Citrus huanglongbing in Venezuela: partial distribution and the relative incidence of *Candidatus* Liberibacter asiaticus in central-northern states

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ABSTRACT

Huanglongbing (HLB), is the most devastating citrus disease in the world. Two of the three known HLB-associated (*Candidatus* Liberibacter spp.) species are present in the Americas. Among them, '*Ca*. L. asiaticus' (*C*Las) was detected in Venezuela in 2017, although early characteristics HLB symptoms such as yellow shoots were first noticed in 2016. In this study, plant material from suspect citrus and from knowing HLB hosts, were collected in 17 municipalities in the states of Aragua, Carabobo, Yaracuy and Portuguesa (Venezuela) between August and November 2017, to determine *C*Las relative incidence and host range. Samples were then evaluated by PCR, using primers targeting the β-operon locus of ribosomal proteins. Out of 113 HLB-symptomatic citrus plants, 87 (76.9 %) were positive for *C*Las. *Citrofortunella microcarpa*, *Swinglea glutinosa*, and *Murraya paniculata* known host plants of the vector, the Asian citrus psyllid *Diaphorina citri* Kuwayama, were also found infected in all regions analyzed. Further spread of the pathogen is expected, given the nationwide distribution and abundance of the vector. Given the HLB-infection rates found in this study, additional surveys in eastern and western states are recommended to evaluate the spread and severity of the disease which will greatly assist the design of efficient management strategies based on research data.

Keywords: Citrus greening, detection, distribution, HLB, PCR, Venezuela.

El huanglongbing de los cítricos en Venezuela: distribución parcial e incidencia relativa de *Candidatus* liberibacter asiaticus en la región centro-norte

RESUMEN

El "Huanglongbing" (HLB) es actualmente la enfermedad más devastadora de los cítricos. Dos de las tres especies de bacterias que se asocian al HLB (*Candidatus* Liberibacter spp.), han sido encontradas en las Américas. En Venezuela, la especie '*Ca*. L asiaticus' (*C*Las) fue detectada en 2017, aunque los síntomas tempranos característicos asociados a la enfermedad, tales como brotes amarillos fueron notificados por productores en varias regiones del país desde el año 2016. En este estudio, se colectó material vegetal proveniente de árboles de cítricos, así como de hospederos conocidos del HLB, en 17 municipios de los estados Aragua, Carabobo, Yaracuy y Portuguesa (Venezuela) entre Agosto y Noviembre de 2017, con el objetivo de determinar la incidencia relativa y el rango de hospederos de *C*Las en la región central del país. Las muestras fueron evaluadas mediante PCR, utilizando cebadores para amplificar la región del locus de las proteínas ribosomales en el operón-β de la bacteria. De un total de 113 plantas de cítricos sintomáticas, 87 (76.9 %) resultaron positivas para *C*Las. Adicionalmente, se encontraron plantas de *Citrofortunella microcarpa*, *Murraya paniculata* y *Swinglea glutinosa* (hospederas del vector del HLB, el psílido asiático de los cítricos, *Diaphorina citri* Kuwayama) infectadas con *C*Las en todos los estados incluidos en el estudio. Dado el porcentaje de infección que revela esta investigación, se recomienda realizar muestreos en los estados orientales y occidentales, a fin de conocer la diseminación e incidencia de la enfermedad en Venezuela y formular estrategias eficientes de manejo y control basadas en evidencia científica.

Palabras clave: enverdecimiento de los cítricos, detección, distribución, HLB, PCR, Venezuela.

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INTRODUCTION

Huanglongbing (ex-greening, HLB) is currently the most economically damaging disease of citrus trees and is distributed in more than 40 countries, causing important economic losses (Li *et al.* 2020, Singerman and Rogers 2020). The disease is associated with three nonculturable and phloem restricted alpha proteobacteria species: '*Candidatus* Liberibacter asiaticus' (*C*Las), '*Ca*. Liberibacter americanus' (*C*Lam) and '*Ca*. Liberibacter africanus' (*C*Laf) (Jagoueix *et al.* 1994, Teixeira *et al.* 2005, Bové 2006). *C*Las and *C*Lam are transmitted by the Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), and *C*Laf is transmitted by the African citrus psyllid *Trioza erytreae* Del Guercio (Hemiptera: Psylloidea: Triozidae) (Bové 2006). *C*Lam and *C*Las are found in South America, being *C*Las the predominant species (Lópes *et al.* 2009).

