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VARIACIÓN INTRA-HUERTA DE LA RESISTENCIA A IMIDACLOPRID EN ADULTOS DE *Diaphorina citri* Kuwayama (HEMIPTERA: LIVIIDAE).

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La presente tesis titulada: **Variación intra-huerta de la resistencia a imidacloprid en adultos de *Diaphorina citri* Kuwayama (Hemiptera: Liviidae).** realizada por la estudiante: **ANA KAREN RAMÍREZ SÁNCHEZ**, bajo la dirección del Consejo Particular indicado, ha sido aprobada por el mismo y aceptada como requisito parcial para obtener el grado de:

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VARIACIÓN INTRA-HUERTA DE LA RESISTENCIA A IMIDACLOPRID EN ADULTOS DE *Diaphorina citri* KUWAYAMA (HEMIPTERA: LIVIIDAE).

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RESUMEN

El psílido asiático de los, *Diaphorina citri* Kuwayama, vector de HLB, es una de las plagas más dañinas de los cítricos a nivel mundial. El uso de insecticidas es la táctica de combate dominante debido a la rapidez de su acción y efectividad biológica. Sin embargo, su uso irracional ha derivado en numerosos casos de resistencia. Con la finalidad de manejar adecuadamente este fenómeno, se hacen estudios de resistencia, la mayoría a nivel regional o estatal. Erróneamente se considera que la respuesta a insecticidas es homogénea en superficies tan amplias. Por tanto, dichos estudios tienen utilidad limitada a nivel de huerto, donde se toman las decisiones de uso de insecticidas. La variación de la resistencia a insecticidas a nivel intra-huerto tiene el potencial de proporcionar información valiosa para el agricultor, pues es indicativo sobre el proceso de desarrollo de la resistencia. La variación en la respuesta es alta cuando la población está en la fase exponencial de desarrollo de la resistencia y baja en caso de ser susceptible o encontrarse cerca del límite de su capacidad biológica para sobrevivir y reproducirse exitosamente a pesar de las aplicaciones de insecticidas. Por tanto, los objetivos de esta investigación fueron estimar la intensidad y variación de la resistencia a imidacloprid a nivel de huerto en una población de *D. citri*. en ocho ha de limón persa *Citrus latifolia* Tan, en Martínez de la Torre, Veracruz, México. Dicho huerto se dividió en ocho secciones de tamaño similar y en cada una de ellas se recolectaron 500 adultos. En forma separada, se reprodujeron para obtener la F_1 y se realizaron los bioensayos en adultos. El estudio se efectuó dos veces en temporadas diferentes (noviembre de 2020 y mayo de 2021). En los individuos del primer muestreo, la respuesta relativa a la CL_{50} (RR_{50}) varió de 518× a 16,701×; siendo las secciones 2 y 5 las que tuvieron los valores más altos. Para el segundo experimento los valores más elevados de RR_{50} se observaron en las secciones 5, 6 y 3. El rango de variación dentro del huerto para la CL_{50} (RR_{50}) fue de 635× a 6,626×. Los valores de RR_{95} se pudieron estimar solo en dos secciones del primer experimento: 7,421× (sección 7) y 58,958× (sección 8), respectivamente. Para el resto de las secciones de ambos muestreos, la concentración máxima que se pudo preparar fue de 100,000 mg/L y alcanzó mortalidades ≤87.9%. La variación de la respuesta a imidacloprid a nivel intra huerto fue más baja que la variación observada de los individuos de las diferentes secciones del huerto en referencia a la población susceptible. Para imidacloprid en adultos de *D. citri*, se detectaron los niveles de resistencia más elevados a nivel mundial y se presentó una disertación sobre sus causas y consecuencias en el manejo de este vector a nivel de agroecosistema.

Palabras clave: limón persa, susceptibilidad, Huanglongbing

INTRA-ORCHARD VARIATION OF RESISTANCE TO IMIDACLOPRID IN ADULTS OF *Diaphorina citr*i KUWAYAMA (HEMIPTERA: LIVIIDAE).

