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Host and Vectors of *Xylella fastidiosa* in Parras, Coahuila Vineyards, Mexico

Hospederos y Vectores de *Xylella fastidiosa* en Viñedos de Parras, Coahuila, México

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ABSTRACT

Xylella fastidiosa is a phytopathogenic bacterium that causes important diseases in different crops such as Pierce's disease in grapevine (*Vitis vinifera*), citrus variegated chlorosis, among others. The bacterium spreads very fast through vector insects, mainly leafhoppers (Cicadellidae) and spittlebugs (Cercopidae). The objective of present investigation was to determine the vectors and hosts of *Xylella fastidiosa* in vineyards located in Parras valley, Coahuila. Samples of 22 species were collected, including ornamental plants, weeds, shrubs, fruit trees, evergreen and deciduous forest; At the same time, the insects found in the sampling areas were collected and identified. The detection of *X. fastidiosa* in plants and insects was performed by PCR using the primers RST31/RST33. In the same way, identification of *X. fastidiosa* subsp. *multiplex*

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RESUMEN

Xylella fastidiosa es una bacteria fitopatógena que causa enfermedades de importancia en diferentes cultivos como la enfermedad de Pierce en vid (*Vitis vinifera*) y la clorosis variegada de los cítricos, entre otras. La bacteria se propaga de una manera muy rápida mediante insectos vectores, principalmente chicharritas (Cicadellidae) y salivazos (Cercopidae). La presente investigación tuvo como objetivo determinar los vectores y hospederos de *Xylella fastidiosa* en viñedos ubicados en el valle de Parras, Coahuila. Se colectaron muestras de 22 especies, entre ellas plantas de ornato, malezas, arbustos, árboles frutales, forestales perennes y caducifolios; al mismo tiempo, se colectaron e identificaron los insectos encontrados en las zonas de muestreo. La detección de *X. fastidiosa* en plantas e insectos se realizó mediante PCR utilizando los primers RST31/RST33. De igual forma, se llevó a cabo la identificación de *X. fastidiosa* subsp. *multiplex* con los primers ALM1/ALM2 y XF2542-L/XF2542-R en muestras vegetales que resultaron positivas en la primera prueba. Se detectó la presencia de *X. fastidiosa* en plantas de

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with primers ALM1/ALM2 and XF2542-L/XF2542-R in plant samples that were positive in first test was carried out. The presence of *X. fastidiosa* was detected in commercial vine and wild grape plants, while *X. fastidiosa* subsp. *multiplex* was identified in apricot and ash trees. Among the insects collected, six genera and four species of leafhoppers were identified, however, only *X. fastidiosa* was detected in *Homalodisca vitripennis*.

KEY WORDS

Xylella, Pierce disease, *Homalodisca.*

Introduction

Xylella fastidiosa is a phytopathogenic bacterium that causes important diseases in different crops such as Pierce´s disease of grapevine (*Vitis vinifera*), coffee leaf scorch in coffee (*Coffea arabiga*), quick decline syndrome of olive (*Olea europea*), almond leaf scorch (*Prunus dulcis*), the citrus variegated chlorosis, alfalfa dwarfism (*Medicago sativa*) and leaf scorch in urban trees (de Lima *et al.,* 1998; Purcell *et al*., 1999; Li, *et al.,* 2001; Almeida & Purcell, 2003; Sisterson *et al*., 2010; Costa *et al*., 2004). The bacterium has been found dormant in asymptomatic hosts such as california mugwort (*Artemisia douglasiana*) and barnyard grass (*Echinochloa cruz-galli*) that serve as inoculum reservoirs (Hopkins & Purcell, 2002). The symptoms presented by infected plants with *X. fastidiosa* usually appear first in some leaves and then in almost all the foliage. The affected area is delineated by a narrow chlorotic halo that becomes especially clear in autumn. It presents premature defoliation and malformation in new leaves, the fruits grow abnormally, the stems can show internal and external discoloration; in some cases, there is a regressive death and abnormal growth eventually ending in host death (Janse & Obradovic, 2010). *X. fastidiosa* spreads naturally by insect vectors mainly leafhoppers (Cicadellidae) and spittlebugs (Cercopidae) very fast because there is no latency period for transmission, some related species as *X. fastidiosa* vectors are: red head leafhopper (*Xyphon fulgidum*) (Catanach *et al*., 2013), green leafhopper (*Draeculacephala minerva*), blue-green leafhopper (*Graphocephala atropunctata*), alfalfa leafhopper (*Phera lacerta*) (Burks & Redak, 2003a), glassy winged leafhopper (*Homalodisca vitripennis*) and *Oncometopia nigricans*, among others (Hill and Purcell, 1997; Brlansky *et al*., 2002;