HLB infection causes phloem damage, plugging of sieve pores, and interference with sucrose transport, resulting in starch accumulation (Kim *et al.* 2009). Subsequently, this accumulation blocks nutrient transport and alters the metabolism of carbohydrates, further causing starvation of sink tissues (Cimò *et al.* 2013) resulting in phenotypic changes in trees, such as deterioration of the root system and various nutrient deficiency symptoms including blotchy chlorosis and mottling of leaves; yellowing of shoots; vein corking; lopsided, half green bitter fruits; aborted seeds and finally, dead (Bové 2006, da Graça *et al.* 2016). Symptom expression is dependent on the age and nutritional status of the infected tree (Shen *et al.* 2013).

Worldwide, citrus severe losses have been caused by pre-harvest fruit drop, reduced fruit quality, and tree dead (Munir *et al.* 2017, Fu *et al.* 2019). The disease has no cure, and no resistant germplasm has been found. By 2014, HLB was estimated to have reduced the statewide sweet orange crop by 100 million boxes per year (4 million metric tons) in Florida, USA (Spreen *et al.* 2014). Besides, production costs have increased due to aggressive insecticide and fertilizer applications (Tansey *et al.* 2017).

Citrus production in Venezuela had remained constant for decades, with a harvested area of 30,000-45,000 ha for annual production up to 500,000 tons per year (Fermín *et al.* 2009). Citrus production provides a source of income for farmers, as well as a ready source of vitamins for home-consumption.

The main producing areas comprise the states of Carabobo, Yaracuy, Aragua, and Miranda in the central region of the country; Monagas and Sucre in the east, and Táchira and Zulia in the west (Moreno *et al.* 2006). In terms of production, the most important citrus varieties are oranges (60.05 %), mainly destined for the internal market, with an increasing demand for industrial processing (Aular and Casares 2011). However, production has been on the decline from 2016 to the present, fallen by more than 90 % (FEDEAGRO 2020).

Several biotic and abiotic factors have been responsible for the decreasing yields with pests and diseases among the most important (reviewed in Morales *et al.* 2020), raising concerns of the negative impact of the decline on food security and the local economy. It is no coincidence that the major decline since 2016 corresponds with the first reports of early HLB-like symptoms such as leaf yellowing and yellow shoots in trees, alerted by citrus producer organizations and issued by researchers working at Instituto Nacional de Investigaciones Agrícolas, Centro Nacional de Investigaciones Agropecuarias (INIA-CENIAP) to the respective phytosanitary authorities (Morales and Schmidt 2016). It is worth mentioning that researchers from INIA-CENIAP also reported the presence of the Asian citrus psyllid *D. citri* in 1999 on citrus farms in the northwestern state of Falcón (Cermelli *et al.* 2000). It was later determined that the insect was widespread in the country with high infestation rates (Velázquez 2007). This threat was further worsened due to the closeness of countries where the insect vector and the HLB disease have been detected (Colombia and Brazil), putting Venezuela at high phytosanitary risk.

Because of recurrent complaints from producers facing heavy citrus crop losses in recent years, the Universidad Central de Venezuela (UCV), the Instituto Venezolano de Investigaciones Científicas (IVIC), and the Instituto Nacional de Salud Agrícola Integral (INSAI), started monitoring, collecting, and analyzing samples from commercial citrus fields, urban areas and nurseries from central Venezuela states during 2017. The presence of *C*Las in HLB-symptomatic citrus samples was then confirmed by conventional PCR and DNA sequencing (Marys *et al.* 2020).

Accurate diagnosis and distribution analysis are required to develop effective disease management strategies for HLB-infected plants. For all the reasons mentioned above, the present study aimed to assess the presence and geographic distribution of *C*Las associated with HLB in some of the main citrus producing regions in central Venezuela, as well as to detect the bacteria in citrus relatives that could act as host plants reservoir through the evaluation of leaf tissue using conventional PCR.

MATERIALS AND METHODS

Branches from trees with apparent HLB symptoms were collected from 66 orange plants (*Citrus sinensis* L.) Osbeck and 47 samples from Volkamer lemon (*Citrus volkameriana* Pasq.). Samples from *Murraya paniculata* (L.) Jack, *Swinglea glutinosa* (Blanco) Merr. and *Citrofortunella microcarpa* (Bunge) Wijnands (locally known as "jazmín de azahar"; "limoncillo" and "calamansi or calamondín", respectively) growing close to citrus orchards were also sampled in 17 municipalities in the states of Aragua, Carabobo, Yaracuy and Portuguesa (Figure 1). Symptoms included yellow shoots (Figure 2a), leaves with blotchy mottle, some of which had corky veins (Figure 2b), and lopsided, poorly colored fruits (Figure 2c). Some plants showed severe health decline (Figure 2d). Some fruits from suspected trees were also collected.

