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DISEÑO DE UNA METODOLOGÍA RÁPIDA PARA DETERMINAR RESISTENCIA DE *Diaphorina citri* Kuwayama A INSECTICIDAS

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DISEÑO DE UNA METODOLOGÍA RÁPIDA PARA DETERMINAR RESISTENCIA DE *Diaphorina citri* Kuwayama A INSECTICIDAS

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Colegio de Postgraduados, 2022

RESUMEN

Diaphorina citri Kuwayama (Hemiptera: Liviidae), es una plaga ampliamente distribuida en las zonas citrícolas del mundo. Causa daños directos al extraer la savia de los brotes tiernos, provocando desordenes fisiológicos e inhibición de la fructificación. El daño indirecto se deriva de la transmisión de la bacteria *Candidatus* Liberibacter asiaticus, misma que provoca la enfermedad del Huanglongbing. Los árboles infectados no viven más de díez años. El combate de este vector se sustenta principalmente en el uso de insecticidas. En consecuencia, la resistencia a dichos productos está ampliamente distribuída. Para manejar este fenónemo se realizan bioensayos con insecticidas en las poblaciones objeto de combate y su respuesta se compara con una población susceptible de la misma especie. Las poblaciones que se utilizan como referente de susceptibilidad se recolectan de campo y se reproducen en laboratorio por varias generaciones para "asegurarse" de que si existiera algún nivel de resistencia, éste se pierda. Se evaluó la susceptibilidad a insecticidas en adultos de una población silvestre de *D. citri* que se ha mantenido libre de contacto con agroquímicos, por al menos 18 años. Dicha población fue más sensible a clorpirifós, malatión y spinosad que aquellas documentada en la literatura. Se generó metodología para estimar, de manera confiable, la respuesta de una población de *D. citri* con tan solo 30 minutos de exposición al insecticida y con individuos directamente recolectados en campo. Esta investigación contribuyó a reducir en 99.9% el tiempo que, a nivel mundial, se requiere para estimar en poblaciones de campo, la resistencia del psílido asiático a los insecticidas clorpirifos, malatión, imidacloprid y spinosad.

Palabras clave: psílido asiático, plaga, cítricos, bacteria, huanglongbing

DESIGN OF A METHODOLOGY TO DETERMINING RESISTANCE OF *Diaphorina citri* Kuwayama TO INSECTICIDES

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ABSTRACT

Diaphorina citri Kuwayama (Hemiptera: Liviidae) is a pest widely distributed in citrus growing areas of the world. It causes direct damage by extracting sap from the young shoots, causing physiological disorders and inhibition of fruit set. Indirect damage derives from the transmission of the bacterium *Candidatus Liberibacter* asiaticus, which causes the Huanglongbing disease. Infected trees do not live more than ten years. Combating this vector is mainly based on the use of insecticides. As a result, resistance to these products is widely distributed. To manage this phenomenon, bioassays with insecticides are carried out on the target populations and their response is compared with a susceptible population of the same species. The populations used as a reference for susceptibility are collected from the field and reproduced in the laboratory for several generations to "make sure" that if any level of resistance exists, it is lost. Susceptibility to insecticides was evaluated in adults of a wild population of *D. citri* that has been out of contact with agrochemicals for at least 18 years. This population was more sensitive to chlorpyrifos, malathion and spinosad than those documented in the literature. Methodology was generated to reliably estimate the response of a *D. citri* population with only 30 minutes of exposure to the insecticide and with individuals directly collected in the field. This research contributed to reduce by 99.9% the time required worldwide to estimate the resistance of the Asian psyllid to the insecticides chlorpyrifos, malathion, imidacloprid and spinosad in field populations.

Key words: asian psyllid, pest, citrus, bacteria, huanglongbing

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INTRODUCCIÓN GENERAL

México ocupa el quinto lugar en producción de cítricos a nivel mundial con una superficie establecida de 593,097 ha, mismas que generan una derrama económica de 10 mil millones de pesos anuales (SIAP 2021). La mayor producción nacional de limón mexicano (*Citrus aurantifolia* Swingle) (2,851,426 ton), se concentra en los estados de Colima, Michoacán y Veracruz (SIAP 2021). Las especies con mayor demanda son el limón mexicano y la naranja (*Citrus x sinensis* (L) Osbec), con 204,683 y 342,592 ha establecidas, respectivamente. Particularmente, en el Valle de Apatzingán, Michoacán se encuentran establecidas 63,741 ha de limón mexicano (SIAP 2021).

La demanda de cítricos en México ha conllevado al establecimiento intensivo de huertos. Aunado a eso, han surgido problemas fitosanitarios. Entre las plagas más importantes que se han presentado en limón mexicano se encuentran los trips *Frankliniella occidentalis* Pergande (Thysanoptera:Thripidae) (Miranda-Salcedo et al. 2020), la mosca blanca *Trialeurodes vaporariorum* Westwood (Heteroptera: Aleyrodida) (Aguilar-Astudio et al. 2020), el minador de la hoja *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae) (Fuentes-Guardiola et al. 2017) y el psilido asiático de los cítricos *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) (Yzquierdo-Alvarez et al. 2021). Siendo *D. citri* la plaga de mayor importancia a nivel nacional.

En México, el psílido asiático de los cítricos se documentó por primera vez a principios de 2002 en los estados de Quintana Roo y Campeche (Martínez y Cortez 2008). Para 2008, esta plaga estaba dispersa en la frontera norte de Baja California. En Michoacán, la presencia de *D. citri* se registró por primera vez en 2006 en el municipio de Tepalcatepec en el Valle de Apatzingán (Martínez 2008, López-Arroyo et al. 2010).

Esta especie de insecto causa daños directos e indirectos. El daño directo proviene de su alimentación de brotes tiernos. Además, segrega una sustancia blanca que recubre la superficie de las hojas y permite el crecimiento de un complejo de especies de hongos como *Capnodium citri* Berk y Desm., y *Cladosporium sp*. (Michaud 2004, Pérez-Artiles el al. 2017). El psílido asiático de los cítricos, al alimentarse, inyecta toxinas que inhiben el crecimiento de los brotes y deforman las hojas, provocando una disminución de la capacidad fotosintética. Estos efectos adversos afectan el desarrollo normal del follaje, de la floración y pueden ocasionar reducciones del rendimiento de hasta 50%, incluso matar el árbol (Bin et al. 2019). Los árboles viejos son más tolerantes al daño que los jóvenes porque pierden menos hojas (Ruiz 2019). El daño indirecto más crítico se deriva de la transmisión de la bacteria *Candidatus* Liberibacter asiaticus, que causa la enfermedad de Huanglongbing (HLB) o dragón amarillo (Ruiz 2019).

El estatus actual de *D. citri* en México en el mundo indica que se trata de una especie cuarentenada debido a que es transmisor de la bacteria *Candidatus* liberibacter asiaticus (SENASICA 2022). La enfermedad del HLB ha provocado la muerte de mas de 63 millones de árboles Ámerica, Sudafrica y Brasil (SADER 2021). Actualmente existe investigación sobre su manejo en campo. Se tienen estudios sobre su dinámica y fluctuación poblacional (Pardo 2013), ciclo biológico, así como la relación que tiene con cada uno de sus hospederos y preferencias de oviposición (Fernández y Miranda 2005). Se ha avanzado en el uso racional de insecticidas (Pérez-Zarate et al. 2020; Pardo et al. 2018a), conocimiento del parasitismo natural y agentes de control biológico (Pardo et al 2018b), También se han realizado estudios moleculares sobre genes que codifican enzimas responsables de la resistencia (Killiny et al. 2014).