vid comercial y vid silvestre, mientras que en árboles de chabacano y fresno se identificó a *X. fastidiosa* subsp. *multiplex*. Entre los insectos colectados se identificaron seis géneros y cuatro especies de cicadélidos, sin embargo solo se detectó a *X. fastidiosa* en *Homalodisca vitripennis.*

PALABRAS CLAVE

Xylella, Enfermedad de Pierce, *Homalodisca.*

Introducción

Xylella fastidiosa es una bacteria fitopatógena que causa enfermedades de importancia en diferentes cultivos como la enfermedad de Pierce en vid (*Vitis vinifera*), la crespera del café (*Coffea arabiga*), el síndrome de decaimiento súbito del olivo (*Olea europea*), la quemadura de la hoja del almendro (*Prunus dulcis*), la clorosis variegada de los cítricos, enanismo de la alfalfa (*Medicago sativa*) y quemaduras de la hoja en arboles urbanos (de Lima *et al.,* 1998; Purcell *et al*., 1999; Li, *et al.,* 2001; Almeida & Purcell, 2003; Sisterson *et al*., 2010; Costa *et al*., 2004). La bacteria se ha encontrado de forma latente en hospederos asintomáticos como artemisa (*Artemisia douglasiana*) y pasto pata de gallo (*Echinochloa cruz-galli*) que sirven como reservorios de inóculo (Hopkins & Purcell, 2002). Los síntomas que presentan las plantas infectadas por *X. fastidiosa* generalmente se manifiestan primero en algunas hojas y luego en casi todo el follaje. El área afectada está delineada por un estrecho halo clorótico que se vuelve especialmente claro en otoño. Se presenta defoliación prematura y malformación en hojas nuevas, los frutos crecen anormalmente, los tallos pueden mostrar decoloración interna y externa; en algunos casos ocurre una muerte regresiva y crecimiento anormal terminando eventualmente en la muerte del hospedero (Janse & Obradovic, 2010). *X. fastidiosa* se propaga naturalmente mediante insectos vectores principalmente chicharritas (Cicadellidae) y salivazos (Cercopidae) de manera muy rápida debido a que no hay periodo de latencia para su transmisión, algunas especies relacionadas como vectores de *X. fastidiosa* son: chicharrita cabeza roja (*Xyphon fulgidum*) (Catanach *et al*., 2013), chicharrita verde (*Draeculacephala minerva*), chicharrita verde-azulada (*Graphocephala atropunctata*), chicharrita de la alfalfa (*Phera lacerta*) (Burks & Redak, 2003a), chicharrita de alas cristalinas (*Homalodisca vitripennis*) y *Oncometopia nigricans*, entre otros (Hill & Purcell, 1997; Brlansky *et al*., 2002; Díaz, 2003; Hoddle, 2004; Blackmer, 2006; Daane

Díaz, 2003; Hoddle, 2004; Blackmer, 2006; Daane *et al*., 2011). The bacterium is not trans-ovarial or trans-stag, it feeds and reproduces in salivary of adult insects which can remain infective throughout their lives (Almeida *et al*., 2005; Redak *et al*., 2004). The bacterium detection is mainly carried out by serological techniques such as ELISA and molecular techniques such as PCR in which specific primers are used, such as RST3/; RST33 (Minsavage *et al*., 1994) and XF1968-R/XF1968-L (Firrao & Bazzi, 1994). The objective of present investigation was to determine the vectors and hosts of *Xylella fastidiosa* in wine-growing zone of Parras, Coahuila, Mexico.

Material and Methods

Plant material collection.