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ABSTRACT

The Asian citrus psyllid, Diaphorina citri Kuwayama, vector of HLB, is one of the most damaging pests of citrus worldwide. The use of insecticides is the dominant combat tactic due to their rapid action and biological effectiveness. However, its irrational use has led to numerous cases of resistance. In order to adequately manage this phenomenon, resistance studies are carried out, most of them at the regional or state level. It is mistakenly considered that the response to insecticides is homogeneous in such large surfaces. Therefore, such studies are of limited utility at the orchard level, where insecticide use decisions are made. The variation of resistance to insecticides at the intra-orchard level has the potential to provide valuable information for the farmer, since it is indicative of the resistance development process. The variation in response is high when the population is in the exponential phase of resistance development and low when it is susceptible or near the limit of its biological capacity to survive and successfully reproduce despite insecticide applications. Therefore, the objectives of this research were to estimate the intensity and variation of resistance to imidacloprid at the orchard level in a population of *D. citri*, in eight ha of Persian lemon *Citrus latifolia* Tan, in Martínez de la Torre, Veracruz, Mexico. This orchard was divided into eight sections of similar size, and in each of them, 500 adults were field-collected. Separately, they were reproduced to obtain the F_{1}, and the bioassays were carried out in adults. The study was carried out twice in different field collections (November 2020 and May 2021). In the individuals of the first sampling, the relative response to the CL_{50} (RR_{50}) varied from 518× to 16,701, being sections 2 and 5, the ones with the highest values. For the second experiment, the range of variation within the orchard for the LC_{50} (RR_{50}) was from 635× to 6,626×, and the highest values of RR_{50} were observed in sections 5, 6, and 3. The RR_{95} values could be estimated only in two sections of the first experiment: 7,421× (section 7) and 58,958× (section 8), respectively. For the rest of the sections of both samples, the maximum concentration that could be prepared was 100,000 mg/L and reached mortalities ≤87.9%. The variation of the response to imidacloprid at the intra-orchard level was lower than the variation observed in the individuals of the different sections of the orchard compared to the susceptible population. For imidacloprid in adults of *D. citri*, the highest levels of resistance worldwide were detected, and a dissertation was presented on its causes and consequences in the management of this vector at the agroecosystem level.

Keywords: Persian lemon, susceptibility, Huanglongbing

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CONTENIDO

LISTA DE CUADROS

INTRODUCCIÓN GENERAL

La producción de cítricos a nivel global excede los 143,755 millones de toneladas anuales (FAO 2021). México es el cuarto productor mundial con una superficie de 594,362 hectáreas. Nuestro país produce 8.6 millones de toneladas de cítricos con un valor estimado en 33,648 millones de pesos (FAO 2021, SIAP 2022). El limón persa, *Citrus latifolia* Tan., representa el 19% de la superficie cultivada de los cítricos, con un valor de 3 mil 800 millones de pesos. Veracruz es uno de los estados líderes en la producción y exportación de limón persa, con alrededor de 15 mil productores y 45 mil hectáreas, de las 82 mil que hay a nivel nacional (Yzquierdo-Alvarez et al. 2021, SIAP 2022).

No obstante, la riqueza citrícola del país, dicha industria se encuentra amenazada por patógenos que transmiten algunas especies de insectos y que han devastado la producción citrícola de otros países. En respuesta, los gobiernos y productores inviertan grandes sumas de dinero para su manejo (SENASICA 2022). Entre los patógenos que destacan por sus efectos adversos se encuentran el virus de la tristeza (VTC) que lo transmite por el pulgón café de los cítricos (*Toxoptera citricida* Kirk.), la leprosis de los cítricos [*Citrus leprosis* virus C (CiLV-C)] transmitida por el ácaro *Brevipalpus* sp. y el Huanglongbing (HLB) (*Candidatus* Liberibacter spp.) asociado a *Diaphorina citri* Kuwayama, su vector (Halbert y Manjunath 2004, Bové 2006).