Ante la problemática derivada de los daños que esta plaga ocasiona a los cítricos, los productores hacen aplicaciones desmedidas de insecticidas, con poca o ninguna asesoría técnica. En consecuencia, dicha plaga ha desarrollado resistencia a la mayoría de los compuestos autorizados para el combate en México (Pardo et al. 2018a). De acuerdo con Mota-Sánchez y Wise (2021), *D. citri* a nivel mundial ha desarrollado resistencia a ocho ingredientes activos: acetamiprid, bifentrina, clorfenapir, clorpirifós, imidacloprid, malatión, nitenpiram y tiametoxam. Estos registros de resistencia han sido principalmente en Pakistán, Estados Unidos de América, México y China. Los centros de investigación y universidades han desarrollado metodologías para la medición de resistencia. Estas metodologías se basan en procesos convencionales de laboratorio.

Los estudios de resistencia a insecticidas en condiciones de laboratorio implican la recolección en campo de la especie objetivo, su reproducción en laboratorio para obtener en F_1 los individuos necesarios para realizar los bioensayos donde se compara la respuesta de dicha población de campo con una susceptible de referencia. Idealmente, los insectos que se usan como referente de comparación de la respuesta a insecticidas deben estar libres, en su genoma, de alelos de resistencia. Para cumplir con este objetivo es menester tener una raza de laboratorio certificada. Este proceso es muy costoso, consume mucho tiempo, requiere de especialistas y lugares especiales destinados a su crianza. Todos estos inconvenientes han conllevado a que existan pocos casos de investigaciones que utilicen como referente de susceptibilidad razas como New Orleans y Rockefeller del mosquito *Aedes aegypti* L. (Diptera: Culicidae). Una opción práctica y aceptada en la comunidad científica consiste en usar, como referentes de susceptibilidad, razas de insectos que se han reproducido por muchas generaciones (no hay una cantidad definida) en condiciones de laboratorio y libres de presión de selección con insecticidas. Otra posibilidad sería recolectar en campo una población de insectos de la especie de interés que sea susceptible a insecticidas, reproducirla a F_1 y realizar bioensayos de manera simultánea con los insectos de la población de campo sospechosa de haber desarrollado resistencia. Desafortunadamente, esta posibilidad es inexistente en la inmensa mayoría de los casos, dado que es virtualmente imposible encontrar una población de campo que no haya sido sometida a presión de selección. Como parte de los trabajos de campo de la presente investigación se encontraron individuos de *D. citri* en un área silvestre, alimentándose de *Murraya paniculata* Swingle, una especie que se ha utilizado de manera ornamental, aislada al menos 15 km de plantas de cítricos u otros hospederos y que no ha sido seleccionada por insecticidas. Su vuelo corto, asegura que, en caso de que hubiera tenido intercambio genético con individuos de la misma especie, pero sometidos a aplicaciones comerciales, es insignificante. Se aprovechó la oportunidad de evaluar su respuesta a los insecticidas malatión, spinosad, clorpirifos e imidaclorprid. y compararla con otras poblaciones susceptibles cuyos datos de bioensayo se han publicado en revistas indexadas.

Cada metodología de cría y exposición al tóxico tiene características especiales en función de la especie de plaga y del insecticida. Esta es la manera en que se ha inferido la evolución de la resistencia a insecticidas y sus resultados han servido de guía para tomar decisiones en campo (Pardo et al 2018a, Chen y Stelinski 2017a, Naeem et al. 2016, IRAC 2014, Vázquez-García 2013, Tiwari et al. 2011). Todos los estudios antes mencionados requieren al menos un mes en arrojar datos confiables. El tiempo que tarda el investigador en obtener datos valiosos para guiar las decisiones de manejo de insecticidas en condiciones de campo pierden su utilidad debido a que se conocen demasiado tarde. Incluso, la disponibilidad de la información existe hasta que los resultados se publiquen; lo cual podría tardar un año adicional. Además, las publicaciones científicas, si bien es cierto, contienen información importante, por su naturaleza es muy complicado que los agricultores las usen como insumo en las decisiones de manejo de insecticidas. Para contribuir a resolver este problema que se presenta no solo en México, sino en el mundo, en este trabajo se estableció como objetivo diseñar de una prueba rápida para determinar resistencia de *Diaphorina citri* Kuwayama a nivel parcelario.

CHAPTER I. SUSCEPTIBILITY OF A MEXICAN FIELD-COLLECTED WILD POPULATION OF DIAPHORINA CITRI (HEMIPTERA: LIVIIDAE) TO SELECTED INSECTICIDES

1.2. ABSTRACT

The Asian citrus psyllid, *Diaphorina citri* (Kuwayama) (Hemiptera: Liviidae), is a severe pest of citrus orchards worldwide. Its control is based mainly on the use of conventional insecticides, and resistance to many of those compounds is widespread. Phenotypic bioassays to detect resistance compare the response of a field-collected population with a laboratory-reared population that is susceptible to insecticides. This comparison usually does not involve a susceptible field-collected counterpart, since its existence is currently rare. We found an isolated field population of *D. citri* living on a wild host, orange jasmine (*Murraya paniculata* [L.] Jack). Considering its lifetime fly capacity, gene flow with any insecticide-treated population was nonexistent or negligible. Thus, we determined the response in fourth-instar nymphs and unsexed 2 to 5-d-old adults in bioassays of commercial formulations of the commonly-used insecticides chlorpyrifos, malathion, imidacloprid, and spinosad. In the bioassays, insects were placed on leaf discs previously immersed for 10 s in the respective insecticide concentrations. For adults, the lowest concentration-mortality response was with chlorpyrifos (LC_{50} of 0.72 mg L^{1} and LC_{95} of 1.02 mg L^{1}). The highest toxicity response was with malathion (LC_{95} of 0.05 mg L^{1}). The highest toxicity response with fourth-instar nymphs was observed with spinosad (LC_{50} of 0.007, LC_{95} of 0.021 mg L^{1}). The estimated LC_{50} and LC_{95} values for chlorpyrifos, malathion and spinosad were lower than those documented worldwide for these insecticides in susceptible populations of *D. citri*.

1.3. INTRODUCTION

The Asian citrus psyllid, *Diaphorina citri* (Kuwayama) (Hemiptera: Liviidae), is one of the most serious pests of citrus crops causing millions of dollars in losses worldwide (Giuseppe 2017). In China, it reportedly destroyed 200,000 ha of citrus (Deng 2019). *Diaphorina citri* has been so devastating in Mexico that it threatens to end the Mexican citrus industry (Díaz-Padilla et al. 2014). It causes direct damage as it feeds on tender shoots, but it also transmits the bacterium *Candidatus* Liberibacter asiaticus which causes Huanglongbing disease in citrus and other plants (Ruiz 2019, López-San Juan 2021).

The most common control tactic to decrease Asian citrus psyllid populations and the disease they transmit has been the use of conventional insecticides (Qureshi and Stansly 2007, 2008, Rogers 2008). Monitoring the response to insecticides by this vector is of great importance to evaluate the efficacy of resistance management strategies. These studies require the use of an insecticide-susceptible population to compare its response with those from the field. However, it is difficult to find populations of *D. citri* fully susceptible to insecticides. The municipality of Múgica, state of Michoacán, Mexico, has an area of 378.2 km^2 and contains a sparse wild population of orange jasmine (*Murraya paniculata* [L.] Jack) on which we found a wild population of *D. citri* that lives and reproduces year-round (Hernández-Landa et al. 2013). This area is at least 5 km away from the closest suitable citrus plant host (e.g., lemon, *Citrus limon* [L.] Burm,) of this pest. In their study of the dispersal capacity of *D. citri*, Hall and Hentz (2011) found a single individual on a trap 150 m away from the release point. Arakawa and Mivamolo (2007) estimated that individuals of *D. citri* (4 d old) were able to fly, on their own, 26.9 min at an average of 1.6 km h^{1}, reaching a maximum distance of 0.7 km.