The plant material collection was carried out in area surrounding 4 vineyards in Parras, Coahuila, Mexico during 2016. Leaves and shoots of 22 plant species with symptoms like to those caused by *X. fastidiosa* were collected.

Insect collection.

By using an entomological striking net through a series of 500 nets carried out in periphery of vineyards, insects were collected on the weeds and were also manually collected in bushes, fruit tres and forest. The insects were preserved in ethanol 70 %.

Insects identification.

The insects were separated and grouped by morphological observations, the identification was made with help of taxonomic keys (DeLong & Davidson, 1935; Young, 1958; Blocker, 1967) and male genitalia extraction.

DNA extraction from plants and insects.

For this investigation, the CTAB DNA extraction method (Doyle & Doyle, 1987) with modifications was used. 50 to 100 mg of tissue were taken from each plant and in the case of insects, the whole specimen was considered. From plants, the main veins and the petiole of each leaf were selected, these were macerated with 300 µL of CTAB buffer, placed in eppendorf tubes and 1 mL of CTAB buffer was added, 100 µL of 5M NaCl was added, they were incubated 95 °C for 90 min, centrifuged at 12,000 rpm for 1 min and the supernatant was recovered. 500 µL of chloroform isoamyl alcohol

et al., 2011). La bacteria no es trans-ovarial ni trans-estadial, se alimenta y se reproduce en el salivario de los insectos adultos los cuales pueden permanecer infectivos a lo largo de toda su vida (Almeida *et al*., 2005; Redak *et al*., 2004). La detección de la bacteria se realiza principalmente por técnicas serológicas como ELISA y técnicas moleculares como PCR en la que se utilizan primers específicos como los RST3/; RST33 (Minsavage *et al*., 1994) y XF1968-R/XF1968-L (Firrao & Bazzi, 1994). El objetivo de la presente investigación fue determinar los vectores y hospederos de *Xylella fastidiosa* en la zona vitivinícola de Parras, Coahuila, México.

Material y Métodos

Colecta de material vegetal.

La colecta de material vegetal se realizó en el área circundante a 4 viñedos de Parras, Coahuila, México durante el 2016. Se colectaron hojas y brotes de 22 especies de plantas con síntomas similares a los ocasionados por *X. fastidiosa*.

Colecta de insectos.

Utilizando una red entomológica de golpeo a través de una serie de 500 redeos efectuados en la periferia de los viñedos se colectaron insectos en malezas y de manera manual se colectaron en los arbustos, árboles frutales y forestales. Los insectos fueron conservados en etanol al 70 %.

Identificación de insectos.

Los insectos fueron separados y agrupados mediante observaciones morfológicas, la identificación se realizó con ayuda de claves taxonómicas (DeLong & Davidson, 1935; Young, 1958; Blocker, 1967) y extracción de genitales masculinos.

Extracción de DNA de plantas e insectos.

Para esta investigación, se utilizó el método de extracción de CTAB DNA (Doyle & Doyle, 1987) con modificaciones. De cada planta se tomaron de 50 a 100 mg de tejido y en el caso de los insectos, se consideró al espécimen completo. De las plantas se seleccionaron las nervaduras principales y el peciolo de cada hoja, estos se maceraron con 300 µL de buffer CTAB, se colocaron en tubos eppendorf y se agregó 1 mL de buffer CTAB, se agregaron 100 µL de NaCl 5M, se incubaron a 95 °C por 90 min, se centrifugaron a 12,000 rpm por 1 min y se recuperó el sobrenadante. Se agregaron 500 µL de cloroformo alcohol isoamílico (24:1), se mezclaron por inversión y se

(24:1) were added, mixed by inversion and centrifuged at 12,000 rpm for 10 min; the supernatant was recovered and 500 µL of cold isopropanol was added. The samples were incubated overnight at -20 °C, then centrifuged at 12,000 rpm for 15 min, decanted and the pellet obtained was washed with 1 mL of ethanol 70 % by centrifuging at 12,000 rpm for 5 min. The pellet was resuspended in 100 µL of nuclease-free sterile water.