El HLB es una de las enfermedades más destructivas de los cítricos a nivel mundial y responsable de la muerte de millones de árboles (Roistscher 1996, Halbert y Manjunath 2004). Se trata de un tipo de bacteria que afecta a todas las partes de la planta. Los síntomas de esta enfermedad tardan al menos seis meses en manifestarse. Inicialmente aparece un brote con amarillamiento en hojas que contrasta con el verde de la planta. Los síntomas son más evidentes durante otoño-invierno, y se expresan como amarillamiento, así como moteado intenso. Los árboles enfermos producen frutos amargos y deformes, no aptos para el consumo. Los árboles infectados mueren en aproximadamente entre 2 y 3 años (Lee 2015, Li et al. 2018). En México, el HLB es una enfermedad relativamente reciente. Los registros indican que se encuentra presente en 351 municipios de 25 entidades federativas del país (SENASICA 2022). De acuerdo con las estimaciones oficiales, las pérdidas a nivel nacional exceden el 50% de la producción proyectada, lo que representa un fuerte impacto negativo no sólo para las unidades productivas, sino para los trabajadores que dependen de esta industria como fuente de sustento económico (Salcedo et al. 2010).

En el mundo se han empleado diversos métodos y estrategias de control para enfrentar tanto a insectos vectores como a la enfermedad que transmiten. Algunas de las acciones consisten en la detección y eliminación oportuna de los árboles enfermos, el uso plantas provenientes de viveros certificados, así como el abatimiento de la densidad de población del insecto vector con el uso de agentes de control biológico (*Tamarixia radiata* Waterston (Hymenoptera: Eulophidae) e insecticidas (Qureshi y Stansly 2007, SENASICA, 2022). En México, para llevar estas acciones se estableció un esquema de manejo regional, a través de Áreas de Manejo Epidemiológico Fitosanitario (AMEFIs) que opera en 24 estados con la finalidad de mantener bajas las densidades poblaciones de esta especie de psílido (SENASICA 2022).

No obstante, la táctica común entre los citricultores para el manejo de las poblaciones de *D. citri* es mediante el uso agroquímicos. Para la selección de insecticidas se debe tomar cuenta su efectividad biológica, período residual y programas de rotación por modos de acción (Sandoval-Rincón et al. 2010). Entre los insecticidas que se utilizan están los grupos de organofosforados (dimetoato, clorpirifós, acefate, fosmet, metamidafós); avermectinas (abamectina); piretroides (bifentrina, fenpropatrín, betaciflutrina, lambda cihalotrina); spinosinas (spinetoram); derivados de los ácidos tetrónicos y tetrámico (spirotetramat); sulfoximinas (sulfoxaflor); butenolides (flupyradifurone); diamidas (ciantraniliprol); neonicotinoides (dinotefuran, thiacloprid, thiametoxam, e imidacloprid) entre otros (Villanueva-Jiménez et al. 2011, Ruiz-Galván et al. 2015, García-Méndez et al. 2019). Sin embargo, uno de los productos más utilizado para el manejo de *D. citri* en todo el mundo ha sido imidacloprid (Serikawa 2012, Ruiz-Galván et al. 2015, Fletcher et al. 2018). Este compuesto entró al mercado en 1991 y pertenece al grupo de los neonicotinoides. Este insecticida actúa en el sistema nervioso al estimular los receptores nicotínicos de acetilcolina, lo que lo hace eficaz contra los insectos y menos tóxico para los peces o los mamíferos (Pandey et al. 2009). En el cultivo actúa de forma sistémica cuando se aplica al suelo, se absorbe por la raíz y viaja a través del xilema, hasta llegar a los tejidos como hojas, incluso al polen (Fletcher et al. 2018, Motaung, 2020).