After collection, samples were labeled, saved in plastic bags, and stored at -20 °C. Total DNA was extracted using the AccuPrep GMO DNA Extraction Kit (Bioneer, Inc) according to the manufacturer's instructions. The leaf samples and fruit cortex were first rinsed with tap water and then sterilized using 70 % ethanol. Midribs were excised aseptically using scissors into smaller pieces and 200 mg of each sample was ground in extraction buffer using a pre-cooled mortar and pestle. Single, conventional PCR amplification of ribosomal proteins of *C*Las (703 bp amplicon) was carried out with primers A2/J5 targeting the β-operon, according to the protocol and PCR conditions designed by Hocquellet *et al.* (1999).

The PCR reaction mixture (40 µl) contained: 1 µM of each of the primers, 200 µM of each of the four dNTP, 2 mM MgCl2, 20 mM Tris–HCl pH 8.4, 50 mM KCl, 1.5 U

Figure 1. HLB-positive states in central Venezuela (2017). Credits: E. Marys

Figure 2. HLB-like symptoms observed in citrus plants during sampling: **A**. yellow shots; **B**. blotchy mottle leaves; **C.** lopsided, poorly colored fruits; **D**. severe health decline of trees. Credits: E. Marys

of Taq polymerase (Thermo Fisher Scientific, Inc.), and 1 µl of DNA preparation. A master cycle thermocycler (Eppendorf) was used with the following program for DNA amplification with primers A2/J5: initial denaturation at 95 °C for 3 min, 35 cycles each at 92 °C for 20 s, 62 °C for 20 s and 72 °C for 45 s. Following amplification, 10 µl aliquots of each PCR product were analyzed by agarose-gel electrophoresis at 1.5 % in 1X TBE buffer (Tris base, boric acid, and 0.5 M EDTA [pH 8.0]). A 100 bp DNA ladder set (Invitrogen, Carlsbad, CA, USA) was included to determine fragment size.

Twenty-five PCR-positive products from different geographical sites and hosts were purified using the QIAquick PCR Purification Kit (Qiagen) according to manufacturer's instructions and directly submitted for sequencing to Macrogen (South Korea). Sequences were compared with others *Ca*. Liberibacter spp. sequences available in the GenBank database using Basic Local Alignment Search Tool (BLAST) algorithm to identify closely related sequences (NCBI-BLAST 2020). The nucleotide sequences of two amplicons were deposited in GenBank under accession numbers: MG418842.1 and MG418841.1 (Marys *et al.* 2020).

RESULTS AND DISCUSSION

In Venezuela, the presence of *C*Las was previous detected by molecular means (Marys *et al.* 2020). However, our present report depicts the more extensive study performed in the country to survey HLB distribution in different geographical locations in the main central citrus growing regions (Figure 1). HLB-like symptoms such as leaf mottling, yellowing of leaf, yellow shoots, Zn-deficiency-like symptoms, vein clearing, twig dieback, and lopsided fruits, which resembled symptoms reported in other parts of the world (Bové 2006, Li *et al.* 2020), were encountered in all regions surveyed (Figure 2). Survey results showed a wide distribution of *C*Las in the main central citrus production region of the country, *C*Las being diagnosed in all 17 municipalities located in the four surveyed states (Table 1).

A proper understanding of HLB symptoms is necessary for the preliminary steps for disease survey and investigation. To manage the disease transmission, routine monitoring of HLB-like symptoms is needed. However, there is a lack of precision in detecting HLB based on visual assessment due to the similarity of symptoms to nutrient deficiencies. Also, there might be confusion between other plant disorder symptoms and HLB infection, leading to misidentification. In this work, some of the symptomatic leaf samples did not give positive PCR results (Figure 3), which could be due either to the uneven distribution pattern of the HLB bacterium in the sampled trees or to the fact that sampled trees were affected by nutritional deficiencies or by other pests producing symptoms that can be confused with those of HLB. Among them, "citrus variegated chlorosis" caused by *Xylella fastidiosa* and "citrus stubborn disease" caused by *Spiroplasma citri* have been described in the country (Hernández *et al.* 1993, EFSA-HPL 2014), and could account for the 20 % symptomatic HLB-negative samples found in this study. Those situations clearly show that PCR detection of "*Ca*. Liberibacter" is the prime choice for HLB diagnosis.