***X. fastidiosa* detection in plants and insects.**

The bacterium detection was carried out by PCR using the primers RST31 (5'-GCGTTAAT TTTCGAAGTGATTCGATTGC-3') and RST33 (5'-CACCATTCGTATCCCGGTG-3') (Minsavage *et al*., 1994). The procedure for PCR reaction consisted of 1 cycle at 95 °C for 5 min, 35 cycles of amplification: 95 °C for 30 s, 60 ° C for 30 s, 72 ° C for 45 s and the final extension at 72 ºC for 7 minutes. The amplified products were analyzed by electrophoresis in a 1.5 % agarose gel, ethidium bromide was used for staining.

***X. fastidiosa* subsp. *multiplex* detection.**

The samples that were positive *for X. fastidiosa* were again analyzed by PCR for the subspecies identification using the specific primers for the subsp. *multiplex* XF1968-L (5'-GGAGGTTTACCGAAGACA GAT-3') and XF1968-R (5'ATCCACAGTAAAACCACATGC-3'), ALM1 (5'CTGCAG AATTGGAAACTTCAG-3') and ALM2 (5'-GCCACACGTGATCTATGAA-3') (Hernández-Martínez *et al*., 2006). The amplification consisted of 1 cycle at 94 °C for 5 min, 40 cycles at 94 °C for 1 min, annealing temperature 55 °C for 1 min, 72 °C for 1 min and the final extension was one cycle at 72 °C for 10 min. The amplified products were analyzed by electrophoresis in a 1.5 % agarose gel.

Results

Insects identification and *X. fastidiosa* detection in insects.

Six genera and four species of leafhoppers were identified through morphological observations and with the use taxonomic keys. One of the leafhoppers was identified as *Homalodisca vitripennis* (Germar) (Hemiptera: Cicadellidae: Cicadellinae) commonly known as glassy winged sharpshoot (Figure 1). Of the genera analyzed by PCR, only *X. fastidiosa* was detected in *H. vitripennis* (Table 1), which is reported

centrifugaron 12,000 rpm durante 10 min; se recuperó el sobrenadante y se agregaron 500 µL de isopropanol frío. Las muestras se incubaron toda la noche a -20 °C, posteriormente se centrifugaron a 12,000 rpm durante 15 min, se decantaron y la pastilla obtenida se lavó con 1 mL de etanol al 70 % centrifugando a 12,000 rpm por 5 min. La pastilla se re suspendió en 100 µL de agua estéril libre de nucleasas.

Detección de *X. fastidiosa* en plantas e insectos.

La detección de la bacteria se realizó mediante PCR utilizando los primers RST31 (5´-GCGTTAAT TTTCGAAGTGATTCGATTGC-3´) y RST33 (5´CACCATTCGTATCCCGGTG-3´) (Minsavage *et al*., 1994). El procedimiento para la reacción PCR consistió en 1 ciclo a 95 °C por 5 min, 35 ciclos de amplificación: 95 °C por 30 s, 60 °C por 30 s, 72 °C por 45 s y la extensión final a 72 ºC por 7 minutos. Los productos amplificados se analizaron por electroforesis en un gel de agarosa al 1.5 %, para la tinción se utilizó bromuro de etidio

Detección de *X. fastidiosa* subsp. *multiplex*.

Las muestras que resultaron positivas para *X. fastidiosa* se analizaron nuevamente por PCR para la identificación de subespecie utilizando los primers específicos para la subsp. *multiplex* XF1968-L (5′-GGAGGTTTACCGAAGACAGAT-3′) y XF1968-R (5′ATCCACAGTAAAACCACATGC-3′), ALM1 (5′-CTGCAGAAATTGGAAACTTCA G-3′) y ALM2 (5′-GC CACACGTGATCTATGAA-3′) (Hernández-Martínez *et al*., 2006). La amplificación consistió en 1 ciclo a 94 °C por 5 min, 40 ciclos a 94 °C por 1 min, temperatura de anillamiento 55 °C por 1 min, 72 °C por 1 min y la extensión final fue de un ciclo a 72 °C por 10 min. Los productos amplificados se analizaron por electroforesis en un gel de agarosa al 1.5 %

Resultados

Identificación de insectos y detección de *X. fastidiosa* en insectos.