No obstante, la alta eficacia inicial de este insecticida ocasionó que los agricultores realicen aplicaciones calendarizadas, provocando aumento de casos de resistencia en campo. Hasta la fecha se documentan 32 especies resistentes a imidacloprid, siendo una de ellas *D. citri* con 19 casos a nivel de campo registrados en todo el mundo por Arthropod Pesticide Resistance Database (APRD) (2022). El primer reporte de resistencia se documentó en Estados Unidos de América (Florida). En ese país, los productores elevaron hasta 35 veces la dosis inicial de imidacloprid (Tiwari et al. 2011b). En Punjab, Pakistán, el imidacloprid mostró un nivel muy alto de resistencia en 12 poblaciones de *D. citri* recogidas en campo, los cuales oscilaron entre 236,6× y 759,5× (Naeem et al. 2016). En México, en el estado de Tecomán, Colima se encontró una resistencia de 12× (García-Méndez et al. 2019). El dato más alto de resistencia en el país fue en el estado de Michoacán, donde Vásquez-García et al. (2013) registraron una resistencia de 4,265.6×.

La resistencia de las plagas a los insecticidas es un problema creciente debido a que los agroquímicos se consideran una parte integral de la agricultura de producción de alto rendimiento (Miller et al. 2010). Como un esfuerzo para racionalizar el uso de agroquímicos, se han realizado monitoreos para detectar el desarrollo de la resistencia. La información que se obtiene, se usa para la selección de los productos a emplear (Khan et al. 2013). Sin embargo, existen factores que determinan la tasa de desarrollo de resistencia, incluidos los genéticos, biológicos, ecológicos y operacionales (Georghiou y Taylor, 1976), mismos que deben ser comprendidos para un mejor manejo de insecticidas.

D. citri ha demostrado ser una especie de insecto con gran capacidad para desarrollar resistencia a insecticidas (Tiwari et al. 2011b, Vásquez-García et al. 2013; Chen y Stelinski 2017, Pardo et al. 2018) ya que cuenta con genes que expresan cambios en el receptor o enzimas como las monooxigenasas del citocromo P450 (Tiwari et al. 2011a); glutatión S-transferasas (GST) y esterasas (EST), los cuales juegan un papel importante en el metabolismo de moléculas neonicotinoides, organofosforados, piretroides, entre otros (Tian et al. 2019, Tiwari et al. 2011a). Además, las características de *D. citri*, como su alta capacidad de reproducción (630 a 1900 huevos por hembra) (Chavan y Summanwar 1993, Liu y Tsai 2000), ciclo de vida corto (de huevo a adulto puede completarse en dos o tres semanas en climas tropicales) (Hodkinson 2009), y la polifagia (hospederos principales a especies del género *Citrus*) (Stelinski 2019) han sido determinantes para que la resistencia evolucione con facilidad.

No obstante, el flujo genético dentro y entre las poblaciones es un aspecto importante para el desarrollo de la resistencia. Los alelos de resistencia o susceptibilidad que se intercambian debido a la dispersión de esta plaga, dan como resultado variación compleja en los niveles de respuesta a nivel de agroecosistema (Grafius 1995, Pasteur y Raymond 1996, Castle et al. 2009). Chevillone et al. (1995), estudiaron el flujo de genes de resistencia a clorpirifós en poblaciones de *Culex pipiens* L. (Diptera: Culicidae), en diferentes áreas geográficas al sur de Francia y norte de España. Demostraron que existe intercambio de genes de resistencia entre las poblaciones. Atribuyeron este fenómeno a la movilidad de esta especie de mosquito, a la variación en el uso de insecticidas, así como un posible efecto diferencial de los genes de resistencia sobre la aptitud biológica. Arias et al. (2019), estimaron alta resistencia a flubendiamida y lufenurón en *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae). Estas especies lograron dispersarse a diferentes regiones, esparciendo los alelos resistentes, alterando la respuesta a dichos insecticidas en otros cultivos, incluyendo aquellos donde no se usaba el combate químico. Hunucmá una ciudad pequeña en el estado de Yucatán, México, se dividió aleatoriamente en 24 cuadrantes, con el fin de monitorear la frecuencia de haplotipos kdr, mutación en el gen del canal de sodio asociado con la resistencia a piretroides y organoclorados, en *Aedes aegypti* L. (Diptera: Culicidae). En este estudio detectaron variación genética significativa entre los sitios seleccionados, relacionándolo con la aplicación de insecticidas en las diferentes áreas estudiadas, la adaptación local del vector y el tiempo. La interacción de estas variables dieron resultado un mosaico irregular de respuesta a la presión de selección (Grossman et al. 2019).

