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DISTRIBUCIÓN Y RANGO DE HOSPEDEROS DEL VIRUS DE LA LEPROSIS DE LOS CÍTRICOS (CITOPLASMÁTICO Y NUCLEAR) TRANSMITIDO POR EL COMPLEJO *Brevipalpus* spp. EN LOS PRINCIPALES ESTADOS CITRÍCOLAS DE MÉXICO

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DISTRIBUCIÓN Y RANGO DE HOSPEDEROS DEL VIRUS DE LA LEPROSIS DE LOS CÍTRICOS (CITOPLASMÁTICO Y NUCLEAR) TRANSMITIDO POR EL COMPLEJO *Brevipalpus* spp. EN LOS PRINCIPALES ESTADOS CITRÍCOLAS DE MÉXICO

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Colegio de Postgraduados, 2018

RESUMEN

La leprosis de los cítricos es ocasionada por dos tipos de virus, citoplasmático y nuclear, los cuales son transmitidos por un complejo de ácaros del género *Brevipalpus.* El objetivo del trabajo fue determinar la distribución de estos virus en las especies cítricas con mayor importancia económica en México y determinar si existe una relación de especificidad o preferencia entre la presencia del virus de la leprosis y los diversos hospederos, pues se tiene la hipótesis de que dichos virus se encuentran ampliamente distribuidos en el país, sobre especies de cítricos dulces. Para el estudio se realizaron muestreos de febrero a diciembre de 2017 en 16 Estados de la República Mexicana (Campeche, Chiapas, Hidalgo, Jalisco, Morelos, Nuevo León, Oaxaca, Puebla, Querétaro, Quintana Roo, San Luis Potosí, Sinaloa, Tamaulipas, Tabasco, Veracruz y Zacatecas) en huertas de *Citrus sinensis, C. aurantium*, *C. reticulata, C. latifolia*, *C. paradisi* y *C. reticulata, C. limmeta* y *C. aurantifolia.* La presencia del virus se determinó con análisis de transcripción reversa y reacción en cadena de la polimerasa (RT-PCR). En total se obtuvieron 106 muestras positivas para el virus citoplasmático y 81 para el nuclear y sus variantes. Los resultados indican que la distribución de esta enfermedad se ha incrementado de manera exponencial y se encuentra actualmente en cítricos dulces y agrios en todos los estados citrícolas del país que se evaluaron.

Palabras clave: RT-PCR**,** enfermedad, cítricos, virus

HOST RANGE AND DISTRIBUTION OF THE CITRUS LEPROSIS VIRUS (CITOPLASMATIC AND NUCLEAR TYPES) TRANSMITTED BY THE SPECIES COMPLEX OF THE GENUS *BREVIPALPUS* IN THE MAIN CITRUS PRODUCERS STATES IN MEXICO

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Colegio de Postgraduados, 2018

ABSTRACT

The citrus leprosis is caused by two viruses, cytoplamastic and nuclear; which are transmitted by mites within the genus *Brevipalpus*. Currently, these viruses have been mainly associated only with sweet citrus species. However, considering their current distribution, it is likely that other citrus species are also infected. Therefore, the aim of our study was to determine the distribution of these two viruses on the most important citrus species orchards in Mexico. For this, a systematic sampling was carried out from February to December 2017 in 16 states: Campeche, Chiapas, Hidalgo, Jalisco, Morelos, Nuevo León, Oaxaca, Puebla, Querétaro, Quintana Roo, San Luis Potosí, Sinaloa, Tamaulipas, Veracruz and Zacatecas. The following citrus species were studied *Citrus sinensis, C. aurantium, C. reticulata, C. latifolia, C. paradisi* y *C. reticulata, C. limmeta y C. aurantifolia*. The presence of these viruses was determined using RT-PCR reactions. Overall, 106 samples were positive to CiLV-C and 81 to CiLV-N and its variants. Our results showed that the distribution of the citrus leprosis has increased greatly, infecting sweet and sour citrus species in all Mexican states sampled.

Palabras clave: RT-PCR**,** disease, citrus, virus

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CONTENIDO

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INTRODUCCIÓN GENERAL

Hace más de cien años se reportó la enfermedad de la leprosis de los cítricos en Estados Unidos. Inicialmente se le denominó "corteza escamosa" debido a los síntomas ocasionados en la corteza verde de los árboles (Fawcett, 1911). Posteriormente, síntomas similares se observaron simultáneamente en América del Sur, pero se le denominó leprosis de los cítricos (Bastianel *et al.*, 2010). La enfermedad es inducida por un virus, que causa defoliación, caída prematura de frutos, muerte regresiva de ramas, reducción en calidad y cantidad de frutos, así como la muerte de árboles (SENASICA, 2017).

Existen dos tipos de virus asociados a la leprosis de los cítricos, los cuales se ubican en dos géneros: *Cilevirus* y *Dichorhavirus* (ICTV, 2018). El primero, el Citrus leprosis virus C (CiLV-C) es de ARN en sentido positivo y se localiza en el citoplasma; el segundo, el Orchid fleck dichorhavirus es de ARN en sentido negativo, se encuentra en el núcleo y tiene dos variantes relacionadas, el Citrus leprosis virus tipo nuclear (CiLV-N) y el Citrus necrotic spot virus (CiNSV) (Kitajima *et al*., 2003; Rodrigues *et al*., 2003; Cruz-Jaramillo *et al.*, 2014; Roy *et al.*, 2015).

Actualmente, el CiLV-C ocurre en Argentina, Belice, Bolivia, Brasil, Colombia, Costa Rica, Guatemala, Honduras, México, Nicaragua, Panamá, Paraguay, Uruguay y Venezuela. El CiLV-N se encuentra en Brasil, Colombia, México, Panamá; en tanto que CiNSV sólo en Colombia y México (Cruz-Jaramillo *et al*., 2014; Roy *et al.*, 2015). En México, la enfermedad se detectó por primera vez en 2004, en ocho municipios del sur del estado de Chiapas (Robles, 2010). Desde entonces, se inició la campaña contra la leprosis de los cítricos, en la cual de 2005 a 2009 se

invirtieron $59,129,183 de pesos para confinarla y controlarla en los primeros estados afectados como Chiapas, Tabasco y Veracruz (SENASICA, 2010), con la finalidad de proteger a los 24 estados productores de cítricos, que en conjunto cuentan con una superficie sembrada de 565,483 hectáreas, dando una producción superior a los 7.7 millones de toneladas con un valor aproximado de 17,309 millones de pesos (SIAP, 2016).

Los virus asociados a la enfermedad de la leprosis de los cítricos son transmitidos por ácaros del género *Brevipalpus* (Rodrigues *et al*., 2000). Para el caso de México, diversos estudios reportan poblaciones de las especies *Brevipalpus californicus, B. papayensis* y *B. yothersi,* siendo esta última la más común y diversa genéticamente, por lo que se recomienda que esta información se utilice para nuevas áreas de investigación, incluidos estudios de comportamiento y ecología, pues probablemente una especie sea más efectiva en la transmisión de CiLV-N, además es de suma importancia considerar a la especie de *B. yothersi* en el diseño de programas de monitoreo y control debido a su abundancia en el país (Sánchez-Velázquez *et al.,* 2015; Salinas-Vargas *et al.,* 2016).

Las plantas hospedantes en las que se ha detectado a CiLV-C en México son *Citrus sinensis, C. paradisi, C. reticulata, Swinglea glutinosa* y *Commelina benghalensis*. OFV, CiNSV y CiLV-N se han detectado, principalmente, en *C. aurantifolia, C. aurantium, C. latifolia, C. limetta, C. limon, C. paradisi, C. reticulata, C. sinensis* y *C. tangerina* (SENASICA, 2017).

Debido a la importancia que tiene la leprosis de los cítricos en nuestro país, los resultados del presente estudio ayudarán a generar estrategias de control eficaces contra el vector del virus causante de la leprosis de los cítricos, así como generar métodos de diagnóstico y muestreo en un programa de vigilancia federal, con la finalidad de reducir el impacto negativo que pueda ocasionar el virus de la leprosis a nivel nacional.

OBJETIVOS

Objetivo general

Determinar la distribución del virus de la leprosis tipo citoplasmático y nuclear, en diferentes especies de cítricos de las principales zonas productoras del país.