Se identificaron seis géneros y cuatro especies de cicadélidos mediante observaciones morfológicas y con el uso de claves taxonómicas. Uno de los cicadélidos se identificó como *Homalodisca vitripennis* (Germar) (Hemiptera: Cicadellidae: Cicadellinae) conocida comúnmente como chicharrita de alas cristalinas (Figura 1). De los géneros analizados mediante PCR solo se detectó a *X. fastidiosa* en *H. vitripennis* (Tabla 1) misma que se

Figure 1. Images of *Homalodisca vitripennis*. A) Dorsal view; B) Side view.

Figura 1. Imágenes de *Homalodisca vitripennis.* A) Vista dorsal; B) Vista lateral.

Table 1.
PCR test results in insects for *Xylella fastidiosa* detection.
Tabla 1.
Resultados de la prueba de PCR en insectos para la detección de *Xylella fastidiosa.*

Cicadellid	PCR Result
Homalodisca vitripennis	Positive
Empoasca dilitara	Negative
Balclutha mexicana	Negative
Balclutha abdominalis	Negative
Aceratogallia spp.	Negative
Erythroneura spp.	Negative

as the main vector of the bacterium. In relation to the above, Almeida & Purcell (2003) showed that *H. vitripennis* is a *X. fastidiosa* vector through a greenhouse transmission experiment. Likewise, Hopkins & Purcell (2002) confirmed that *H. vitripennis* is the main vector transmitter of bacterium when relating the coincidence of patterns of high insects populations with the appearance of new outbreaks of Pierce's disease in Napa Valley, Sonoma and Temecula in United States. The literature does not mention other genera of leafhoppers identified in this research as insects vectors of *X. fastidiosa*.

X. fastidiosa detection in plants.

Of the 22 plants species analyzed, *X. fastidiosa* was detected in vine plants (samples vine 1 and wild vine), in apricot (samples 1, 2 and 3) and in ash

reporta como el principal vector de la bacteria**.** En relación con lo anterior, Almeida & Purcell (2003) demostraron que *H. vitripennis* es vector de *X. fastidiosa* mediante un experimento de transmisión en invernadero. Asimismo, Hopkins & Purcell (2002) confirmaron que *H. vitripennis* es el principal vector transmisor de la bacteria al relacionar la coincidencia de patrones de altas poblaciones del insecto con la aparición de nuevos brotes de la enfermedad de Pierce en el valle de Napa, Sonoma y Temecula en Estados Unidos. La literatura no menciona a los demás géneros de cicadélidos identificados en esta investigación como insectos vectores de *X. fastidiosa.*

Detección de *X. fastidiosa* en plantas.

De las 22 especies de plantas analizadas se detectó *a X. fastidiosa* en plantas de vid (muestras vid 1 y vid silvestre), en chabacano (muestras 1, 2 y 3) y en fresno

Table 2.
PCR test results in plants for *X. fastidiosa* detection.

Tabla 2.
Resultados de la prueba de PCR en plantas para la detección de *X. fastidiosa.*

Plant	Scientific name	PCR Result
Wild vine	*Vitis* sp.	**Positive**
Eucalyptus	*Eucalyptus* sp.	Negative
Rose	*Rosa* sp.	Negative
Pear	*Pyrus communis*	Negative
Peach	*Prunus domestica*	Negative
Oriental persimmon	*Diospyrus kaki*	Negative
Carnation	*Dianthus caryophyllus*	Negative
Chinese palm	*Yucca filifera*	Negative
Pecan	*Carya illinoensis*	Negative
Vine 1	*Vitis vinifera*	**Positive**
Vine 2	*Vitis vinifera*	Negative
Jupiter tree	*Lagerstroemia indica*	Negative
Cedar	*Cedrela* sp.	Negative
Japanese privet	*Ligustrum japonicum*	Negative
Olive	*Olea europea*	Negative
Lily	*Lilium candidum*	Negative
Johnson grass	*Sorghum alepense*	Negative
Apricot 1	*Prunus armeniaca*	**Positive**
Apricot 2	*Prunus armeniaca*	**Positive**
Apricot 3	*Prunus armeniaca*	**Positive**
Mandarin	*Citrus reticulata*	Negative
Bouganvilla	*Bougainvillea* sp.	Negative
Avocado	*Persea americana*	Negative
Fig	*Ficus carica*	Negative
Ash 1	*Fraxynus* sp.	**Positive**
Ash 2	*Fraxynus* sp.	**Positive**
Ash 3	*Fraxynus* sp.	Negative
Ash 4	*Fraxynus* sp.	Negative