En *D. citri*, no se sabe si existe un efecto en la resistencia derivado de su poca movilidad. Los adultos de esta especie se desplazan hasta 150 m de distancia en huertos de cítricos con clima óptimo y durante el invierno solo llegan a 2 m (Hall y Hentz 2011, Stelinski 2019). Sin embargo, hay evidencias que indican que los diferentes niveles de resistencia se deben al manejo de insecticidas que cada citricultor realiza (García-Méndez et al. 2019, Naeem et al.2016). El conocimiento del patrón de dispersión, de este vector y la aplicación de insecticidas para su combate que hay entre el huerto son cruciales para implementar programas de manejo efectivos.

Por tanto, este estudio tiene como objetivo determinar la intensidad y variación de la resistencia a imidacloprid en adultos de *D. citri* presentes en un huerto citrícola de 8 ha.

CHAPTER I. INTRA-ORCHARD VARIATION OF RESISTANCE TO IMIDACLOPRID IN ADULTS OF *Diaphorina citri* KUWAYAMA (HEMIPTERA: LIVIIDAE).

1.1 ABSTRACT

Diaphorina citri Kuwayama is the most severe pest of citrus worldwide. It has a high capacity to develop insecticide resistance. We estimated the level of resistance and the intra-plot variation of resistance to imidacloprid in collected individuals from an orchard (8 ha) of Persian lemon *Citrus latifolia* Tan., from Martínez de la Torre, Veracruz, Mexico. This orchard was divided into eight sections of similar size. Adults were sampled from each section to assess their response to imidacloprid in F_1. We carried out two field samplings: in November 2020 and May 2021. In the individuals from the first sampling, the relative response at the LC_{50} level (RR_{50}) varied from 518× to 16,701×; sections 2 and 5 had the highest LC_{50} values. In the second experiment, the highest LC_{50} values were observed in sections 5, 6, and 3; The range of intra-orchard variation at the LC_{50} level (RR_{50}) was from 635× to 6,626×. The RR_{95} values could be estimated in two sections of the first experiment: 7,421× (section 7) and 58,958× (Section 8), respectively. For the rest of the sections of both samplings, the maximum concentration that could be prepared was 100,000 mg/L and reached ≤87.9% mortality. The range of variation at the LC_{50} among sections (FRR_{50}) was low: 1 to 32.17× in the first sampling; and from 1 to 10.43× in the second. The resistance detected to imidacloprid is the highest registered worldwide for *D. citri*. The causes and implications of the observed response for the rational management of insecticides are discussed.

Keywords: Asian citrus psyllid, *Candidatus,* Liberibacter, insect vector, insecticide resistance