Objetivos específicos

Determinar la prevalencia del virus de la leprosis tipo citoplasmático en naranja dulce (*Citrus sinensis*), toronja (*C. paradisi*), mandarina (*C. reticulata*), limón persa (*C. latifolia*), limón mexicano (*C. aurantifolia*) y limón (*Citrus limon*) en los estados de Chiapas, Tabasco, Veracruz, Querétaro, Campeche, Hidalgo, Nuevo León, Oaxaca, Puebla, San Luis Potosí, Jalisco, Tamaulipas, Nuevo León y Quintana Roo.

Determinar la prevalencia del virus de la leprosis tipo nuclear, considerando al OFV, CiNSV y CiLV-N en naranja dulce (*Citrus sinensis*), toronja (*C. paradisi*), mandarina (*C. reticulata*), limón persa (*C. latifolia*), limón mexicano (*C. aurantifolia*) y limón (*Citrus limon*) en los estados de Chiapas, Tabasco, Veracruz, Querétaro, Campeche, Hidalgo, Nuevo León, Oaxaca, Puebla, San Luis Potosí, Jalisco, Tamaulipas, Nuevo León y Quintana Roo.

HIPÓTESIS

La prevalencia y distribución de los virus tipo citoplasmático y nuclear está relacionada con la especie de cítrico.

LITERATURA CITADA

Bastianel, M., Novelli, M.V., Kitajima, W.E., Kubo, K.S., Bassanezi, S.R., Machado M.A.& Freitas, A.J. 2010. Citrus leprosis. Centennial of an unusual mite-virus pathosystem. Plant Disease 94(3): 284-292.

Cruz-Jaramillo, J.L., Ruiz, M.R., Rojas, M.L., López, B.J.A., Morales-Galván, O., Chavarin-Palacio, C., Ramírez-Pool, J.A. & Xoconostle-Cazares, B. 2014. Caracterización de un *Dichorhavirus* propuesto asociado con la enfermedad de la leprosis de los cítricos y análisis de la respuesta del huésped. Virus 6:2602 – 2622.

Fawcett, H.S. 1911. Corteza escamosa o rojez de cítricos. Florida. Agricultural. Exp. Stn. Toro. 106: 1-41.

ICTV. 2018. Comité internacional de taxonomía de virus: Disponible en: https://talk.ictvonline.org/search-124283882/?q=citrus%20leprosis%202018 (consulta mayo de 2018).

Kitajima, E.W. & Machado, M.A. 2003. Development of a molecular tool for the diagnosis of leprosis, a major threat to citrus production in the Americas. Plant Disease 87:1317-1321.

Robles, G.P.L. 2010. Protocolo para la detección y el manejo de la leprosis de los cítricos. Dirección General de Sanidad Vegetal. 25 p.

Rodrigues, J.C.V., Machado, M.A., Kitajima, E.W. & Müller, G.W. 2000. Transmission of citrus leprosis virus by *Brevipalpus phoenicis*. pp. 174–178. *In*: Proc. 14th Conf. Int. Org. Citrus Virol, Univ. Calif. Press, Riverside.

Rodrigues, J.C.V., Kitajima, E.W., Childers, C.C. & Chagas, C.M. 2003. Citrus leprosis virus vectored by *Brevipalpus phoenicis* (Acari: Tenuipalpidae) on citrus in Brazil. Experimental and Applied Acarology 30:161-179.

Roy, A., Stone, A., Shao, J., Otero, C.G., Wei, G., Choudhary, N., Achor, D., Levy, L., Nakhla, M.K., Hartung, J.S., Schneider, W.L., & Brlansky, R.H. 2015. Identification and molecular characterization of nuclear citrus leprosis virus, a member of the proposed *Dichorhavirus* Genus infecting multiple citrus species in Mexico. Phytopathology 105 (4):564-75.

Salinas-Vargas, D., Santillán-Galicia, M.T., Guzmán-Franco, A.W., Hernández-López, A., Ortega-Arenas, L.D. & Mora-Aguilera, G. 2016. Análisis de variación genética de *Brevipalpus yothersi* (Acari: Tenuipalpidae) en poblaciones de cuatro especies de plantas hospederas de cítricos. PLoS ONE 11 (10): e0164552.

Sánchez-Velázquez, E.J., Santillán-Galicia, M.T., Novelli, V.M., Nunes, M.A., Mora-Aguilera, G. & Valdez-Carrasco, J.M. 2015. Diversidad y variación genética entre poblaciones de *Brevipalpus* de Brasil y México. PLoS ONE 10 (7): e0133861.

SENASICA**.** 2010. Protocolo técnico para la detección y manejo de la leprosis de los cítricos. Dirección General de Sanidad Vegetal. 29 p.

SENASICA. 2017. La enfermedad de la leprosis de los cítricos es asociada con varios virus. Revista de Vigilancia Pasiva Fitosanitaria. 4. (32): 1-4.

SIAP. 2016. Anuarios Estadísticos de la Producción Agrícola en México. Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación. Consultado en línea en noviembre 2016: http://www.siap.gob.mx

CAPÍTULO I. PREVALENCE OF VIRUSES WITHIN THE CITRUS LEPROSIS COMPLEX, AND THEIR INTERACTIONS WITH CITRUS HOST SPECIES AND GEOGRAPHICAL ORIGIN

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1.1 ABSTRACT

We studied the distribution and host range of viruses infecting citrus orchards in Mexico. The viruses were distributed within two genera: *Cilevirus*, which includes the Citrus leprosis virus cytoplasmic type (CiLV-C); and *Dichorhavirus*, which includes two strains related strains to the Orchid fleck dichorhavirus (OFV), the Citrus necrotic spot virus (CiNSV) and Citrus leprosis virus nuclear type (CiLV-N). The study was done in orange, lime, sweet lime, mandarin and grapefruit orchards in 15 Mexican states. Our aim was to determine the presence of these viruses in different citrus species, and assess whether there was a relationship between virus diversity and citrus species or geographical distribution. Virus presence was determined using molecular techniques. All four viruses were detected in the majority of sampling sites. Only a few samples were collected from mandarin, sweet lime and grapefruit so these data were excluded from statistical analysis. The most common viruses were CiLV-C and OFV. A significant effect of citrus species was found for CiLV-C and OFV. Orange had significantly more leaves infected with CiLV-C than lime, and this effect was opposite for OFV. The proportion of leaves infected by either CiLV-C or OFV was significantly affected by geographical origin; higher rates of infection by CiLV-C were found in states with dry climates compared with OFV infections, were the highest infection rates were found in states with high humidity. Using molecular techniques some asymptomatic leaves proved to be positive for the viruses of interest. For this reason we suggest that field monitoring should be done using molecular techniques to identify the presence of these viruses earlier and before symptoms appear.

1.2 INTRODUCTION

Citrus leprosis is a viral disease of significant economic importance that mainly affects orange (*Citrus* × *sinensis* (L.) Osbeck) and mandarin (*Citrus reticulata* Blanco) (Bastianel *et al*. 2006; SENASICA 2013). Mite species within the genus *Brevipalpus* are thought to be the main vectors of the viruses that are the causative agents of this disease (Kitajima *et al*. 2011; Hartung *et al*. 2015). In Brazil, (one of the main citrus-producing countries in the world [Liu *et al*. 2012]), citrus leprosis is the most devastating disease of citrus, and the costs associated with reducing populations of the vector (*Brevipalpus* spp) are approximately 90 million US dollars per year (Rodríguez *et al*. 2003). Currently, citrus leprosis disease occurs in most Southern and Central American countries and, since 2005, it has also been recorded in Mexico (Izquierdo-Castillo *et al*. 2011).

There are several causative agents of citrus leprosis disease (Locali-Fabris *et al*. 2006; Roy *et al*. 2014; Cruz-Jaramillo *et al*. 2014). These include: Citrus leprosis virus cytoplasmic type (CiLV-C) from the genus *Cilevirus*; two strains of the Orchid fleck virus (OFV) which belong to Citrus leprosis virus nuclear type (CiLV-N); and Citrus necrotic spot virus (CiNSV) from the genus *Dichorhavirus* (Ramos-González *et al*. 2016).