(samples 1 and 2) (Table 2). The identification was made by observing a band of 733 bp in electrophoresis gel. The results found in this study are similar to those described in the database of European Food Safety Authority (EFSA) in 2016, which indicate that *X. fastidiosa* subsp. *fastidiosa* was detected in vine plants, while *X. fastidiosa* subsp. *multiplex* is present in ash and apricot trees, as well as in olive trees.

(muestras 1 y 2) (Tabla 2). La identificación se realizó mediante la observación de una banda de 733 pb en el gel de electroforesis. Los resultados encontrados en este estudio son similares a los descritos en la base de datos de la Autoridad Europea de Seguridad Alimentaria (European Food Safety Authority-EFSA) en el 2016, éstos señalan que *X. fastidiosa* subsp. *fastidiosa* se detectó en plantas de vid, mientras que *X. fastidiosa*

***X. fastidiosa* subsp. *multiplex* detection.**

The identification of *X. fastidiosa* subsp. *multiplex* was carried out using the primers XF1968-L / XF1968-R and ALM1/ALM2. The bacterium was detected in apricot plants (samples 1, 2 and 3) and ash (samples 1 and 2) by observing an amplification of 638 and 521 bp corresponding to the aforementioned primers. The PCR multiprimers test with primers pairs: XF1968-L/ XF1968-R, XF2542-L/XF2542-R and ALM1/ALM2 used to differentiate *X. fastidiosa* in infected vine, almond and flower laurel plants was developed by Hernández-Martínez *et al*. (2006). In their assay, the subspecies were differentiated based on the set of primers they amplified; based on this, they reported that the samples they amplified with the three pairs of primers correspond to *X. fastidiosa* subsp. *multiplex*.

Conclusions

It was shown that *X. fastidiosa* is affecting different hosts in wine-growing area in Parras, Coahuila. In addition to detecting *X. fastidiosa* in commercial vine plants and wild grapevine, *X. fastidiosa* subsp. *mutiplex* in other hosts such as ash and apricot was identified. Likewise, *X. fastidiosa* was detected in *H. vitripennis* which is a potential vector of the bacterium in Parras Valley, Coahuila, Mexico.

Detección de *X. fastidiosa* subsp. *multiplex*.

La identificación de *X. fastidiosa* subsp. *multiplex* se llevó a cabo utilizando los primers XF1968-L/XF1968-R y ALM1/ALM2. Se logró detectar a la bacteria en plantas de chabacano (muestras 1, 2 y 3) y fresno (muestras 1 y 2) mediante la observación de un amplificado de 638 y 521 pb correspondientes a los primers antes mencionados. La prueba de PCR multiprimers con los pares de primers: XF1968-L/ XF1968-R, XF2542-L/XF2542-R y ALM1/ALM2 utilizados para diferenciar *X. fastidiosa* en plantas infectadas de vid, almendro y laurel de flor fue desarrollada por Hernández-Martínez *et al.* (2006). En su ensayo, las subespecies fueron diferenciadas en base al conjunto de primers que amplificaron; en base a esto, reportaron que las muestras que amplificaron con los tres pares de primers corresponden a *X. fastidiosa* subsp. *multiplex*.

Conclusiones

Se demostró que *X. fastidiosa* se encuentra afectando a diferentes hospederos en la zona vitivinícola de Parras, Coahuila. Además de detectar a *X. fastidiosa* en plantas de vid comercial y vid silvestre, se identificó a *X. fastidiosa* subsp. *mutiplex* en otros hospederos como fresno y chabacano. De igual forma, detectó a *X. fastidiosa* en *H. vitripennis* el cual es un potencial vector de la bacteria en el valle de Parras, Coahuila, México.

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