1.2 RESUMEN

Diaphorina citri Kuwayama es la plaga más dañina de los cítricos a nivel mundial. Tiene una alta capacidad para desarrollar resistencia a los insecticidas. Se estimó el nivel de resistencia y la variación intra parcela de resistencia a imidacloprid en individuos colectados de una huerta (8 ha) de limón persa Citrus latifolia Tan., de Martínez de la Torre, Veracruz, México. Este huerto se dividió en ocho secciones de tamaño similar. Se tomaron muestras de adultos de cada sección para evaluar su respuesta a imidacloprid en F_1. Realizamos dos muestreos de campo: en noviembre de 2020 y mayo de 2021. En los individuos del primer muestreo, la respuesta relativa al nivel de CL_{50} (RR_{50}) varió de 518× a 16,701×; las secciones 2 y 5 tuvieron los valores más altos de CL_{50}. En el segundo experimento, los valores más altos de CL_{50} se observaron en las secciones 5, 6 y 3; El rango de variación dentro del huerto al nivel de LC_{50} (RR_{50}) fue de 635× a 6,626×. Los valores de RR_{95} se pudieron estimar en dos secciones del primer experimento: 7.421× (sección 7) y 58.958× (sección 8), respectivamente. Para el resto de las secciones de ambos muestreos, la concentración máxima que se pudo preparar fue de 100.000 mg/L y alcanzó una mortalidad de ≤87,2%. El rango de variación de la CL_{50} entre secciones (FRR_{50}) fue bajo: 1 a 32.17× en el primer muestreo; y de 1 a 10.43× en el segundo. La resistencia detectada a imidacloprid es la más alta registrada a nivel mundial para *D. citri*. Se discuten las causas e implicaciones de la respuesta observada para el manejo racional de los insecticidas.

Palabras clave: psílido asiático de los cítricos, *Candidatus*, Liberibacter, insecto vector, resistencia a insecticidas

1.3 INTRODUCTION

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is a vector for the bacteria *Candidatus* Liberibacter var. asiaticus and *Candidatus* Liberibacter *americanus*, causal agents of the Huanglongbing disease (HLB) (Halbert and Manjunath, 2004, Bové 2006). This disease has caused losses estimated at US$1 billion per year in the US state of Florida (Li et al. 2020, da Costa et al. 2021). In Mexico, it has economically affected more than 67,000 citrus producers, who harvest more than seven million tons, with an approximate value of 500 million US dollars (SENASICA 2018). To mitigate its dispersion, actions have been taken to prevent the trade of infected plant material (Qureshi and Stansly 2007, SENASICA 2018). However, their field combat has focused mainly on conventional insecticides. In most cases, growers do not have rational management programs for these products (Boina et al. 2009, García-Méndez et al. 2019, Chen and Stelinski 2017). In the face of biological efficacy problems, they usually increase the dose and frequency of applications. This scenario raises the cases of insecticide resistance (Hawkins et al. 2018) and the risks to the environment 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 vector expressed 35× resistance, under field conditions, to imidacloprid (Tiwari et al. 2011b). 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 in this insect species: 2,435× to chlorpyrifos and 4,265× to imidacloprid, respectively (Vásquez-García et al. 2013, Pardo et al. 2018). These researches demonstrated the ability of this species to live and reproduce in environments treated with commercial doses of 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 (Pasteur and Raymond 1996, Collins and Schlipalius 2018), 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 three years of study, they observed that summer resistance 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 (Naeem et al. 2016, García-Méndez et al. 2019). 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 intra-orchard 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's contribution of resistant individuals to the rest of the 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 carried out 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 taken. Therefore, the objectives of this study were to estimate, in adults of *D. citri*, the intensity of resistance to imidacloprid and its variation at the intra-orchard level.

1.4 MATERIALS AND METHODS

1.4.1. 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 been reproduced 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 (20.091184°N, -97.0662280°W), from an eight-hectare orchard with five-year-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 x 45 x 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 years 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 to carry out the bioassays. The infested plants were kept under greenhouse conditions at a temperature of 25 ± 5 °C and 60 ± 5 % relative humidity. Two field collections were made: November 2020 and May 2021. The response to imidacloprid was estimated separately for each section and sampling date.

1.4.2. Insecticides

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

1.4.3. Bioassays

The immersion method proposed by the Insecticide Resistance Action Committee (IRAC 2019) was used with slight modifications. From the middle stratum of four years old orange trees, *Citrus sinensis* (L.) Osbeck var. Valencia, not exposed to pesticides, we collected leaves to obtain discs (Ø 4.0 cm). Then, dipped for 30 s in the respective insecticide concentration. Subsequently, they were left to dry for one hour at room temperature to remove excess water. Later, placed downwards in a Petri dish containing a 3 mm layer of agar-agar (Merck®, Darmstadt, Germany).