Characteristic symptoms of citrus leprosis disease in oranges include colourless lesions on branches, leaves and fruits which affect the commercial value of fruits significantly (Bastianel *et al*. 2010). Mexico produces several citrus species including orange, mandarin, lime (*Citrus latifolia* [Yu. Tanaka] Tanaka), key lime (*Citrus x aurantifolia* [Christm.] Swingle) and grapefruit (*Citrus* × *paradise* Macfad). Recently, Salinas-Vargas *et al*. (2016) reported the presence of *Brevipalpus yothersi* (Baker) and *Brevipalpus californicus* (Banks) in Mexican citrus orchards, where the

former species was the most abundant on all citrus species and in all localities. *Brevipalpus californicus* was found predominantly on orange and mandarin. In contrast, *B. yothersi* was found on all citrus species (Salinas-Vargas *et al*. 2016). Although citrus leprosis in other countries has been reported mainly in sweet citrus species like orange and mandarin, and limes are considered to be immune (Bastianel *et al*. 2006; 2008), the presence of *B. yothersi* on all citrus species in Mexico (Salinas-Vargas *et al*. 2016), makes it important to accurately determine the presence of the leprosis viruses in all these citrus species under Mexican field conditions.

Globally, Mexico is the fifth largest producer of orange and the third largest producer of lime (SIAP 2017). Therefore, it is important to determine the status of the various viruses associated with citrus leprosis disease on the key citrus species grown in Mexico. In this study our aim was to determine which viruses were present infecting citrus orchards; then, to determine whether there were relationships between the diversity of viruses found and citrus species or geographical origin. We sampled in 15 Mexican states from the most economically important citrus specie: orange, mandarin, lime, sweet lime (*Citrus* × *limetta* Risso) and grapefruit. Virus detection was achieved using molecular techniques.

1.3 MATERIAL AND METHODS

1.3.1 Sampling site and data collection

Orange, grapefruit, mandarin, lime and sweet lime crops were sampled during 2017 from orchards in 15 states: Campeche, Chiapas, Hidalgo, Jalisco, Morelos, Nuevo León, Oaxaca, Puebla, Querétaro, San Luis Potosí, Sinaloa, Tamaulipas, Veracruz, Quintana Roo and Zacatecas. In each state, five orchards were sampled, except Chiapas and Tamaulipas were seven and two orchards were sampled, respectively (Table 1). The sampling methodology was the same for all citrus species and locations. In each orchard, 25 trees were sampled from five points: the four corners and the central part of the orchard. From each tree, 20 leaves were randomly sampled, five from each cardinal point and 1.5 m above the ground. The collected leaves were deposited in plastic bags, labelled, placed inside cool boxes and transported to the laboratory. Once in the laboratory, all leaves were inspected under a stereomicroscope to identify the presence of symptoms associated with citrus leprosis disease. All leaves were then washed in sterile distilled water and left at room temperature for 15 min to remove excess moisture. Despite collecting 20 leaves per tree, we selected five leaves per tree for molecular detection of viruses. Selection was based on the presence of symptoms, or randomly when no symptoms were evident. From each selected leaf, two circular sections of 1cm diameter, each containing a lesion associated with citrus leprosis, were excised using a metal cork borer; when no lesions were evident, two circular sections of 1cm diameter were excised from randomly-selected positions within the leaf. All sections were deposited individually into 2 mL Eppendorf tubes containing 1 mL of RNAlater™ (SIGMA-ALDRICH®, St. Louis, Mo, USA), and stored at -20 °C until required.

1.3.2 Molecular analysis

1.3.3 RNA extraction

For virus detection we used RNA extraction. Eppendorf tubes containing 100 mg of infected leaf tissue were submerged in liquid nitrogen for 15 minutes. The frozen tissue was ground using a pellet pestle rod (Daigger and Company Inc., Vernon Hills. Il. USA) and RNA extracted using the RNeasy Plant Mini Kit (QIAGEN, GmbH, Hilden, Germany) following the manufacturer's instructions. The concentration of RNA in the samples was estimated using a NanoDrop™ and stored at -20 °C until required.

1.3.4 RT-PCR reactions and sequencing

Virus detection was achieved by reverse transcriptase and PCR reactions. Reverse transcriptase reactions were done using the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc. Waltham, MA, USA). Each RT reaction was done in a final volume of 20µL containing 4 µL of 5X reaction buffer, 1 mM of each dNTP, 5 µM of random hexamer primer, 1 µL of RevertAid M-MuLV RT enzyme (200U/µL), 10 µL nuclease-free water and 2 µL of RNA template (approx. 80 ng). Tubes were incubated at 25 °C for five min, and then 60 min at 42 °C followed by a final step of 5 min at 70 °C in a MyCycler™ thermal cycler (BIO-RAD Laboratories Inc., Hercules, CA, USA).

The presence of four different viruses was evaluated in each leaf sample. Detections were done by PCR reactions using the complementary DNA (cDNA obtained in the RT reactions previously described as a template. Presence of CiLV-C virus was detected using the primers MPF

and MPR (Locali-Fabris et al. 2006); CiLV-N virus was detected using the primers NPF and NPR (Roy et al. 2014); CiNSV was detected using the primers CNSV2F and CNSV2R (Cruz-Jaramillo et al. 2014); and OFV was detected using the primers OFVF and OFVR (Ali et al. 2014). OFV was included in this study because reports suggest that it is transmitted by mites in the genus *Brevipalpus* (Kitajima et al. 2003). For all pair of primers, PCR reactions were done in a final volume of 25 µL containing 2.5 µL of 10X PCR buffer (Tris-Cl, KCl, (NH4)2SO4, 15mM MgCl2; pH 8.7), 0.18 µM of each primer, 0.2 mM of each dNTP, 0.2 µL of Taq polymerase (5 U/µL) (QIAGEN, GmbH, Hilden, Germany) and 3 µL of cDNA. Thermal conditions for each primer pair were as follows: CiLV-C - one cycle of 5 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 57 °C and 1 min a 72 °C, followed by a final extension of 72 °C for 10 min; OFV: one cycle of 5 min at 95 °C, 35 cycles of 1 min at 95 °C, 1 min at 61 °C and 1 min at 72 °C, followed by a final extension of 72 °C for 10 min; CiLV-N - one cycle of 5 min at 95 °C, 35 cycles of 1 min at 95 °C, 1 min at 61 °C and 1 min at 72 °C, followed by a final extension of 72 °C for 10 min; and CiNSV - one cycle of 5 min at 94 °C, 35 cycles of 35 s at 94 °C, 20 s at 62 °C and 45 s at 72 °C, followed by a final extension of 72 °C for 10 min. All PCR reactions were done in a MyCycler^{TM} thermal cycler. PCR products were visualized on 1.5 % agarose gels in 1X TAE. The gels were stained with ethidium bromide (10 mg mL^{-1}) and photographed. The expected band sizes were 339 bp for CiLV-C, 681 bp for CiLV-N, 480 bp for CiNSV and 160 bp for OFV. The presence of these viruses in the plant tissues was confirmed by the size of the PCR products. However, some PCR products were sent to Macrogen Inc. (Geumchen-gu, Seoul, Korea) for direct sequencing to confirm the identity of the viruses. Basic Local Alignment Tool (BLASTN) implemented in GenBank were used to confirm the identity of the sequences belonging to each of the viruses found.

1.3.5 Data analysis

The number of asymptomatic leaves that were positive to one or more of the viruses of interest here were recorded in relation to citrus species and geographic origin (Table 1). The presence per leaf of individual viruses and combinations of two viruses at the same time were recorded for each citrus species and geographic location (Table 2). Based on previous results, a series of logistic regressions was done for each of the most common viruses (CiLV-C and OFV), separately. Also, data for mandarin and sweet lime were excluded from the analysis because of the low number of samples analyzed (Table 2). For each virus (CiLV-C and OFV), the number of infected leaves were compared (they were assumed to follow a binomial distribution with sample size equal to the total number of leaves analyzed), using hierarchical contrasts where first the effect of citrus species (lime and orange) was compared, then, within each citrus species, the effect of geographical origin was assessed. Only treatments with a sample size above 10 leaves were analyzed. The final sample sizes for the different treatments varied between 10 and 25. All analyses were made using GenStat v 8.0 (Payne *et al*. 2005).

Table 1. Citrus species sampled for citrus leprosis viruses in different Mexican states. The number of leaves sampled with symptoms and without symptoms are shown with the number of asymptomatic leaves that were subsequently identified as positive for citrus leprosis viruses.