Based on these findings, we postulated that the *D. citri* population infesting orange jasmine either does not receive immigrant *D. citri* or that immigration of *D. citri* into the area is marginal or nonexistent due to the poor capacity of *D. citri* to disperse.

We, consequently, consider that our target *D. citri* population is fully susceptible to insecticides and lacks the severe genetic bottlenecks characteristic of insect populations maintained under laboratory or greenhouse conditions. Georghiou (1986) noted that, while insect resistance to insecticides was rare in the 1950s, fully susceptible populations of insects are rare the 1980s. Consequently, evaluating the response of a population of *D. citri* that has remained free of selection pressure and with marginal or nonexistent genetic exchange with populations of the same species exposed to commercial applications of conventional insecticides is a rare opportunity to determine the response to insecticides in unselected field-collected populations. Therefore, this study aimed to determine the baseline susceptibility of a wild Mexican field-collected population of *D. citri* to the insecticides spinosad, imidacloprid, chlorpyrifos, and malathion.

1.4. MATERIALS AND METHODS

1.4.1. Insect collection and rearing

In November 2019, approximately 500 adults of *D. citri* were field-collected in the Municipality of Múgica, Mexico (19.04221 - 102.083251), where this species reproduces year-round on wild *M. paniculata* plants. A mouth aspirator was used to collect specimens from the middle stratum of the plants. Specimens were transported to and released on *M. paniculate* plants in a greenhouse maintained at 16-30°C, 60% relative humidity (RH), and on a photoperiod of 12:12 h (L:D). Bioassays were conducted using F_1 individuals from these specimens.

1.4.2. Insecticides

The insecticides selected for the study were commercial formulations commonly used in citrus production and Mexican production systems. These were Lorsban® (chlorpyrifos, 480 g active ingredient [a.i.] L^{1}, concentrate suspension, Dow Agrosciences, Mexico), Malphos® (malathion, 1000 g a.i. L^{1}, emulsifiable concentration, Agrícola Innovación, S. A. de C. V., Mexico), Confidor® (imidacloprid 480 g a.i. L^{1}, concentrated suspension, Bayer Crop Science, México), and Tracer® (spinosad, 350 g a.i. L^{1}, concentrated suspension, Dow Agrosciences, Mexico).

1.4.3. Bioassays

The bioassays were conducted by the dip method proposed by the Insecticide Resistance Action Committee (IRAC) (2019) with some modifications. Leaf discs (30 mm in diameter) were obtained from young shoots (6-8 cm long, 30-40 days old, lower third stratus) of alemow plants (*Citrus macrophylla* Wester). The discs were dipped and immersed for 10 s in 50 mL of different concentrations of the insecticides chlorpyrifos (0.00010, 0.00022, 0.00038, 0.00045, 0.00052, 0.00062, 0.00075, 0.00085 mg L^{1}), malathion (0.010, 0.021, 0.046, 0.055, 0.069, 0.075, 0.080, 0.10 mg L^{1}), imidacloprid (0.10, 0.22, 0.46, 0.58, 0.70, 0.82, 1.00 mg L^{1}), and spinosad (0.001, 0.0016, 0.0035, 0.0050, 0.0070, 0.0087, 0.0100, 0.0120 mg L^{1}). These concentrations were determined based on preliminary experiments to estimate the biological response window or concentration range that caused between 0 and 100% mortality. Then, these intermediate concentrations were included to cover that range. As a control, we used leaf discs of *C. macrophylla* dipped in distilled water.

The treated leaf discs were dried for 1 h on absorbent paper. Then, they were individually placed with the adaxial part in touch with an agar (Bacto™ Agar; Becton and Dickinson Co., Mexico City, Mexico) layer in a Petri dish (40 mm diameter, containing 5 mL of 1.5% agar prepared with distilled water). Adults (2-5 d old, unsexed) and nymphs (fourth instar) from the F_1 generation were used for the bioassays.

The adults were anesthetized for 8 s with CO_2 and transferred in groups of 15 to the abaxial surface of the leaf disc. Before transferring nymphs, the samples were stimulated with a small brush to interrupt their feeding to avoid damaging the stylet. Then, with the help of an 11-2 brush, were transferred to the abaxial part of the treated leaf disc. The Petri dishes were covered and placed upside down, so the individuals were in the normal position. Five replicates were conducted on different days, and each replication included an untreated control.

Mortality was evaluated at 24 h after initial exposure. Adults and nymphs were considered dead if they remained immobile when touched with a brush, as suggested by Pardo et al. (2018). The experimental units were maintained in a controlled environmental chamber at 25 ± 2°C, 75 ± 5% RH, and on a photoperiod of 16:8 h (L:D).

1.4.4. Statistical analysis

The maximum mortality accepted in the untreated control was 10%, and it was corrected using the Abbott (1925) formula. Probit analysis (Finney 1971) was performed using the

Proc Probit procedure of SAS version 9.3 (SAS Institute 2008) to estimate the slope, lethal concentrations (LC_{50}, LC_{95}), fiducial limits (95%), and test of goodness of fit to a straight line. To estimate the relative toxicity (RT) of each insecticide, the highest $LC_{50(95)}$ value was used as a reference, RT = highest $LC_{50(95)}$ value/$LC_{50(95)}$ of the respective insecticide.

1.5. RESULTS AND DISCUSSION

In comparing the LC_{50} and LC_{95} levels of the insecticides in adults, the lowest toxicity was observed with chlorpyrifos (0.72 and 1.02 mg L^{1}, respectively), with a relative toxicity (RT) of 1.0 (Table 1). In comparing the LC_{50} levels, the highest toxicity detected was with malathion and spinosad (0.05 mg L^{1}). Malathion was the most toxic insecticide at LC_{95} level (0.09 mg L^{1}), with a relative toxicity (RT) of 11.3× (Table 1).

In nymphs, the least toxic insecticide based on the LC_{50} and LC_{95} levels was chlorpyrifos (0.72, 1.0 mg L^{1}). Based on the LC_{50} and LC_{95} levels, the highest toxicity was observed with spinosad (0.007 and 0.021 mg L^{1}, respectively), and the RT values were 102.9 and 46.6×, respectively (Table 1).

The first stage of a sound resistance management program consists of estimating the natural variation in response to a new insecticide type before it is available commercially. This practice is common in plant protection with transgenic plants and is known as baseline susceptibility (Rivero-Borja 2020). Unfortunately, the market introduction of novel insecticides is usually not supported by prior knowledge of the geographic variation in target pest population response. The problem is complicated because it is challenging to find fully susceptible populations under field conditions (Stacke et al. 2019). Consequently, there is generally no estimation of the response variability that can be used as a benchmark for comparison through the years in which an insecticide is used (Sudo et al. 2018).

Table 1. Toxicity of several insecticides against adults (2-5 d old, unsexed) and nymphs (fourth instar) of a wild population of the Asian citrus psyllid *Diaphorina citri*.