In this study, the identification of *C*Las was based on the amplification of part of the ribosomal protein gene of the rplA-rplJ operon (β-operon) using primers A2/J5 by PCR, sequencing and analysis and comparison of sequences of other "*Ca*. Liberibacter" deposited in the GenBank. A total of 87 out of 113 citrus samples (76.9 %) tested positive to *C*Las (Table 1), with the conventional PCR results showing amplification of the 730 bp amplicon obtained from symptomatic plants (Figure 3). Sequence comparison indicated that amplicons MG418842.1 and MG418841.1 shared greather than 99 % identity with 16S rDNA sequences of *C*Las in the GeneBank.

High HLB-incidence percentages were found in Aragua (87.5 %), Carabobo (65 %), Portuguesa (100 %) and Yaracuy (77.5 %). The results were somehow expected because these estates are contiguous and because of the high HLB-vector (*D. citri*) populations recorded during surveys (data not shown).

The detection of HLB-infected citrus trees in the western state of Portuguesa suggests a widespread distribution of the disease, and these alarming

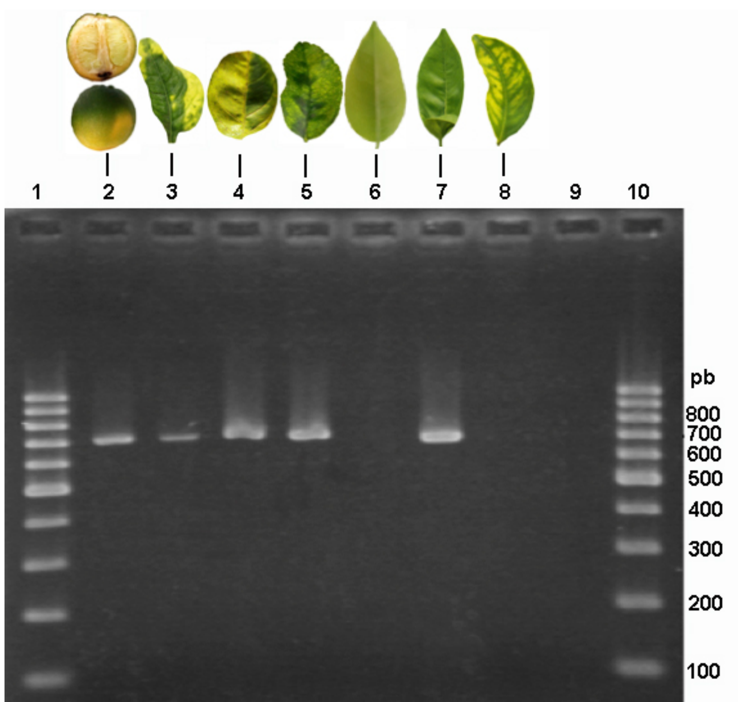

Figure 3. Symptoms and electrophoresis on 1.5 % agarose gel of amplicons with primers A2/J5 from symptomatic lemon cortex (lane 2), orange leaves (lanes 3-5), asymptomatic Rutaceae leaf (lane 6), *Swinglea glutinosa* leaf (lane 7), and lemon leaf (lane 8). Lane 9: negative control. Lanes 1 and 10: 100 bp ladder (Invitrogen, Carlsbad, CA, USA).

Table 1.- Relative incidence of HLB-associated bacteria (CLas) in citrus plants showing HLB-like symptoms in four Venezuelan states using polymerase chain reaction (PCR).

State	Municipality	Samples (n)	PCR results	
			*C*Las (+)	*C*las (-)
Aragua	José Félix Rivas	9	6	9
	Mariño	23	22	1
Carabobo	Montalbán	22	11	11
	Bejuma	7	4	3
	Miranda	5	5	0
Portuguesa	Carlos Arvelo	6	6	0
	Araure	5	5	0
	Veroes	4	1	3
Yaracuy	Manuel Monge	3	3	0
	Bruzual	1	1	0
	Cocorote	4	4	0
	Trinidad	6	6	0
	Arístides Bastidas	1	1	0
	Bolívar	5	3	2
	Nirgua	8	7	1
	San Felipe	4	2	2
Total		113	87	26
Percent			76.9	33

(+) Positive amplification; (-) No amplification; *C*Las: '*Ca*. Liberibacter asiaticus'

results reinforce the need to develop innovative management strategies. Historically, citrus cultivation in Venezuela has been characterized by the lack of rational plans to improve or develop cultivars adapted to our conditions, inappropriate cultural practices, mismanagement of pest and pathogens, and the absence of strategies for sustaining crop germplasms (Fermín *et al.* 2009, Morales *et al.* 2020).