State	Citrus species	No. leaves with symptoms sampled	No. leaves without symptoms sampled	No. leaves without symptoms but with virus
Campeche	Lime	15	5	2
	Mandarin	5	0	-
	Orange	5	2	1
Chiapas	Lime	3	1	0
	Orange	16	8	4
Hidalgo	Orange	25	16	3
Jalisco	Lime	28	0	-
Morelos	Lime	19	5	1
	Orange	6	1	0
Nuevo Leon	Orange	16	5	0
	Grapefruit	9	7	4
Oaxaca	Lime	20	2	1
	Orange	5	2	1
Puebla	Lime	11	1	1
Queretaro	Orange	24	0	-
Quintana Roo	Orange	25	1	0
San Luis Potosi	Orange	25	1	0
Sinaloa	Orange	7	2	0
	Sweet lime	3	0	-
Tamaulipas	Orange	25	2	0
Veracruz	Orange	25	5	3
Zacatecas	Lime	15	4	2
	Sweet lime	5	0	-
	Orange	5	1	1

Table 2. **Citrus leprosis viruses detected using molecular techniques in citrus leaves sampled from different Mexican states**. The number in brackets shows the number of leaf samples analysed per citrus species. Numbers in columns represent the number of leaf samples that were positive for each of the viruses evaluated either alone or as a dual infection. The total number of positive samples selected for logistic regression analysis are shown in bold.

Estate	**Citrus spp**	**No virus**	**CiLV-C**	**CiNSV**	**OFV**	**CiLVN**	**CiLVC + OFV**	**CiLVN + OFV**	**CiNSV + OFV**	**CILVC + CiNSV**	**CiNSV + CiLVN**
Campeche	Lime (n=15)	5	7	0	0	0	3	0	0	0	0
	Mandarin (n=5)	2	1	0	0	0	2	0	0	0	0
	Orange (n=5)	2	3	0	0	0	0	0	0	0	0
Chiapas	Lime (n=3)	2	0	0	0	0	0	0	1	0	0
	Orange (n=16)	8	6	0	2	0	0	0	0	0	0
Hidalgo	Orange (n=25)	19	2	0	3	0	0	0	1	0	0
Jalisco	Lime (n=25)	15	4	0	6	0	0	0	0	0	0
Morelos	Lime (n=19)	14	1	1	2	0	1	0	0	0	0
	Orange (n=6)	4	0	0	1	0	0	0	1	0	0
Nuevo León	Orange (n=16)	1	12	1	0	0	0	0	0	2	0
	Sweet lime (n=9)	4	1	0	0	0	1	0	0	3	0
Oaxaca	Lime (n=20)	13	1	0	2	0	2	0	1	1	0
	Orange (n=5)	2	0	0	3	0	0	0	0	0	0

Puebla	Lime (n=11)	4	0	0	3	0	0	0	4	0	0
Queretaro	Mandarin (n=1)	0	0	0	0	0	0	0	1	0	0
	Orange (n=23)	11	8	0	0	0	0	0	4	0	0
Quintana Roo	Orange (n=25)	15	9	0	0	1	0	0	0	0	0
SLP	Orange (n=25)	16	4	2	2	0	0	0	1	0	0
Sinaloa	Sweet lime	2	0	0	1	0	0	0	0	0	0
	Orange (n=7)	3	3	1	0	0	0	0	0	0	0
Tamaulipas	Orange (n=25)	9	10	0	1	0	4	0	1	0	0
Veracruz	Srange (n=25)	13	9	0	1	1	0	0	1	0	0
Zacatecas	Sweet lime	1	2	1	1	0	0	0	0	0	0
	Lime (n=15)	8	0	3	0	0	1	0	0	2	1
	Orange (n=5)	1	1	2	0	0	0	0	0	0	1
Total no. positive samples		**174**	**84**	**11**	**28**	**2**	**14**	**0**	**16**	**8**	**2**

1.4 RESULTS

For all citrus species studied, we found asymptomatic leaves that were subsequently shown to be positive for the viruses of interest (Table 1). Overall, all four viruses studied were detected in all citrus species (Table 2) regardless of whether they were asymptomatic or not. The most common viruses were CiLV-C and OFV. The greatest percentage of dual infections by these two viruses was in lime (approx. 15%); for orange it was approx. 7% (Table 2).

There was a significant effect of citrus species were compared (χ_1^2=11.72, P<0.001). The greatest proportion of infected leaves were found in orange compared with lime (Fig 1A). When the presence of CiLV-C was compared amongst estates producing lime, significant effects were found (χ_5^2=17.30, P=0.004). The greatest proportion of leaves infected with CiLV-C was in limes of Campeche, with approximately 6% of infection, followed by the other estates with infections never above 2 % (Fig 2A). The presence of CiLV-C had significant differences amongst the estates analysed (χ_{10}^2=29.04, P=0.001). The greatest proportion of infected leaves were found in Nuevo Leon with approximately 8 % of infection followed by the other estates with infections never above 4% (Fig 2B).

When the proportion of leaves infected with OFV was analysed, lime had more infected leaves compared with orange (χ_1^2=4.91, P=0.027) (Fig 1B). When the estates producing limes were compared differences were also found (χ_5^2=13.26, P=0.021). The greatest proportion of infection was found in Puebla with 4% of infection, and the other estates with no infections above 1% (Fig

3A). However, no differences were found amongst the estates producing orange (χ^2_9=0.22, P=0.883). The proportion of orange leaves infected with OFV was never above 2% (Fig 3B).

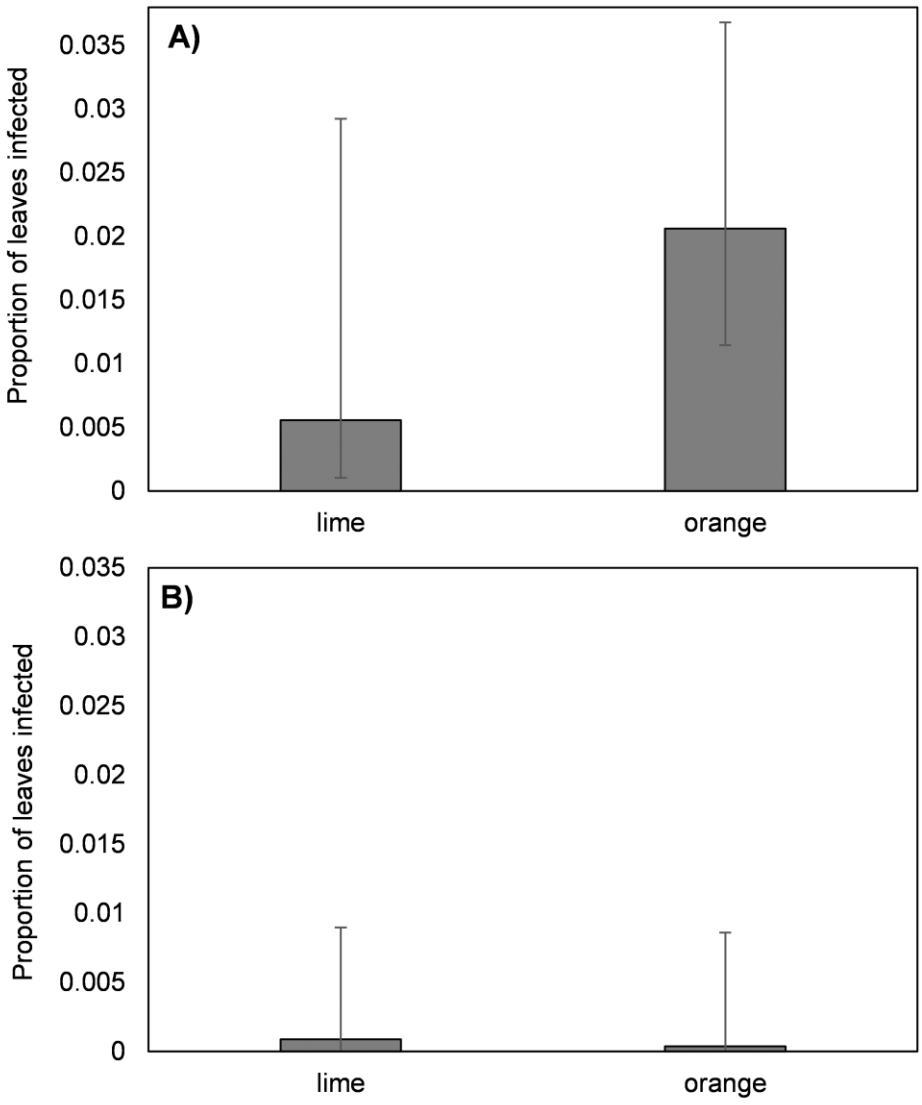

Figure 1. Proportion of leaves infected with the CiLV-C (A) and OFV (B) in lime and orange leaves. Error bars represents 95% confidence intervals back transformed from the logistic scale.