Biological stage	Insecticide	$n^{ς}$	df^{Ω}	$b \pm SE^{\Psi}$	LC_{50}^{α} mg L^{1} FL$^{϶}$ 95% mg L^{1}	LC_{95}^{β} mg L^{1} FL 95% mg L^{1}	$Pr>\chi^{2\Re}$	RT* LC_{50}	RT* LC_{95}
Adult	Malathion	600	5	6.84 ± 0.80	0.05	0.09	0.54	14.4	11.3
					0.050-0.056	0.085-0.107			
	Spinosad	675	6	4.02 ± 0.60	0.05	0.15	0.05	14.4	6.8
					0.052–0.070	0.111-0.295			
	Imidacloprid	600	5	3.95± 0.38	0.44	0.86	0.51	1.63	1.18
					0.405-0.493	0.771-0.991			
	Chlorpyrifos	600	5	5.46± 0.55	0.72	1.02	0.61	1.0	1.0
					0.703-0.751	0.9771.104			
Nymph	Spinosad	600	5	3.64 ± 0.45	0.007	0.021	0.48	102.9	47.6
					0.007-0.008	0.017-0.032			
	Malathion	600	5	5.51 ± 0.47	0.05	0.11	0.23	14.4	9.09
					0.055-0.061	0.104-0.136			
	Imidacloprid	600	5	4.02 ± 0.32	0.43	0.84	0.13	1.67	1.19
					0.403-0.475	0.779-0.938			
	Chlorpyrifos	600	5	5.83± 0.49	0.72	1.00	0.72	1	1
					0.697-0.742	0.9631.056			

^{ς} Treated insects; $^{\Omega}$ Degrees of freedom; $^{\Psi}$ Slope ± standard error; $^{\alpha}$ Estimated concentration that caused 50% mortality (mg i.a. L^{1}); $^{϶}$95% fiducial limits; $^{\beta}$ estimated concentration that caused 95% mortality; $^{\Re}$ probability higher than χ^2; *relative toxicity = highest $LC_{50(95)}$ value/$LC_{50(95)}$ of the respective insecticide.

Thus, researchers reproduce for several generations, under laboratory conditions, the pest species of interest, to allow the phenotypic expression of the resistance alleles to decrease in each generation until they are undetectable by phenotypic bioassays (Chen and Stelinski 2017b, García-Mendez et al. 2019, Pardo et al. 2018). For example, Tiwari et al. (2011) estimated the resistance of *D. citri* in Florida using a field-collected population maintained under laboratory conditions for more than 50 generations as a reference for comparison. Due to the small sample size of individuals that arrive at the laboratory, genetic drift and adaptation processes during generations may significantly modify insecticide responses (Roush and Tabashnick 2012). Thus, generating different results from those observed in a population reared only one generation under this condition.

Tiwari et al. (2011) used the same methodology as we employed and estimated an LC_{95} of 4.81 (0.90 - $1x10^7$) mg L^1 for adults to the insecticide chlorpyrifos; in our population, we observed LC_{95} values of 1.02 (0.9771.104) and 1.0 (0.9631.056) mg L^1 for adults and nymphs, respectively. Vázquez-García (2013) assessed the susceptibility of *D. citri* to different chemical compounds in a field-collected population from the state of Colima, Mexico. These authors documented LC_{95} values of 0.42 (0.20 - 1.5) mg L^1 for adults in response to imidacloprid, while we observed LC_{95} values of 0.86 (0.771-0.991) for adults and 0.84 (0.779-0.938) mg L^1 for nymphs. Vázquez-García (2013) also documented that the LC_{95} for malathion was 13.43 mg L^1; we found an LC_{95} of 0.09 (0.085-0.107) and 0.11 (0.104- 0.136) mg L1 for adults and nymphs, respectively.

1.6. CONCLUSIONS

Thus, our study of the field-collected population *D. citri* showed lower LC_{50} and LC_{95} values for malathion, spinosad, and chlorpyriphos than those reported from previous worldwide studies of susceptible populations. The population we discovered and studied in Michoacán, Mexico, will provide a baseline standard for studies involving response of *D. citri* adults and nymphs to various insecticides.

CHAPTER II. THIRTY-MIN TEST TO DETECT INSECTICIDE RESISTANCE UNDER FIELD CONDITIONS: THE CASE OF THE ASIAN CITRUS PSYLLID

2.1. ABSTRACT

Diaphorina citri Kuwayama is among the most destructive citrus pests worldwide. Its combat is carried out mainly through applications of conventional insecticides. The current methodologies to estimate the resistance to the pesticides used against this pest are unsuccessful because they do not provide timely and trustworthy information to make decisions at the site the spraying is needed. The use of diagnostic doses with 30-min exposure is proposed to estimate the resistance of *D. citri* to imidacloprid, spinosad, malathion, and chlorpyrifos at the orchard level. Under laboratory conditions, in a susceptible *D. citri* colony, we estimated the lowest doses that produced 100% mortality within 30-min of exposure (diagnostic dose). For imidacloprid, spinosad, malathion, and chlorpyrifos, these doses were 7.4, 4.2, 1.0, and 5.5 mg a. i. L^{-1}, respectively. Under field conditions, we applied in five localities of Michoacan state, Mexico (Nueva Italia, Santo Domingo, El Varal, Gambara, and El Señidor) those diagnostic doses and set up an experiment to estimate the field efficacy of these insecticides against *D. citri* populations feeding on *Citrus aurantifolia* Swingle. The results of the diagnostic doses showed a significant correlation with the field biological efficacy of imidacloprid, malathion, and chlorpyrifos ($R^2 \geq 0.93$). Correlation for spinosad could not be estimated because the mortality caused by the diagnostic dose and its field efficacy in all study sites was consistently >98%. For all tested insecticides, field efficacy and resistance can be estimated based on the field diagnostic dose with 30-min exposure. Consequently, the producer has the possibility, at the orchard level, to estimate the performance of evaluated insecticides before carrying out their commercial application.

2.3. INTRODUCTION

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is found in all citrus-growing areas of the world (Wang et al. 2019), with devastating effects in Pakistan (Khan et al. 2018), Malaysia (Leong et al. 2019), China (Wang et al. 2019), United States of America (Johnson et al. 2019), Mexico (Pardo-Melgarejo et al. 2022), and Brazil (Zorzenon et al. 2021). This species feeds on important crops such as the Mexican lemon *Citrus aurantifolia* Swingle (Pardo-Melgarejo et al. 2022), Valencia orange *Citrus sinensis* L Osbeck (Ebert et al. 2020), grapefruit *Citrus paradisi* MacFad (Alves et al. 2021), and mandarin *Citrus reticulata* Blanco (Iqbal et al. 2020). *D. citri* causes both direct and indirect effects. The direct damage is caused by the extraction of large amounts of sap from the plant's phloem, which causes a decrease in fruit quality and yield (Tsai et al. 2009). The indirect damage is the most important and is associated with the ability to transmit one of the most destructive diseases, the HLB (Huanglongbing), also known as the yellow dragon (Rwomushana et al. 2017). The causal agent of this disease is the bacteria *Candidatus* Liberibacter asiaticus and *Candidatus* Liberibacter americanus. Infected trees do not live more than 10 years (Bové 2006). HLB has caused a total loss of citrus trees on millions of hectares worldwide, mainly in North America (Álvarez et al. 2016). The implemented control tactics to decrease the density of *D. citri* populations include using biorational and conventional insecticides (Kumar et al. 2017). Parasitoids and predators have also been used (Flores and Ciomperlik, 2017), but they have not been as successful as expected (Pluke et al. 2008). To reduce vector populations, farmers and government entities use systemic and contact insecticides (Miranda et al. 2018). The disorderly and abundant use of insecticides has led to the development of resistant populations (Tian et al. 2018), compromising the future of citrus production worldwide.

Research centers estimate the dynamics of insecticide resistance as a basis for decision-making in the field (Chen and Stelinski 2017; Pardo et al. 2018; Tian et al. 2018; Rao et al. 2019; Naeem and Freed 2018; Chen et al. 2021). However, these studies are questionable because they do not consider the geographic variation in response to insecticides that populations of this pest show within small geographical areas. This variation is due to the reduced dispersion of *D. citri* and its complex response to insecticides in time and space (Antolines et al. 2021, Hall and Hentz 2011). Therefore, the levels of resistance to insecticides that are estimated over vast areas are not representative of the response of this pest at the orchard level, where the citrus grower must make decisions.

Although these problems were solved, the results of conventional bioassays do not provide reliable information to the farmer because they do not consider the variation in response at the orchard level. The current studies lack scientific bases to infer the existing resistance status at the orchard level.