The age of the citrus trees infected with *C*Las varied between 4 and 40 years, according to individual producers (data not collected). Positive samples were detected in commercial fields, nurseries, and backyards. It is difficult to determine the precise date of the introduction of HLB in Venezuela, taking into account that characteristics HLB symptoms can take six months to several years to develop in infected plants (Coletta-Filho *et al.* 2014).

There are some limitations in this study that could be further improved: 1) surveys should be conducted at frequent intervals and should be accompanied by vector populations dynamics analysis; 2) the number of tree samples to be collected and tested by PCR should be more representative for each state, and 3) among the citrus species cultivated in Venezuela (Fermín *et al.* 2009), only a few species are included in this studio, so further inspection is required for the presence of HLB in other species.

Data from our survey showed that *C*Las was also detected in *C. microcarpa*, *M. paniculata* (used as an ornamental plant) and *S. glutinosa* (used as live barriers) which are also hosts of *D. citri* in all regions surveyed (Table 2). Recently, it was shown that these plants are short-term transient hosts of the pathogen (Cifuentes-Arenas *et al.* 2019). However, under a combination of highly favorable conditions to *C*Las infection and transmission, and an absence of effective quarantine procedures, these plants could eventually serve as carriers of *C*Las to regions free from HLB. Plants from the main nursery stocks in the country located in Turmero, Santiago Mariño municipality, Aragua state, were also positive for HLB infection, thus representing a primary means for the long-distance spread of *C*Las to citrus production zones in western Venezuela.

Finally, there is strong evidence for a significant negative correlation between the vector population with elevation, suggesting that lower vector abundance at higher elevations results in lower HLB incidence (Jenkins *et al*. 2015, Devi *et al*. 2020). Hence, the HLB distribution in different elevations across the country must be promptly investigated, as high-altitude locations could be used for the establishment of insect-proof nurseries devoted to the production of HLB-free clean nursery stock. Detailed assessments of the incidence and distribution patterns of HLB are important for management decisions and control strategies for reducing pathogen transmission, based on a comprehensive inoculum removal and the adoption of better cultural practices that would control HLB incidence to low levels (Bassanezi *et al*. 2020, Yuan *et al*. 2020), as a short-term solution to keep the citrus industry alive in the country.

Table 2.- Detection of CLas by PCR in different plant species grown in various states in Venezuela.

States	Plant species	Samples (n)	PCR result	
			*C*Las (+)	*C*Las (-)
Aragua	*Citrofortunella microcarpa*	7	4	3
	Murraya paniculata	11	6	5
	Swinglea glutinosa	19	17	2
Carabobo	*Citrofortunella microcarpa*	10	6	4
	Murraya paniculata	8	3	5
	Swinglea glutinosa	12	7	5
Yaracuy	*Citrofortunella microcarpa*	13	4	9
	Murraya paniculata	7	4	3
	Swinglea glutinosa	9	5	4
Total		96	56	40

(+) Positive amplification; (-) No amplification; *C*Las: '*Ca*. Liberibacter asiaticus'

CONCLUSIONS

This study is the first to provide evidence for the partial distribution of HLB and associated *C*Las in Venezuela. Future work should determine the extent and occurrence of the disease, which could have reached epidemic levels by now. HLB surveys should be conducted in regions at high elevations, to investigate the incidence of the disease and the vector population dynamics. If low HLB incidence is found at the high mountain range (Andean), the region could provide an ideal environment for the production of clean planting material to rejuvenate citrus production in central and eastern Venezuela at lower altitudes.

Considering the nationwide spread of the Asian citrus psyllid *D. citri* and the high rates of HLB infections found in citrus orchards in central regions, future strategies should include the removal of HLB-symptomatic trees, psyllid control, and replacement with HLB-free trees, as part of a scheme to control HLB in Venezuela. Region-wide implementation of such schemes, based on a comprehensive inoculum removal, and the adoption of better cultural practices would control HLB incidence to low levels and ensure the maintenance of citrus production in the country.

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