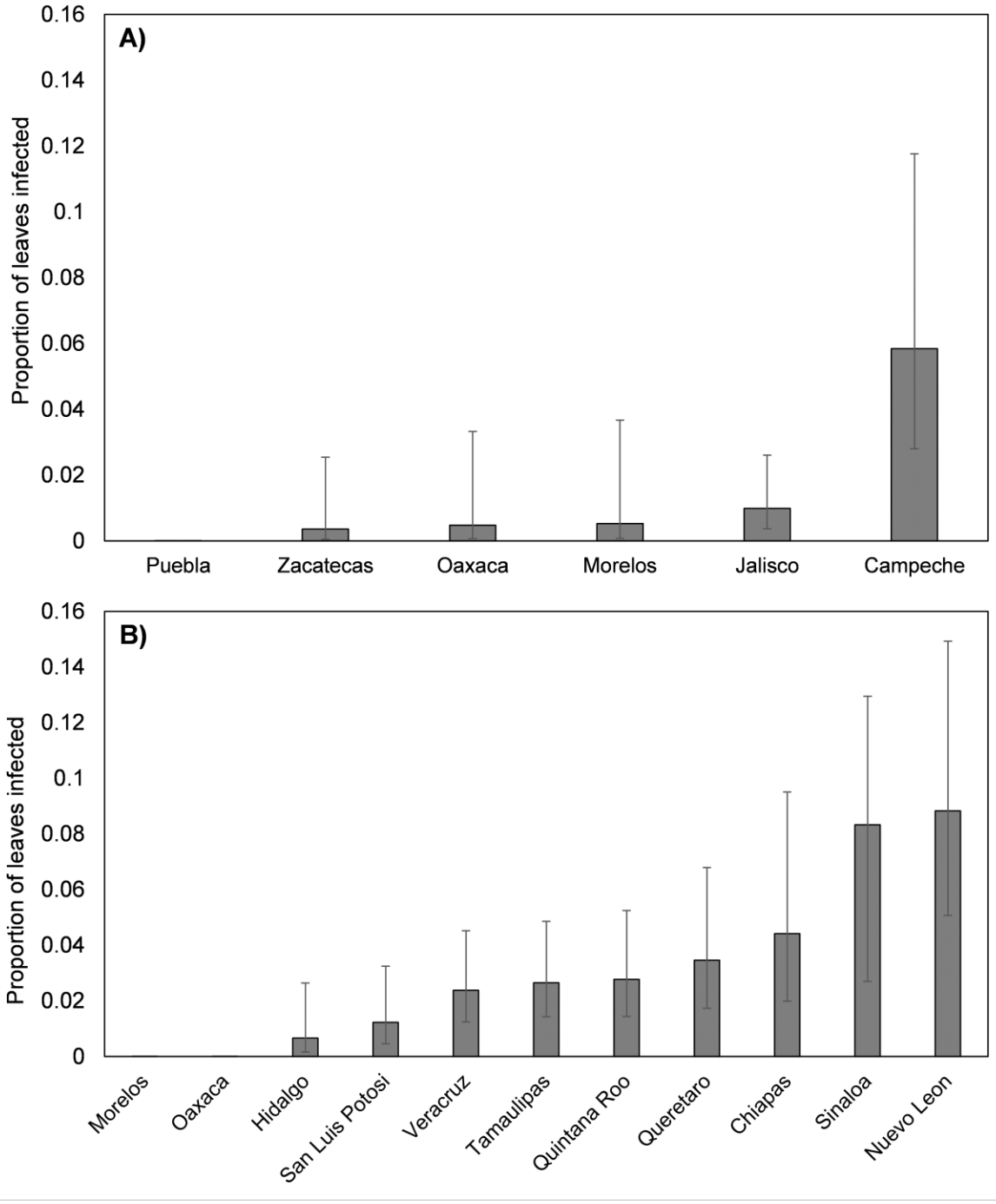

Figure 2. Proportion of leaves infected with the CiLV-C in lime (A) and orange (B) from different geographical origins (states). Error bars represents 95% confidence intervals back transformed from the logistic scale.

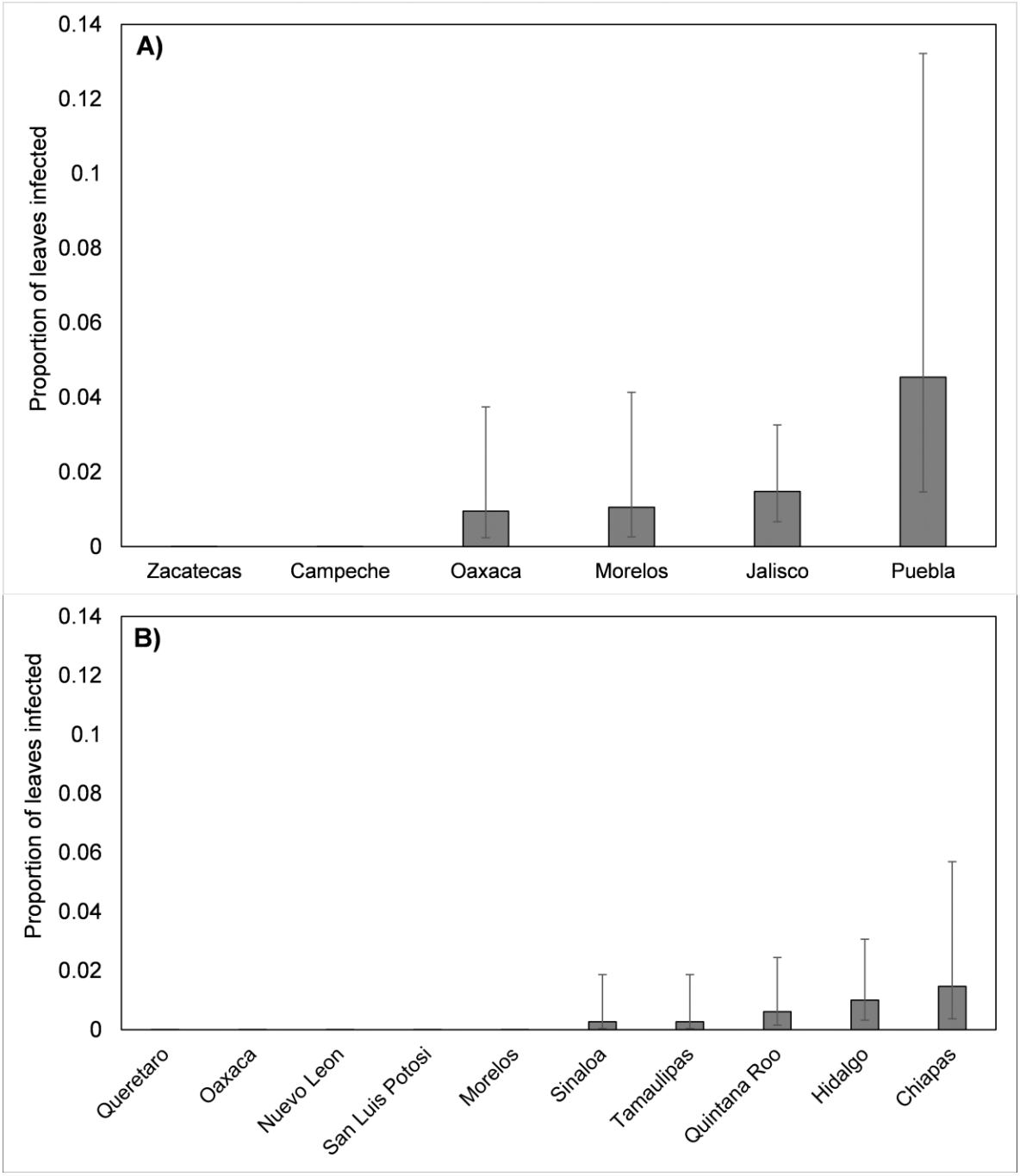

Figure 3. Proportion of leaves infected with the OFV in lime (A) and orange (B) from different geographical origins (states). Error bars represents 95% confidence intervals back transformed from the logistic scale.

