	707/58,958	1.33/7.94
Susceptible	1,220	6	2.1 ± 0.1	1.3 (1.20–1.47)	7.55 (6.30–9.35)	0.98		

* Treated insects.
** Estimated concentration that caused 50% (LC_{50}) or 95% (LC_{95}) mortality (mg a.i./L) and its 95% fiducial limits.
*** Highest mortality observed at 100,000 mg/L.
§ Probability higher than χ^2.
§§ Relative response = highest $LC_{50(95)}$ value/$LC_{50(95)}$ of the respective insecticide.
§§§ Variable relative field response = all high numbers of 50 or 95% relative response ($RR_{50[95]}$) value /lowest $RR_{50(95)}$ value.

Table 2. Variation in resistance to imidacloprid in adult *Diaphorina citri* from eight sections of an 8-ha orchard of Persian lemon, *Citrus latifolia* Tan. Martínez de la Torre, Veracruz, Mexico. Second field collection: May 2021.

Orchard Section	*n**	df	Slope $b \pm$ SE	LC_{50}** 95%FL (mg/L)	LC_{95}** 95%FL (mg/L)	$Pr > \chi^{2}$§	RR_{50}/RR_{95}§§	FRR_{50}/FRR_{95}§§§
1	1,241	7	0.6 ± 0.04	10,207 (7,381–14,653)	>100,000 (72.3)***	0.21	2,526	3.98
2	1,270	7	0.6 ± 0.03	8,359 (5,983–12,122)	>100,000 (75.2)	0.64	2,069	3.26
3	1,260	7	0.4 ± 0.03	10,719 (6,947–17,784)	>100,000 (71.4)	0.91	2,653	4.18
4	1,271	7	0.6 ± 0.03	3,998 (2,988–5,470)	>100,000 (84.3)	0.39	989	1.56
5	1,221	7	0.4 ± 0.05	26,770 (11,208–98,391)	>100,000 (54.8)	0.02	6,626	10.43
6	1,233	7	0.5 ± 0.05	24,094 (12,226–59,884)	>100,000 (54.8)	0.05	5,963	9.39
7	1,228	7	0.6 ± 0.03	2,566 (1,890–3,530)	>100,000 (81.8)	0.32	635	1.0
8	1,234	7	0.6 ± 0.03	3,336 (2,415–4,404)	>100,000 (87.2)	0.27	825	1.30
Susceptible	1,120	6	1.2 ± 0.08	4.04 (2.91–5.66)	91.36 (50.62–205.36)	0.07		

* Treated insects.

** Estimated concentration that caused 50% (LC_{50}) or 95% (LC_{95}) mortality (mg a.i./L) and its 95% fiducial limits.

*** Highest mortality observed at 100,000 mg/L.

§ Probability higher than χ^2.

§§ Relative response = highest $LC_{50(95)}$ value/$LC_{50(95)}$ of the respective insecticide.

§§§ Variable relative field response = all high numbers of 50 or 95% relative response ($RR_{50[95]}$) value /lowest $RR_{50(95)}$ value.

Sections 2 (9,721 mg/L) and 5 (22,212 mg/L) (Table 1). The LC_{95} values could only be estimated for Sections 7 and 8 with 690.5 and 918.5 mg/L, equivalent to RR_{95} of 7,421× and 58,958×, respectively (Table 1). For Sections 1, 2, 3, 4, 5, and 6, the highest dose that could be applied was 100,000 mg/L, which caused mean mortality levels between 64.5 and 87.9% (Table 1). Consequently, the LC_{95} values were considered >100,000 mg/L, and the relative response at the 95% mortality level (RR_{95}) was not calculated.

In the second sampling (May 2021), the LC_{50} values ranged from 2,566 to 26,770 mg/L among the sections, and the variation in response to imidacloprid with RR_{50} values was between 635× and 6,626× (Table 2). The lowest LC_{50} values were observed in Sections 4 (3,998 mg/L), 7 (2,566 mg/L), and 8 (3,336 mg/L) (Table 2). It was impossible to estimate the LC_{95} in any intraorchard sites because the maximum concentration that could be evaluated was 100,000 mg/L. This concentration caused mean mortality levels between 54.8 and 87.2% (Table 2). For the reasons indicated, the information was processed similarly to Sampling 1.

To estimate the intraorchard variation, without considering the response of the susceptible population, the variable "relative field response" was used at the LC_{50} level (FRR_{50}). FRR_{95} values were not calculated for sections where LC_{95} was not estimated. The variation in response to the FRR_{50} values in Sampling 1 (November 2020) ranged from 1.0× (Section 7) to 32.17× (Section 5). In Sampling 2 (May 2021), the FRR_{50} variation was between 1.0× (Section 7) and 10.43× (Section 5). For both field collections, the lowest FRR_{50} values were observed in Section 7 (1.0×) and the highest in Section 5 (10.43×) (Tables 1, 2). In Sampling 1, the FRR_{95} values were calculated only for Sections 7 (1×) and 8 (7.94×). However, in the second sampling, the LC_{95} could not be estimated in any section because the mortality values with the highest concentration of imidacloprid that could be prepared (100,000 mg/l) varied from 54.8 to 87.2%.