In addition, we consider that the time that it takes to estimate the level of response to insecticides is too long. For example, for *D. citri*, nymphs of different instars are generally field-collected to keep them under controlled conditions and obtain enough individuals in the F_1 with the desirable characteristics to carry out the bioassays. Assuming that nymphs of all instars are collected in the field, the availability of nymphs and adults of the F_1 occurs in 20 and 45 days, respectively. Considering that there are no problems in the execution of the bioassay nor the availability of individuals, the complete bioassay will take at least one additional week. We must add the time for statistical processing and preparation of the report. If the dissemination of the results is via a scientific article, it would not be unusual for this process to take another year. In addition, the information is designed in a format that is unintelligible not only for the farmer but also for the vast majority of the technicians who advise them.

Furthermore, the low representativeness of data at the orchard level is contrary to the interests of growers who demand reliable and trustworthy data generated in a short time. Thus, a reliable rapid test to detect resistance under field conditions and at the orchard level is needed. We expect that a test like this would correlate with the field efficacy of the respective insecticide. Thus, the growers may have the scientific evidence to avoid using useless chemicals and contribute to reversing the existing resistance level, if any. Therefore, the objective of this study was to develop a methodology that allows, in 30-minutes, to estimate at the orchard level, resistance to imidacloprid, spinosad, malathion, and chlorpyrifos in field populations of *D. citri* and correlate it with field efficacy studies.

2.4. MATERIALES Y MÉTODOS

2.4.1. Diagnostic dose estimation

2.4.1.1 Susceptible population

In November 2019, between 1500 and 2000 adults of *D. citri* were field-collected from a wild population in an urban area of Nueva Italia, Michoacán, Mexico (19.01837º N 102.10269º W). These adults were feeding on *Murraya paniculate* (L.), an ornamental plant that has not been exposed to insecticides in at least 18 years, so this population is considered susceptible to insecticides (Pardo-Melgarejo et al. 2022).

With the help of an aspirator, adults were captured and placed in an entomological cage (50 x 50 x 50 cm) covered with organza fabric (Capital Textil S.A. de C.V., Ciudad de Mexico, Mexico). Each cage contained four *M. paniculata* plants (≈ 30 cm tall) previously fertilized with a Steiner's solution to stimulate vegetative growth, which is necessary for adult feeding. Cages with infested plants were kept under greenhouse conditions (16-30 ºC and 60% relative humidity (RH), photoperiod of 12:12 h light: darkness) to obtain enough F_1 individuals to carry out the evaluations.

2.4.1.2. Diagnostic dose estimation

With the insecticides chlorpyrifos, malathion, imidacloprid, and spinosad (Table 1), we estimated the lowest dose that produced 100% mortality within 30-min of exposure (diagnostic dose). We used, under laboratory conditions, a susceptible population of *D. citri.* Leaf disks (∅ 38 mm) were obtained from the lower stratum of *M. paniculata* plants, *Citrus macrophylla* Wester (30 - 40 days old), not exposed to pesticides. These leaf discs were dipped for 10 s in a container with 50 mL of the required insecticide concentration. The treated leaf discs were left at room temperature on absorbent paper for 5 min to remove excess water. Subsequently, on a Petri dish (∅ 40 mm), the treated leaf disc was individually placed with the adaxial part upside down. Immediately, it was infested with 15 adults (3 - 4 days old, unsexed), which were previously anesthetized for 6 s with CO_2 and three psi to facilitate handling. Initially, an exploratory evaluation was made to evaluate logarithmically spaced doses (0.1, 1, 10, and 100 mg a. i. L^{-1}) and determine those close to producing 100% mortality within 30-minutes of exposure. Subsequently, with the same criteria, intermediate doses were evaluated until obtaining the lowest dose consistently produced 100% mortality in 30 repetitions (15 adults per repetition) within 30-min exposure to the respective insecticide. Each replicate included an untreated control handled similarly, except that it was dipped in distilled water.

Adults were considered dead when they could not walk or fly when stimulated with a brush with fine bristles. Treated individuals were kept under controlled environmental conditions: 25 ± 2 ºC, 25% RH, and a photoperiod of 12:12 h (light darkness).

2.4.2. Evaluation of the diagnostic dose in the field

2.4.2.1. Field populations

The diagnostic doses obtained for each insecticide in the susceptible population of *D. citri* were evaluated in field populations. Five orchards at Múgica and Parácuaro municipalities of the state of Michoacan, México, were selected with different insecticide management and a minimum distance of 40 km between them (Table 2). The selected orchards were Nueva Italia, Santo Domingo, El Varal, Gambara, and El Señidor (Table 2).

2.4.2.2. Field sampling

Thirty trees were randomly selected from each orchard. From the middle stratum of each tree, a shoot approximately 20 cm long containing nymphs of *D. citri* of different instars was selected. The 30 shoots (one per tree) were cut to obtain the necessary individuals to evaluate the diagnostic dose. This procedure was repeated for each tested insecticide.

2.4.2.3. Diagnostic dose assessment under field conditions

A portable table (1 x 1 m) was used to carry out the bioassays under field conditions. Diagnostic doses (50 mL) of each insecticide were prepared. The infested shoots (30) were dipped for 10 s into the respective diagnostic dose. Subsequently, they were placed on paper towels to remove excess moisture. At 30 min of exposure, mortality was assessed only in fourth instar nymphs. With the help of a brush with fine bristles (number three), the nymphs were touched, and those incapables of moving were considered dead

(Pardo et al. 2018). We evaluated fourth-instar nymphs because it is used for conventional assays, and it is possible to standardize them under field conditions, as required by the principles of the bioassay (Lagunes-Tejeda and Villanueva-Jimenez 1994). Previous studies reported a similar response between 4^{th} instar nymphs and adults (Pardo et al. 2018). For each insecticide, the average percentage of mortality and its standard error were estimated.

Table 2. Field-collected populations of *Diaphorina citri* Kuwayama and characteristics of the orchards where the samples were obtained.

Site	Locality	Municipalty†	Type of management	Geographic coordinates	
1	Nueva Italia	Múgica	Use of biorational and conventional insecticides	18.955726º N	102.201589º W
2	Santo Domingo	Múgica	Semi-organic	18.927102º N	102.089051º W
3	El Varal	Parácuaro	Use of conventional, low use of expensive insecticides	19.995968º N	102.297276º W
4	Gambara	Múgica	Few applications during the year	18.90876º N	102.125280º W
5	El Señidor	Múgica	Low use of biorational, High use of conventional insecticides	19.029607º N	102.175628º W

†Municipalities of the state of Michoacán, Mexico.

2.4.3. Biological efficacy of insecticides in the field

2.4.3.1. Populations

The biological effectiveness tests with these four insecticides were carried out based on the Official Mexican Standard NOM-032-FITO-1995, which establishes the phytosanitary specifications to perform biological effectiveness tests on agricultural pesticides and their technical opinion (SENASICA 2022). According to this official document, the acceptable field efficacy of an insecticide must be >85% (Casler 2015). The studies of biological efficacy were carried out in the same orchards (trees at least eight years old) from which the individuals were collected to evaluate the diagnostic dose under field conditions.

2.4.3.2. Experimental design

The field efficacy studies were carried out with imidacloprid, spinosad, malathion, and chlorpyrifos in a complete randomized block design with nine treatments, including an untreated control (Table 3) and four repetitions. The treatments were randomly assigned to the experimental units of each block using the statistical package R (Casler 2015). The experimental unit was a tree, and the complete experiment included 36 trees.

2.4.3.3. Spraying

Before insecticide applications, we calibrated the equipment to estimate the amount of water and insecticide per hectare. For this purpose, three trees were selected, and the average application time and the average amount of applied liquid per tree were estimated. A 25-liter Honda® gasoline-powered backpack pump, model 2525, with an open-cone TX8 nozzle was used. The applications were made at a pressure of 3 bars. Insecticide treatments included an adherent-dispersant (INEX-A, Polydimethylsiloxane, COSMOCEL, Mexico) at 2 mL/L of water. For each insecticide, the high dose and the low dose recommended on the commercial label were used (Table 3).