1.5 DISCUSSION

Our results showed that all four viruses analyzed are present in Mexican citrus orchards. Unfortunately, the lack of sufficient samples from some species like sweet lime, mandarin or grapefruit did not allow a statistical analysis, as done with orange and lime. The reduced number of samples of these three species was not because of a lack of effort, but because the reduced area where these species were grown.

Overall, 6% of the asymptomatic lime and orange leaves contained virus (Table 1). Despite there being only a small proportion of asymptomatic leaves that were infected, this may represent an important inoculum for transmission if the vectors are present in orchards, which is very likely according to the work of Salinas-Vargas *et al*. (2016). Normally, when a control measure is applied against citrus leprosis, it is directed at the mite vector, *B. yothersi*, and based on observations of evident symptoms (Bastianel et al. 2010; Pedro L. Robles-García, personal communication). However, the presence of infected but asymptomatic leaves suggests that the diagnosis of disease presence to inform control strategies, should be based on molecular techniques rather than visual observation. Asymptomatic infected leaves are likely to be at a very early stage of infection that will eventually develop into evident symptomatology. However, even at an early stage of infection, it is likely that mites will be able to acquire the virus and transmit it to healthy leaves. It has been reported that insect vectors avoid feeding on infected tissue (Sisterson 2008). If they are already carrying the virus (maybe from feeding on asymptomatic leaves) then they will transmit the virus when feeding preferentially on healthy tissue; this is an

effective way of increasing virus transmission. Detecting the disease at an earlier stage will help to target control measures more effectively.

All four viruses were found in all citrus species (Table 2), but the most abundant were CiLV-C virus and OFV. To our knowledge, this is the first report of OFV infecting citrus plants. CiNSV was also present in all citrus species, except mandarin, but due to very low number of samples, we cannot conclude that mandarin is not affected by this virus. This is in line with previous reported for this virus (Cruz-Jaramillo *et al*. 2014). Even without statistical analysis, the low but consistent prevalence of CiNSV across all species (Table 2), suggests that this virus could have been co-existing with citrus species in Mexico for a long time, without an evident effect on the development of the host. The prevalence of CiLV-N was the lowest of all viruses studied. This virus has been reported previously in Colombia and Mexico (Rodriguez et al. 2003; Roy et al. 2015). Although the symptomatology of CiLV-N has been described as similar to CiLV-C (Bastianel et al. 2010), the relationship between CiLV-N and citrus, and its ability to cause damage are still unclear.

Dual infection of leaves was uncommon compared with infection of leaves by only one virus, and only found in lime and orange. We believe this could be a mechanism of the viruses to avoid competition. It is also likely that, even if a plant is initially infected by two viruses, only one virus will ultimately succeed and overgrow the leaf; this has been reported for potyviruses, Watermelon mosaic virus (WMV) and Zucchini yellow virus (ZYMV) in squash plants (*Cucurbita pepo* L.) (Salvaudon *et al*. 2003). Outcomes of dual-infections by viruses causing citrus leprosis, and their interactions with the vector remain to be

investigated, and will provide further insight into the ecology of the virus-vector interaction in citrus orchards.

The presence of CiLV-C was affected by both citrus species (Fig. 1) and geographical origin (Fig. 2). A greatest proportion of leaves infected with CiLV-C were found in orange than lime (Fig. 2), and this is in line with the reports of other researchers (Locali-Fabris et al. 2006). The reason for the effect of geographical origin is unclear. However, some patterns could be observed. The greatest proportion of orange leaves infected with CiLV-C was in the state of Nuevo Leon, where more the 50% of the area is considered to be dry or semidry, and the average temperature is 32 °C (INEGI, 2018). This is in contrast to states such as Quintana Roo, Veracruz and Chiapas, where between 50 and 90% of their area is considered to be humid or semi humid with average temperatures of 33, 23 and 28 °C, respectively (INEGI 2018); in these three states the proportion of orange leaves infected with CiLV-C was low (never exceeding 4%) compared with Nuevo Leon (Fig. 2). Dry environments favour the development of mites (Jeppson *et al*. 1975). Therefore, it is likely that *B. yothersi* could develop much more successfully under the conditions of Nuevo Leon, thus favouring disease transmission. To confirm this, we are currently quantifying the distribution, abundance and species diversity of *Brevipalpus* mites in the same states we sampled for viruses. Surprisingly, the proportion of lime leaves infected with CiLV-C was low in states such as Zacatecas and Jalisco which are considered to be dry (INEGI 2018). Also of interest is the fact that the greatest proportion of lime leaves infected with CiLV-C was in states considered to be humid and hot, such as Campeche.

If our ongoing studies confirm a relationship between high populations of *B. yothersi* in dry states and greater proportions of orange leaves infected with CiLV-C, and the opposite in humid states then *B. yothersi* can be implicated as the main vector in orange. However, for the lime system, *B. yothersi* may not be playing an important role as a vector, and more studies are needed to identify the most important vector in lime. It will also be important to assess whether the CiLV-C virus found in orange is genetically similar, or different, to the virus found in lime.

Overall OFV and CiLV-C were found in the same states but the proportion of both orange and lime leaves infected with OFV was low compared with the proportion infected with CiLV-C (Figs. 2, 3). However, results from Campeche and Puebla were interesting. Lime trees from Campeche seemed to be more susceptible to infection by CiLV-C but far less susceptible to infection by OFV, than lime trees in the other states (Figs. 2A, 3A). In contrast, lime trees from Puebla were more susceptible to infection by OFV than infection by CiLV-C; infact no infection by CiLV-C was recorded from lime in Puebla (Figs. 2A, 3A). Although it is thought that OFV is transmitted by *Brevipalpus* species (Bastianel *et al*. 2010), it is particularly associated with *B. californicus* (Kondo *et al*. 2003) rather than the most abundant species in Mexican citrus orchards, *B. yothersi* (Salinas-Vargas *et al*. 2016). The presence of different *Brevipalpus* species in our study may accoun for the results we observed. As mentioned before, we are currently analysing the *Brevipalpus* species distribution in the same states studied here and detecting viruses within the mites found; results will be presented in a separate publication and will provide further insights into the relationship between citrus leprosis and its vector.

In conclusion, all four viruses studied here were present in the citrus species evaluated. The most common viruses found were CiLV-C and OFV. Orange was the species most commonly infected by CiLV-C, whereas lime was more susceptible to infection by OFV. We suggest that molecular diagnostic tools for these viruses would be better than visual diagnosis as a decision-making tool for implementation of control options against the vector.

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Compliance with ethical standards

The authors declare that they followed all ethical responsibilities required by the European Journal of Plant Pathology when submitted this manuscript. No conflict of interest declared by the authors. The research did not involve any human or animals participants.

1.6 REFERENCES

Ali, R. N., Dann, A. L., Cross, P. A. & Wilson, C. R. (2014). Multiplex RT-PCR detection of three common viruses infecting orchids. *Archives of Virology* 159, 3095-3099.

Bastianel, M., Freitas-Astúa, J., Kitajima, E. W., & Machado, M. A. (2006). The citrus leprosis pathosystem. *Summa Phytopathologica* 32, 211-220.

Bastianel, M., Novelli, V. M., Kitajima, E. W., Kubo, K. S., Bassanezi, R. B., Machado, M. A. & Freitas-Astúa, J. (2010). Citrus Leprosis: Centennial of an unusual mite-virus pathosystem. *Plant Disease* 94, 284-292.

Cruz-Jaramillo, J. L., Ruiz-Medrano, R., Rojas-Morales, L., López-Buenfil, J. A., Morales-Galván, O., Chavarín-Palacio, C., Ramírez-Pool, J. A. & Xoconostle-Cázares, B. (2014). Characterization of a proposed Dichorhavirus associated with the Citrus Leprosis disease and analysis of the host response. *Viruses* 6, 2602-2622.

Hartung, J. S., Roy, A., Fu, S., Shao, J., Schneider, W. L., & Brlansky, R. H. (2015). History and diversity of citrus leprosis virus recorded in herbarium specimens. *Phytopathology* 105, 1277-1284.