Initially, the concentration in 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 the different intra-orchard sections. If the evaluated concentrations did not cover the range from 0 to 100%, additional repetitions were carried out, 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 mg/L.

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 x 14.9 cm) with double closure (SCJohnson a Family company, Racine Wisconsin, USA). 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 (Ø 2.0 cm in 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 ± 5 °C, 60 ± 5 % relative humidity, and a photoperiod of 12: 12 h light darkness. Mortality was recorded 24 h after exposure to the toxicant. 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 (1925).

1.4.4. Statistical analysis

Using Statistical Analysis System 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).

1.5 RESULTS

In the bioassays from individuals field collected on both dates, we observed significant variation in the response at the LC_{50} level (Tables 1 and 2) among sections. In the first experiment (field collection: November 2020), the LC_{50} of the intra-orchard sections was 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× and 16,701×, respectively (Table 1). The highest LC_{50} values were observed in sections 2 (9,721 mg/L) and 5 (22,212 mg/L), respectively (Table 1). The LC_{95} values could only be estimated for sections 7 and 8: 690.5 and 918.5 mg/L, equivalent to RR_{95} of 7,421× and 58,958×, respectively (Table 1). For sites 1, 2, 3, 4, 5, and 6, the highest dose that could be applied was 100,000 mg/L, which caused average mortality levels between 64.5 and 87.9%, respectively (Table 1). Consequently, the LC_{95} values were considered >100,000 mg/L, and the relative response at the 95% mortality level (RR95) was not calculated.

In the second experiment (field collection: May 2021), among sections, the LC_{50} ranged from 2,566 to 26,770 mg/L, and the variation in response to imidacloprid at 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), respectively (Table 2). It was impossible to estimate the LC_{95}

in any intra-orchard sites because the maximum concentration that could be evaluated was 100,000 mg/L. This concentration caused average mortality between 54.8 and 87.2% (Table 2). For the reasons indicated, the information was processed similarly to experiment 1.

To estimate the intra-orchard 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 experiment 1 (November 2020) ranged from 1.0× (section 7) to 32.17× (section 5). In experiment 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×), respectively (Tables 1 and 2). In experiment 1, the FRR_{95} values were calculated only for sections 7 (1×) and 8 (7.94×). However, in the second experiment, the LC_{95} could not be estimated in any section since the mortality values with the highest concentration of imidacloprid that could be prepared (100,000 mg/l) varied from 54.8 to 87.2%.

1.6 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 and 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 intra-orchard level, in this document, 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 before, 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 field relative response variable at the intra-orchard level.

Imidacloprid is one of the most used insecticides in citrus against the Asian psyllid (Serikawa et al. 2012, Ruiz-Galván et al. 2015, Fletcher et al. 2018). As a result, this pest has developed the biological capacity to live and reproduce in citrus environments treated with this product (Vázquez-García et al. 2013, Naeem et al. 2016, Langdon and Roger 2017). 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 °C and 28 °C, the life cycle of *D. citri*, from egg to adult, is completed in 14-17 days. During the 195 days that elapsed from the beginning of the first field collection (November 2020) to the second one (May 2021), we estimate that in this orchard, nine to 10 generations were developed. During this time, the resistance level did not drop. On 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 micro-evolutionary phenomenon does not come from the use of agrochemicals following the recommendations printed on the respective commercial label. Instead, it is a consequence of abuse, as suggested by Georghiou (1986)

However, there are "levels of abuse." In the orchard under study, the owner delegates responsibility for citrus production to people without experience or interest in adequately managing pests. For the combat of *D. citri*, in this orchard, during the last five years, they have used imidacloprid exclusively and carried out 12 to 15 applications annually. The commercial label of this product recommends to apply 30-40 ml of formulated product/100 liters of water. They use 100 mL of formulated product/100 L of water. Although the intense use of high doses of imidacloprid, they are not exerting control of this vector. We estimate that imidacloprid field efficacy is around 30-50%, which is unacceptable.