Table 3. Treatments used to evaluate their biological efficacy against adults of Diaphorina citri Kuwayama under field conditions

	Commercial dosages mL FP 100 L^{-1} water (mg a. i. 100 L^{-1} water)	
Insecticide	**Low**	**High**
Imidacloprid	30 (10.5)	40 (14.0)
Spinosad	15 (7.2)	20 (9.6)
Malathion	100 (10.0)	125 (12.5)
Chlorpyrifos	75 (33.7)	150 (52.5)
Untreated control		

FP = Formulated Product

2.4.3.4. Evaluations

Live adults per experimental unit were evaluated before application and after 72 h. From the tree in the experimental unit, a quadrant of 1 x 1 m was randomly selected from the medium stratum. Within that quadrant, two tender shoots were randomly selected, and the number of living adults was recorded.

2.4.3.5. Statistical analysis

Data were analyzed with ANOVA followed by a multiple mean comparison test (Tukey, $\alpha = 0.05$) using the statistical analysis software Statistical Analysis System® (SAS, 2011). When necessary to fulfill normality, mortality values were transformed to the square root. Data were processed with the values of the number of live insects per experimental unit, and the percentage of biological efficacy of each treatment was calculated using the Henderson and Tilton formula (Henderson and Tilton 1955). Additionally, we estimated if the observed values were >85% following the Mexican regulation requirements (SENASICA 2022). Thus, the percent mortality of the diagnostic dose repetitions and the percent field efficacy of each insecticide repetition were subjected to one sample *t*-test ($P < 0.05$) using Minitab Statistical Software version 21.2 (Lesik 2018).

2.5. RESULTS

2.5.1. Diagnostic dose in the laboratory

As the dose increased, mortality increased (Table 4) for all the products evaluated. It was possible to reach 100 % mortality within 30 min of exposure to the toxicant. There were no problems with properly diluting the insecticides. The diagnostic doses for imidacloprid, chlorpyrifos, spinosad, and malathion were 7.4, 5.5, 4.2, and 1.0 mg L^{-1}, respectively (Table 4).

Table 4. Evaluated doses of insecticide to find the diagnostic dose that kill 100 % of *Diaphorina citri* Kuwayama adults after 30 min of exposure

Insecticide	**Dosis (mg i. a. L^{-1})**	**Replications**	**n‡**	**Mortality (% ± se§)**
Chlorpyrifos	5.5	30	450	100.0 ± 0.0
	3.8	5	75	94.6 ± 0.5
	3.5	5	75	86.6 ± 0.3
	3.1	5	75	70.6 ± 0.3
	2.7	5	75	49.3 ± 0.2
	2.5	5	75	29.3 ± 0.1
	1.9	5	75	14.6 ± 0.2
	1.6	5	75	5.3 ± 0.2
	1.5	5	75	2.6 ± 0.1
Malathion	1.0	30	450	100.0± 0.0
	0.85	5	75	96.0 ± 0.1
	0.75	5	75	85.3 ± 0.1
	0.67	5	75	72.0 ± 0.2
	0.61	5	75	54.6 ± 0.0
	0.45	5	75	32.0 ± 0.0
	0.38	5	75	17.3 ± 0.3
	0.32	5	75	6.6 ± 0.0
Imidacloprid	7.4	30	450	100.0 ± 0.0
	7.3	5	75	97.3 ± 0.1
	7.1	5	75	84.0 ± 0.1
	7.0	5	75	66.6 ± 0.0
	6.9	5	75	48.0 ± 0.1
	6.6	5	75	29.3 ± 0.2
	6.4	5	75	13.3 ± 0.0
	6.2	5	75	6.6 ± 0.0
	5.9	5	75	1.3 ± 0.4
Spinosad	4.2	30	450	100.0 ± 00
	4.18	5	75	93.3 ± 0.2
	4.14	5	75	84.0 ± 0.2
	4.10	5	75	70.6 ± 0.2
	3.82	5	75	58.6 ± 0.2
	3.62	5	75	44.0 ± 0.2
	3.56	5	75	34.6 ± 0.2
	3.32	5	75	29.3 ± 0.2
	3.11	5	75	22.6 ± 0.3
	3.00	5	75	12.0 ± 0.3

‡ Number of individuals per experiment
§ Standard Error

2.5.2. Field studies: diagnostic dose - biological effectiveness on *D. citri*

Based on the calibration results of the field efficacy studies, we used 1,750 liters of water plus insecticide per hectare. There were significant statistical differences among treatments in the experiments of Nueva Italia (F = 39.25; df = 8,3; P = 6.09^{-12}), Santo

Domingo (*F* = 600; *df* = 8,3; *P* = 2^{-16}), El Varal (*F* = 15.97; *df* = 8,3; *P* = 6.93^{-8}), Gambara (*F* = 125.8; *df* = 8,3; *P* = 2^{-16}), and El Señidor (*F* = 27.32; *df* = 8,3; *P* = 3^{-10}), respectively. In all cases, the untreated control was significantly different from the rest of the treatments (Tukey, *P* = 0.05).

For Nueva Italia, the diagnostic dose of the tested insecticides caused >94 % mortality, and in the biological field efficacy studies, the level of control was >96 % with all the evaluated insecticides (Table 5). For the population of Santo Domingo, the diagnostic dose of the evaluated insecticides produced > 94 % mortality in all cases, and at 72 h after application, 100 % biological efficacy was observed at low and high doses for all insecticides.

Regarding the population of El Varal, high mortality was observed with the diagnostic dose of imidacloprid and spinosad, with values of 93.7 and 99.6 %, respectively. For these insecticides, both the low dose and the high dose showed a biological efficacy of ≥91.1%. In contrast, the mortality derived from applying the diagnostic dose of chlorpyrifos and malathion was 55.9 and 70.1 %, respectively. The corresponding biological efficacy values of these two insecticides, at the low and the high dose, were between 53.1 and 71.4% (Table 5).

Table 5. Percentage of mortality caused by the diagnostic dose applied under field conditions to fourth instar nymphs and percentage of field biological efficacy of the insecticides against adults of *Diaphorina citri* Kuwayama.