INEGI (2018). Instituto Nacional de Estadística y Geografía. Available at: http://www.inegi.org.mx/ (Accessed July 31st, 2018)

Izquierdo-Castillo, I., Zermeño-Díaz, L. F., Méndez, W. , Otero-Colina, G. ,Freitas-Astúa, J., Locali-Fabris, E. C., de Moraes, G. J., Faier-Calegario, R., Tassi, A. D. & Kitajima, E. W. (2011). Confirmation of the presence of the Citrus leprosis virus C (CiLV-C) in Southern Mexico. *Tropical Plant Pathology* 36, 400-403.

Jeppson, L. R., Keifer, H. H. & Baker, E. W. (1975) Mites injurious to economic plants. University of California Press, 614 pp.

Kitajima, E. W., Chagas, C. M. & Rodrigues, J. C. V. (2003). *Brevipalpus* transmitted plant virus and virus-like diseases: Cytopathology and some recent cases. *Experimental and Applied Acarology* 30, 135-160.

Kitajima, E. W., Chagas, C. M., Harakava, R., Calegario, R. F., Freitas-Astúa, J., Rodrigues, J. C. V., & Childers, C. C. (2011) Citrus leprosis in Florida, USA, appears to have been caused by the nuclear type of Citrus leprosis virus N (CiLV-N). *Virus Review and Research* 16, 23-27.

Kondo, H., Maeda, T. & Tamada, T. (2003). Orchid Fleck Virus: *Brevipalpus californicus* mite transmission, biological properties and genome structure. *Experimental and Applied Acarology* 30, 215-223.

Liu, Y., Heying, E. & Tanumihardjo, A. S. (2012). History, global distribution, and nutritional importance of citrus fruits. *Comprehensive Reviews in Food Science and Food Safety* 11, 530-545.

Locali-Fabris, E. C., Freitas-Astúa, J., De Souza, A. A., Takita, M. A., Astua-Monge, G., Antonioli, R., Kitajima, E. W. & Machado, M. A. (2003). Development of a molecular tool for the diagnosis of leprosis, a major threat to citrus production in the Americas. *Plant Disease* 87, 1317-1321.

Locali-Fabris, E. C., Freitas-Astúa, J., Souza, A. A., Takita, M. A., Astua-Monge, G., Antonioli-Luizon, R., Rodrigues, V., Targon, M. L. & Machado, M. A. (2006) Complete nucleotide sequence, genomic organization and phylogenetic analysis of Citrus leprosis virus cytoplasmic type. *Journal of General Virology* 87, 2721-2729.

Payne, R., Murray, D., Harding, S., Baird, D. & Soutar D. (2005). GenStat for Windows, 8th Edition, Introduction. VSN International. Hemel Hempstead, UK.

Ramos-González, P. L., Sarubbi-Orue, H., González-Segnana, L., Chabi-Jesus, C., Freitas-Astúa, J. & Kitajima, E. W. (2016). Orchid Fleck Virus infecting orchids in Paraguay: First report and use of degenerate primers for its detection. *Journal of Phytopathology* 164, 342-347.

Rodriguez, J. C. V., Kitajima, E. W., Childers, C. C. & Chagas, C. M. (2003). Citrus Leprosis Virus vectored by *Brevipalpus phoenicis* (Acari: Tenuipalpidae) on citrus in Brazil. *Experimental and Applied Acarology* 30, 161-179.

Roy, A., Leon, M. G., Stone, A., Schneider, W. L., Hartung, J. S. & Brlansky, R. H. (2014). First Report of Citrus leprosis virus nuclear type in sweet orange in Colombia. *Plant Disease* 98, 1162.

Roy, A., Stone, A., Shao, J., Otero-Colina, G., Wei, G., Achor, D., Choudhary, N., Levy, L., Nakhla, M. K., Hartung, J. S., Schneider, W. L. & Brlansky, R. L. (2015). Identification and characterization of nuclear *Citrus leprosis virus*, an unassigned *Dichorhavirus* genus associated with citrus leprosis disease in Mexico. *Phytopathology* 105, 564-575.

Salinas-Vargas, D., Santillán-Galicia, M. T., Guzmán-Franco, A. W., Hernández-López, A., Ortega-Arenas, L. D. & Mora-Aguilera, G. (2016) Analysis of genetic variation in *Brevipalpus yothersi* (Acari: Tenuipalpidae) populations from four species of citrus host plants. *Plos One* 11: e0164552.

Salvaudon, L., De Moraes, C. M. & Mescher, M. C. (2003). Outcomes of co-infection by two potyviruses: implications for the evolution of manipulative strategies. *Experimental and Applied Acarology* 30, 135-160.

SENASICA (2013). Leprosis de los cítrico (Citrus leprosis virus C). Dirección General de Sanidad Vegetal - Sistema Nacional de Vigilancia Epidemiológica Fitosanitaria. México, D.F. Ficha Técnica No.35 27 p.

SIAP. 2017. Anuarios Estadísticos de la Producción Agrícola en México. Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación. http://www.siap.gob.mx. Acceded on November 2017.

Sisterson, M. S. (2008). Effects of insect-vector preference for healthy or infected plants on pathogen spread: Insights from a model. *Journal of Economic Entomology* 101, 1-8.

DISCUSIÓN GENERAL Y CONCLUSIÓN

Aun cuando en México la enfermedad de la leprosis de los cítricos, causada por el virus citoplasmático, se encontraba confinada al sur y centro del país en los Estados de Chiapas, Tabasco, Veracruz y Querétaro, actualmente la distribución del virus se ha incrementado y con ello su importancia (SENASICA, 2012; SCOPE, 2013). Los últimos estudios realizados por el SENASICA (2017), ubican al CiLV-C en los Estados de Chiapas, Campeche, Guerrero, Hidalgo, Jalisco, Morelos, Nayarit, Oaxaca, Puebla, Quintana Roo, San Luis Potosí, Sinaloa, Tabasco, Tamaulipas, Veracruz, Zacatecas y Querétaro; en este último estado, el agente causal se reporta restringido a plantas de traspatio; sin embargo, en este estudio, los muestreos realizados en Querétaro se hicieron en huertas comerciales, hallando en diversas muestras el virus tipo citoplasmático (CiLV-C), así como en el estado de Nuevo León.

En esta investigación el muestreo se llevó a cabo sobre diferentes especies de cítricos (naranja, limón, toronja, lima y mandarina), en donde se detectó mayor presencia de algunos virus hacia ciertas especies; por ejemplo, el CiLV-C en naranja y OFV en limón persa, lo que coincide con lo reportado por Salvo (1997), Domingues y Rodrigues (1999), Locali *et al*. (2006) y Bastianel *et al.* (2008), quienes mencionan que la naranja dulce y los mandarinos tienden a ser más susceptibles a CiLV-C, lo cual puede deberse a que los ácaros del género *Brevipalpus,* potenciales vectores del virus, tienen cierta tendencia a hallarse sobre dichos hospederos (Rodrigues *et al.,* 2003). Dicha información coincide con lo obtenido en este estudio, pues cómo se constató este virus se encuentra presente en todas las zonas de

importancia citrícola y con gran preferencia sobre *C. sinensis*, pues en los 16 estados muestreados el virus siempre estuvo presente.

En cuanto a los cítricos agrios como los limones (*C. limon*), se consideró que éstos eran inmunes al virus de la leprosis (Bastianel *et al*., 2004); no obstante, en la presente investigación, observamos síntomas característicos de leprosis en cítricos ácidos como limón persa (*C. latifolia*) y limón mexicano (*C. aurantifolia*). Además, 39 muestras recolectadas en los diferentes estados, fueron positivas al virus tipo citoplasmático y nuclear. Deducimos que los virus se encuentran afectando cítricos agrios sin inducir alteraciones visibles, pues la mayoría de las muestras positivas con dichos virus fueron asintomáticas. Dicha observación coincide con Bastianel *et al*. (2004), quienes demostraron que plantas de cítricos asintomáticos albergan el virus de la leprosis de los cítricos.

En toronjas (*C. paradisi)*, Locali *et al*. (2003), mostraron niveles variables de resistencia al virus de la leprosis tipo citoplasmático; sin embargo, en este estudio, muestras aparentemente sanas provenientes de los estados de Nuevo León y Tamaulipas resultaron positivas a los virus CiLV-C, OFV y CiNSV.

En cuanto a lima dulce, de acuerdo a investigaciones realizadas por Roy *et al.* (2015), también observaron síntomas de leprosis, aun cuando el agente causal que encontraron fue el CiLV-N en los estados de Querétaro, Jalisco y Michoacán. A diferencia de nuestro estudio, en el cual la presencia de CiLV-N en las muestras fue poco frecuente; aún en Zacatecas, que

colindan con Jalisco, en donde las muestras resultaron positivas a CiLV-C, OFV y CiNSV, pero no se encontró a CiLV-N.