This scenario configures 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 of insecticides. Unfortunately, the strategies of rational insecticide management do not fully consider the potential existence of "red spots" and their impact on pest management. 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 a "bite" to use specific commercial brands of insecticides in favor of 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.

1.7 ACKNOWLEDGMENT

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Table 1. Variation in resistance to imidacloprid in adults of *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), from eight sections of an eight-ha orchard of Persian Lemon, *Citrus latifolia* Tan. Martínez de la Torre, Veracruz, Mexico. First field collection: November 2020.

Orchard section	*n**	df**	*b* ± SE^{+}	$CL_{50}$$^{++}$ 95%LF (mg/L)	$CL_{95}$$^{++}$ 95%LF (mg/L)	Pr > $\chi2$§	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)	<.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, **degrees of freedom, ***highest mortality observed at 100,000 mg/L, $^{+}$slope and its standard error, $^{++}$estimated concentration that caused 50% (LC_{50}) or 95% (LC_{95}) mortality (mg a.i. L^{-1}) and its 95% fiducial limits, §probability higher than $\chi2$; §§relative response= highest $LC_{50(95)}$ value/$LC_{50(95)}$ of the respective insecticide; §§§variable relative field response= all high numbers $RR_{50(95)}$ value /lowest $RR_{50(95)}$ value.

Table 2. Variation in resistance to imidacloprid in adults of *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), 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**	*b* ± SE+	CL_{50}++ 95%LF (mg/L)	CL_{95}++ 95%LF (mg/L)	Pr >χ^{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, **degrees of freedom, ***highest mortality observed at 100,000 mg/L, +slope and its standard error, ++estimated concentration that caused 50% (LC_{50}) or 95% (LC_{95}) mortality (mg a.i. L^{-1}) and its 95% fiducial limits, §probability higher than χ2; §§relative response= highest $LC_{50(95)}$ value/$LC_{50(95)}$ of the respective insecticide; §§§variable relative field response= all high numbers $RR_{50(95)}$ value /lowest $RR_{50(95)}$ value.

CONCLUSIÓN

Se observó variación en la respuesta de adultos de D. *citri* a imidacloprid entre las ocho secciones de un huerto de limón persa, *Citrus latifolia* Tan, en las dos fechas de muestreo (noviembre de 2020 y mayo de 2021). En los individuos recolectados en la primera fecha de muestreo, la respuesta relativa a la CL_{50} (RR_{50}) varió de 518× a 16,701×; siendo las secciones 2 y 5 las que tuvieron los valores más altos. Para el segundo experimento el rango de variación dentro del huerto para la CL_{50} (RR_{50}) fue de 635× a 6,626× y los valores más elevados de RR_{50} se observaron en las secciones 5, 6 y 3. Los valores de RR_{95} se pudieron estimar solo en dos secciones del primer experimento: 7,421× (sección 7) y 58,958× (sección 8), respectivamente. Para el resto de las secciones de ambos muestreos, la concentración máxima que se pudo preparar fue de 100,000 mg/L y alcanzó mortalidades ≤87.9%. Por tanto, no se pudo estimar los valores de CL_{95} y RR_{95}. El rango de variación de la CL_{50} entre secciones (FRR_{50}) fue bajo: 1 a 32.17× en el primer muestreo; y de 1 a 10,43× en el segundo. La resistencia detectada a imidacloprid es la más alta registrada a nivel mundial para *D. citri.* La variación de la respuesta a imidacloprid a nivel intra huerto fue más baja que la variación observada de los individuos de las diferentes secciones del huerto en referencia a la población susceptible. Para imidacloprid en adultos de *D. citri*, se detectaron los niveles de resistencia más elevados a nivel mundial.

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