			Field efficacy studies			
		Diagnostic Dose §	**Number of living individuals[a]**		**Biological efficacy (%)**	
Populatión ‡	**Treatment**	**(Mortality %)**	**Low dose (mg a. i. ha^{-1})**	**High dose (mg a. i. ha^{-1})**	**Low dose (mg a. i. ha^{-1})**	**High dose (mg a. i. ha^{-1})**
Nueva Italia	Chlorpyrifos	94.7 ± 0.1 *	0.25 ± 0.03 b	0.25 ± 0.03 b	96.8 *	97 *
	Malathion	97.9 ± 0.1 *	0.0 b	0.0 b	100 *	100 *
	Imidacloprid	95.7 ± 0.3 *	0.25 ± 0.03 b	0.0 b	96.6 *	100 *
	Spinosad	98.9 ± 0.1 *	0.0 b	0.0 b	100 *	100 *
	Untreated control	0.0 ± 0.0	8.25 ± 1.1 a			-
Santo Domingo	Chlorpyrifos	97.3 ± 0.1 *	0.0 b	0.0 b	100 *	100 *
	Malathion	95.7 ± 0.1 *	0.0 b	0.0 b	100 *	100 *
	Imidacloprid	94.7 ± 0.2 *	0.0 b	0.0 b	100 *	100 *
	Spinosad	98.9 ± 0.0 *	0.0 b	0.0 b	100 *	100 *
	Untreated control	0.0 ± 0.0	10 ± 1.3 a		-	-
El Varal	Chlorpyrifos	55.9 ± 1.0 ns	5.5 ± 0.7 b	3.5 ± 0.4 bc	53.1 ns	61.1 ns
	Malathion	70.1 ± 1.0 ns	4.2 ± 0.5 bc	3 ± 0.4 bc	63.0 ns	71.4 ns
	Imidacloprid	93.7 ± 0.2 *	0.7 ± 0.1 c	0.75 ± 0.1 c	91.1 *	92.7 *
	Spinosad	99.6 ± 9.0 *	0.25 ± 0.03 c	0.0 ± 0.0 c	97.4 *	100 *
	Untreated control	0.0 ± 0.0	11.7 ± 1.5 a			-
Gambara	Chlorpyrifos	97.9 ± 0.1*	0.0 b	0.0 b	100 *	100 *
	Malathion	97.1 ± 0.1 *	0.0 b	0.0 b	100 *	100 *
	Imidacloprid	95.3 ± 0.1 *	0.0 b	0.0 b	100 *	100 *
	Spinosad	99.9 ± 0.1 *	0.0 b	0.0 b	100 *	100 *
	Untreated control	0.0 ± 0.0	13.2 ± 1.7 a			
El Señidor	Chlorpyrifos	54.9 ± 0.3 ns	4.5 ± 0.5 b	3.7 ± 0.6 b	59.0 ns	63.9 ns
	Malathion	64.0 ± 1.0 ns	6 ± 0.5 b	4.7 ± 0.4 b	52.1 ns	61.1 ns
	Imidacloprid	58.1 ± 1.0 ns	4 ± 0.8 b	3.2 ± 0.6 b	66.4 ns	66.7 ns
	Spinosad	98.1 ± 1.0 *	0.25 ± 0.03 c	0.0 ± 0.0 c	98.0 *	100 *
	Untreated control	0.0 ± 0.0	11 ± 1.4 a			-

‡ Populations from the Mexican state of Michoacan
§ Observed mortality after 30 min of exposure
¶ Field biological efficacy calculated using the Henderson-Tilton equation

¶¶ Standard Error
[a]72 h after treatment
*Significantly higher than 85% (one sample *t*-test, ($P < 0.05$)
ns = No significantly lower than 85% (one sample *t*-test, ($P < 0.05$))
Within the column “number of living individuals,” the values with the same lowercase letters are not significantly different (Tukey, $\propto = 0.05$). The diagnostic doses studies were performed in laboratory conditions, with the fourth instar nymph of *D. citri*, and the biological efficacy studies were performed under field conditions with adults.

In the Gambara orchard, the diagnostic dose of four tested insecticides resulted in a mortality >95 %, and in all cases, the field efficacy was 100%. In the El Señidor population, the diagnostic dose of spinosad led to 98% mortality. In its low and high doses, the biological effectiveness of spinosad in the field was 98 and 100%, respectively. In this population, the mortality produced by the diagnostic dose of chlorpyrifos, malathion, and imidacloprid was 54.9, 64.0 and 58.1 %, respectively (Table 5). For these insecticides, in their low and high commercial doses, the biological efficacy values were between 52.1 and 66.4 % (Table 5).

We observed a significant correlation between the results of the diagnostic dose and those of the field efficacy studies. There was a high positive correlation between the diagnostic dose values and the percent of field efficacy for chlorpyrifos (r = 0.9661, P = 0.000; n = 2400), malathion (r = 0.9974, P = 0.002; n = 2736), and imidacloprid (r = 0.9564, P = 0.032; n = 2644), respectively (Figure 1). The spinosad correlation in all populations was not estimated because, in the diagnostic dose and the biological efficacy, the values were ≥97.4 % (Table 5).

2.6. DISCUSSIONS

The field populations of *D. citri* were susceptible to spinosad in all studied orchards. For this insecticide, the diagnostic dose caused >97.4 % mortality, and the field efficacy was 100 %. Spinosad is highly prone to resistance (Khan et al. 2018, Sayyed et al. 2004) but its use in the studies sites is scarce due to the high price to the end user. An application of spinosad per hectare to control Asian citrus psyllid (500 mL/ha) costs US$100. The treatment per hectare of chlorpyrifos, malathion, and imidacloprid costs $15, $10, and 12 American dollars, respectively.

In the Nueva Italia orchard, the results of the diagnostic dose (>94% mortality) and the field efficacy studies (> 96 % control) in all tested insecticides indicated effectiveness against *D. citri.* According to information provided by the producer, they use more biorational insecticides than conventional ones. Thus, using biorational insecticides reduces the selection pressure with conventional ones.

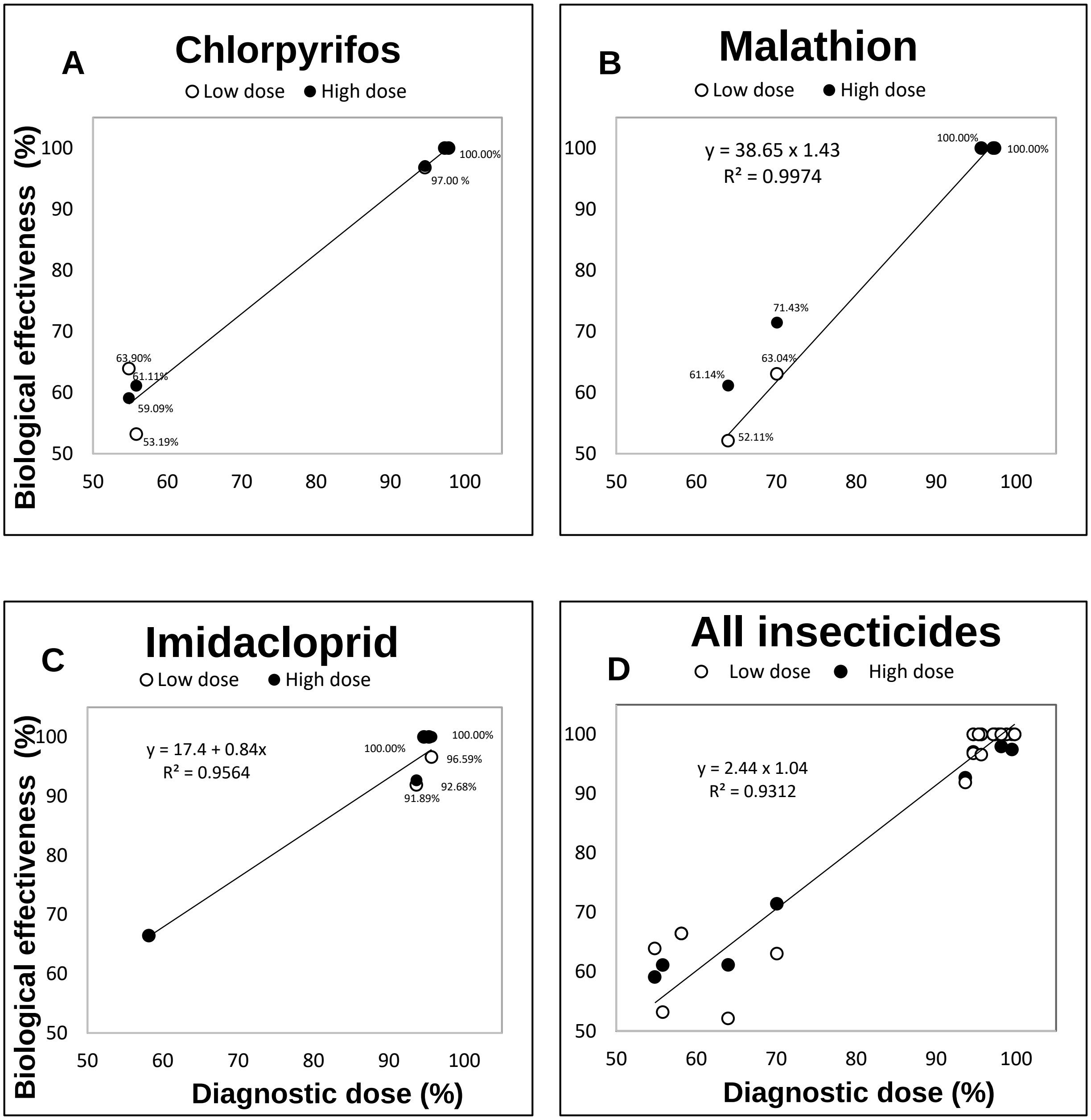

Figure 1. Simple linear correlation between the mortality rates and field efficacy of insecticides used against *Diaphorina citri* Kuwayama from the field applied biological effectiveness test (low and high dose versus diagnostic dose). Chlorpyrifos: low dose = 75 mL 100 L^{-1}, high dose = 150 mL/100 L^{-1}. Malathion: low dose = 100 mL 100 L^{-1}, high dose = 125 mL 100 L^{-1}.