Se sabe que la dispersión principal del virus es a través del ácaro vector *Brevipalpus* spp., pero migra hacia áreas libres mediante la movilización de plántulas asintomáticas (NAPPO, 2014), lo cual coincide con los resultados del presente estudio, pues en muestras de naranja, lima, limón persa, limón mexicano y toronja, que no presentaban los síntomas característicos de leprosis, se detectó al virus de tipo citoplasmático y nuclear con sus dos variantes. Esta posible vía de dispersión resulta de interés, pues, aunque en México no se tienen informes de infecciones naturales por el OFV en cítricos, y el CiLV-N se reporta sólo en el estado de Querétaro (Roy *et al.*, 2015), observamos que en 15 de los 16 estados evaluados, hubo muestras positivas al OFV. Además, se observó que las pocas muestras positivas al CiLV-N, fueron de los estados de Veracruz, Quintana Roo y Zacatecas.

Los virus de tipo nuclear (OFV) y su variante (CiNSV), también se encontraron ampliamente distribuidos en los cultivos de naranja, limón, lima y toronja de todos los estados que se evaluaron. Al respecto, se discute la rápida dispersión y aparición de síntomas sobre diferentes especies de cítricos y sobre áreas donde no encontraban.

De acuerdo con Cruz-Jaramillo *et al*. (2014), las plantas asintomáticas podrían estar más relacionadas al CiNSV, y se estarían replicando en cítricos agrios, como *C. aurantium;* además, menciona que la expresión del virus puede verse alterado bajo algunas circunstancias de estrés. Deducimos entonces que, aunque los virus con mayor distribución son CiLV-C y OFV en especies dulces y agrias, en todas los estados muestreados, hay probablemente preferencia entre el virus de tipo nuclear OFV y su variante CiNSV hacia cítricos agrios, tomando en cuenta la proporción de muestras positivas a estos virus por estados con hospederos agrios, como el limón persa.

En la presente investigación, las muestras que resultaron positivas al OFV fueron asintomáticas, por lo que existe la posibilidad de que esta variante se encontrara en México desde hace ya mucho tiempo atrás, quizás décadas, y asociada a cítricos, pero sin haber sido detectada.

Hartung *et al.* (2015) lograron ensamblar e identificar "contigs" de dos muestras preservadas de México de los años 1955 y 1967, las cuales claramente correspondían al CiLV-N, dicho estudio nos sugiere que la presencia actual del virus tipo nuclear, que, con sus variantes, se tenía ya desde el siglo pasado ampliamente distribuido, pues, actualmente se encuentra de manera prácticamente generalizada en el país, de acuerdo con nuestros muestreos, a pesar de que CiLV-C y CiLV-N se reportaron por primera vez en México en 2005 y 2012 (Cruz-Jaramillo *et al*., 2014 ; Izquierdo *et al*., 2011). Es probable que el CiLV-N sea endémico en México, pero no causa pérdidas serias de producción, por ello es que no

se notaba su presencia. Otra posibilidad es que el virus haya estado en México desde mediados del siglo XX, pero desapareció, como se ha documentado que ocurrió en Florida (Hartung *et al.,* 2015). De ser este el caso, entonces CiLV-N sería una patógeno reemergente en México (Hartung *et al.,* 2015), pero no existen evidencias para demostrarlo.

Tras lo observado en este estudio, se da por hecho que la toma de muestras con síntomas con diferentes características, con fines de diagnóstico, resulta subjetiva al momento de asociarles con la enfermedad de la leprosis, pues varias de estas muestras no dieron respuesta a la presencia de ninguno de los virus; por otro lado, la presencia de éstos en plantas asintomáticas, sugiere que las detecciones deben realizarse mediante técnicas moleculares, y no únicamente basadas en la presencia de síntomas. Dicha información puede resultar de gran relevancia para la epidemiología de la enfermedad y abre las puertas a estudios futuros sobre resistencia a estos virus en cítricos.

LITERATURA CITADA

Bastianel, M., Freitas A., Rodrigues, V., Arrivabem, F., Antonioli, L. & Machado, M. 2004. Resposta do tangor ´Murcott´ a inoculacao do virus da leprose dos citros em campo e em casa de vegetacao. Laranja. 25(2):337-348.

Bastianel, M., Freitas, A.J., Nicolini, F., Segatti, N., Novelli, V.M., Rodrigues, V., Medina, C.L. y Machado M.A. 2008. Response of mandarin cultivars and hybrids to Citrus leprosis virus. *J. Plant Pathol. 90,* 305–310.

Cruz-Jaramillo, J.L., Ruiz-Medrano, R., Rojas-Morales, L., López-Buenfil, J.A., Morales-Galván, O., Chavarin-Palacio, C., Ramírez-Pool, J.A. y Xoconostle-Cazares., B. 2014. Caracterización de un Dichorhavirus propuesto asociado con la enfermedad de la leprosis de los cítricos y análisis de la respuesta del huésped. Virus 6:2602 – 2622.

Domingues, A. y Rodrigues, J. 1999. Ocorrencia de leprose dos citros em Paraiso do Tocantins (TO). Laranja. 20(1):35-50.

Hartung, J.S., Roy, A., Fu, S., Shao, J., Schneider, W.L. & Brlansky, R.H. 2015. History and diversity of Citrus leprosis virus recorded in herbarium specimens. Phytopathology. 105:1277–84.

Izquierdo-Castillo, I., Zermeño-Díaz, L.F., Méndez, W., Otero-Colina, G., Freitas-Astua, J., Locali-Fabris, C.E., de Moraes G.J., Faier, C.R., Tassi, A.D. y Kitajima, E.W. 2011. Confirmación de la presencia del virus de la leprosis Citrus C (CiLV-C) en el sur de México. Trop. Planta Pathol. 36: 400 – 403.

Kondo, H., Maeda, T. & Tamada, T. 2003. Orchid fleck virus: *Brevipalpus californicus* mite transmission, biological properties and genome structure. Exp. Appl. Acarol. 30:215–23.

Locali, E.C., Freitas-Astua, J., de Souza A, A., Takita, M.A., Astua-Monge, G., Antonioli, R., Kitajima, E.W. y Machado, M.A. 2003. Development of a molecular tool for the diagnosis of leprosis, a major threat to citrus production in the Americas. Plant Disease 87:1317-1321.

Organización de Protección de Plantas de América del Norte (NAPPO). 2014. Sistema de alerta fitosanitaria. Detección de la leprosis en el estado de Chiapas,

México. Consultado en línea en mayo de 2017: http://www.pestalert.org/espanol/oprDetail.cfm?oprID=165 [Google Scholar].

Rodrigues, J.C.V., Kitajima, E.W., Childers, C.C. y Chagas, C.M. 2003. Citrus leprosis virus vectored by Brevipalpus phoenicis (Acari: Tenuipalpidae) on citrus in Brazil. Exp. Appl. Acarol. 30:161-179.

Roy, A., Stone, A., Shao, J., Otero-Colina, G., Wei, G., Choudhary, N., Achor D., Levy, L., Nakhla, M.K., Hartung J.S., Schneider, W.L., Brlansky, R.H. 2015. Identification and Molecular Characterization of Nuclear Citrus leprosis virus, a Member of the Proposed Dichorhavirus Genus Infecting Multiple Citrus Species in Mexico. Phytopathology. Apr;105(4):564-75.

Salvo, F.A., 1997. Notas sobre o tratamento fitossanitario em citros. Laranja. 18:155-163.

SCOPE. 2013. Sistema Coordinado para la Vigilancia de Plagas Reglamentadas y su Epidemiología. Consultado en línea el 22 de mayo de 2013: https://scopepublico.zedxinc.com/ cgi-bin/index.cgi.

SENASICA. 2012. Servicio Nacional de Sanidad Inocuidad y Calidad Agroalimentaria. SAGARPA. http:// www.senasica.gob.mx/?id=2527 Fecha de consulta: noviembre 2017.

SENASICA. 2017. Servicio Nacional de Sanidad Inocuidad y Calidad Agroalimentaria. Manual operativo de la campaña contra la leprosis de los cítricos (CiLV). 24 p.