Discussion

The impossibility of exposing test individuals to concentrations >100,000 mg/L was due to the formation of precipitates. In a log dose/probit line, as in any regression, it is incorrect to estimate values outside the range of observed data (Bartley et al. 2019). This is why, in these cases, it was indicated that the LC_{95} was >100,000 mg/L (Tables 1, 2).

Current studies on this insecticide resistance give little importance to the variation among field populations. Susceptible and resistant populations have less variation in their response to insecticides than those in the process of developing this phenomenon (Lenormand 2002, Grossman et al. 2019). Therefore, to estimate the response variation at the intraorchard level, herein we introduced the concept of "relative response of field populations" (FRR) at both the LC_{50} (FRR_{50}) and the LC_{95} (FRR_{95}). This variable makes it possible to estimate the variation in response among field-selected populations without considering the one used as a susceptible reference. As outlined previously, the field population with the lowest $LC_{50(95)}$ value is used as the basis for performing the $FRR_{50(95)}$ calculations. Our data revealed high resistance levels and variation in the relative response to imidacloprid but low variation in the FRR variable at the intraorchard level.

Imidacloprid is one of the most frequently used insecticides in citrus against the Asian psyllid (Fletcher et al. 2018, Ruiz-Galván et al. 2015, Serikawa et al. 2012). As a result, this pest has developed the biological capacity to survive and reproduce in citrus orchard systems treated with this product (Langdon and Roger 2017, Naeem et al. 2016, Vázquez-García et al. 2013). Globally, 19 cases of resistance of *D. citri* to imidacloprid have been documented under field conditions (APRD 2022).

In Pakistan, Neem et al. (2016) found, in *D. citri*, 759.5× resistance to imidacloprid. Vazquez-García et al. (2013) estimated resistance of 4,000× to this insecticide. Based on the literature available, we believe that we have detected, in adults of *D. citri*, the highest levels of resistance to imidacloprid worldwide. These significant response levels are likely associated with the imidacloprid mode of action since they generate the most extreme intensity of response to a selection agent, as suggested by Georghiou (1972).

According to Bragard et al. (2021), at 25 and 28°C, the life cycle of *D. citri*, from egg to adult, is completed in 14–17 d. During the 195 d that elapsed from the beginning of the first field collection (November 2020) to the second one (May 2021), we estimate that in this orchard, 9 to 10 generations developed. During this time, the resistance level did not drop. To the contrary, it increased in Sections 7 and 8 (Table 2). This occurred because the target population continued being selected by commercial applications of imidacloprid.

How is it possible that an insect pest develops, under field conditions, such extreme levels of resistance? We consider that, in most cases, the development of this microevolutionary phenomenon does not arise from the use of agrochemicals following the recommendations on the respective commercial label. Instead, it is a consequence of abuse, as suggested by Georghiou (1986)

Abuse of this type is in several forms. In the orchard under study, the owner delegates responsibility for citrus production to workers without experience or interest in adequately managing pests. To combat *D. citri* in this orchard during the 5 yr prior to this study, imidacloprid has been used exclusively and applied in 12 to 15 sprays annually. The product is labeled for application of 30–40 ml of formulated product per 100 L of water; however, 100 mL of formulated product per 100 L of water is routinely applied. Even with the intense use of high doses of imidacloprid, the vector insect is not being adequately managed, with a field efficacy of only 30–50%, which is unacceptable.

We surmise that this scenario results in the existence of what we call the “red spots” and constitutes a significant source of resistance alleles whose dispersion through the agroecosystem has the potential to invalidate the actions of intelligent management with insecticides. Unfortunately, the strategies of rational insecticide management do not fully consider the potential existence of “red spots” and their impact on pest management. We postulate that this scenario, with different levels of abuse, can be one of the leading causes of the significant number of insect pest resistance under field conditions.

We consider that the “red spots” exist because the owners of the orchards are not in close contact with the production process or the level of supervision is low. In addition, we do not rule out that, in some cases, the technicians responsible for plant health may receive encouragement to use specific commercial brands of insecticides favored by some agrochemical companies. “Red spots” are also the leading cause of the devastating adverse effects of pesticides on the environment and human health. We argue that the evolution of pesticide resistance also has deep roots in the intentional negative behavior of some people directly responsible for selecting the agrochemicals used in plant health. Ethical values in chemical combat must be considered one of the most critical operational factors contributing to developing insect pest resistance. Timely and adequate attention to these considerations would be beneficial to implement actions that avoid or mitigate the adverse effects of the "red spots" on pest control, the environment, and human health.

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