In Santo Domingo orchard, the four evaluated insecticides had acceptable field efficacy (100%), and the diagnostic dose (>90 %) indicated susceptibility to all of them. This site has semi-organic management without certification. Against the Asian citrus psyllid and other insect pests, growers use botanical insecticides, such as garlic (*Allium sativum* L.) and neem extracts (*Azadirachta indica* A. Juss). This orchard also has grasslands that harbor natural enemies to mitigate the increase in the density of pest populations. It is also far from other citrus-growing areas.

In El Varal orchard, the diagnostic dose (>50 %) and the field efficacy studies (62 %) indicate that chlorpyrifos lacks effectiveness against *D. citri*. For malathion, the diagnostic dose also indicated low effectiveness (>70 %), and the field efficacy provided a similar result (72 %). This orchard is under selection pressure with chlorpyrifos and malathion. The efficacy of imidacloprid and Spinosad was acceptable, as revealed by the diagnostic dose (>93 %) and the field efficacy test (100 %). This site has a fertigation system that promotes plant growth and hence favors the increase in density of *D. citri*.

Gambara is a five-hectare orchard. In this site, both the diagnostic dose (>95 % mortality) and the field efficacy (100% control) studies indicated susceptibility to all tested insecticides. To combat *D. citri,* growers use a rotation scheme of insecticides with different modes of action: organophosphates, pyrethroids, neonicotinoids, and avermectins. This resistance management strategy mitigates the changes in response to each type of insecticide.

The field efficacy studies (≤66.4 % control) from the El Señidor orchard indicated that chlorpyrifos, malathion, and imidacloprid were ineffective. The diagnostic dose for these insecticides caused <64 % mortality. They apply them up to 30 times yearly, mainly from April to June. Growers use imidacloprid at a dose of 350 mL of formulated product in 1000 L of water. The density of *D. citri* is favored by the constant vegetative sprouting derived from the agronomic management. Additionally, they use phytohormones such as AGROMIL-PLUS (cytokinins, gibberellins, auxins, San Jerónimo Tepetlacalco, Tlalnepantla, State of Mexico, Mexico) and therefore create the conditions for the population development of *D. citri.*

The high correlation between the diagnostic dose and the field efficacy studies indicates that our proposal is sound and has advantages over conventional phenotypic bioassays. Phenotypic estimation of resistance is usually done by complete bioassays. This type of bioassay includes doses that are not informative because they cause very low levels of mortality but are invaluable for estimating the slope of the Log dose-Probit line.

In the diagnostic dose, we propose that at least 600 individuals are used in 30 repetitions. We do not discard that we used a large sample size. It was the best way to avoid conflict between hypothesis testing and the weakness associated with a small, non-representative sample size.

Our proposal allows us to estimate, quickly and at the plot level, the response of insect pests to the insecticides used to combat *D. citri*. This information can be obtained the same day or day before the commercial application and provides valuable input for the farmer to decide which insecticide to apply. In our experience, if the diagnostic evaluation shows a mortality $>$85 % for a given insecticide, it is feasible to apply it. If the response is between 70 and $\leq$85 %, the population is suspected of resistance, and we suggest not using the respective insecticide. If the diagnostic dose causes $<$70 %, we consider that the target population is resistant, and the farmer must refrain from using the corresponding insecticide. These criteria may change depending on various factors such as the price of the harvest in the market, type of crop, and species of pest, among others.

Once this methodology is generalized at the agroecosystem level, farmers are expected to avoid using ineffective insecticides. This would be the most successful tactic to recover insect pest susceptibility at the area-wide level. The insecticides that fall in the category of "suspects of resistance" should be monitored to estimate the time they could be used again.

In diagnostic dose assessment, all test insects are contacted by the insecticide. However, we recognize that, under field conditions, some insects will survive commercial applications due to coverage failures (Bisset 2002, UK, 1997). A good field application will prevent significant levels of fortuitous survival. The growers must be aware that other factors, such as expired products, low doses, and inadequate coverage, among others, may cause misinterpretations of this rapid test.

The proposed diagnostic doses are intended to exhaust the survival of homozygous susceptible insects. However, we estimate that some individuals may survive despite being homozygous susceptible, but the probability is marginal. Additionally, it is not ruled out that recessive heterozygous may be killed. Perhaps with some insecticides, it will not be possible to obtain total mortality within 30 min of exposure due to problems associated with the dilution of the insecticide or its mode of action. Although the same insecticide, formulation, and concentration are intended to be used against this pest, our diagnostic dose must be validated if intended to be used in other places. It is feasible that this methodology can be applied to other crops and pests, as well as with different insecticides.

2.7. CONCLUSION

We demonstrated the usefulness of a novel methodology to estimate within 30-minute exposure and under field conditions the field performance of imidacloprid, spinosad, malathion, and chlorpyrifos in *D. citri*. Thus, against this pest, the citrus grower has the possibility to know, the same day or a day before the commercial application, which of these insecticides to use or avoid.

2.8. ACKNOWLEDGEMENTS

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CONCLUSIONES GENERALES

La respuesta a insecticidas en una población de *D. citri* que se ha mantenido en forma silvestre libre de aplicaciones de insecticidas presentó mayor susceptibilidad a los insecticidas spinosad, malatión, clorpirifós, que sus similares que se han publicado en revistas científicas indexadas. Sin embargo, hay estudios que reportan mayor susceptibilidad de *D. citri* a imidacloprid, en poblaciones de referencia certificadas. La población de *D. citri* que se encontró en Michoacán, sirve como línea base para futuras investigaciones relacionadas con la medición de la susceptibilidad de *D. citri* en adultos y ninfas.

Las concentraciones de 1.0, 4.20, 5.50 y 7.40 mg i. a. L^{1} de los insecticidas malatión, spinosad, clorpirifós e imidacloprid con exposiciones de 30 minutos en adultos (3 - 5 días de edad) se pueden utilizar como dosis diagnósticas para evaluar la resistencia a dichos insecticidas en condiciones de campo. Este método implica que una exposición de 30 minutos al insecticida, es de bajo costo, práctico, y confiable. Por primera vez se tiene la posibilidad de hacer inferencias robustas sobre el estado de la resistencia a insecticidas y usar la información que se genere como insumo para seleccionar de manera racional el ingrediente activo a utilizar. Considerando la elevada correlación que esta metodología tiene con las pruebas de efectividad biológica tradicionales que se hacen en condiciones de campo, el agricultor también puede evitar el uso de insecticidas inefectivos. Con los ajustes pertinentes, dicha metodología se puede aplicar a una amplia variedad de especies de insectos plaga y de insecticidas. Sin duda alguna, en esta investigación, se desarrolló la herramienta más importante para el manejo racional de insecticidas en condiciones de campo.

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