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TRANSMISIÓN EXPERIMENTAL DE '*CANDIDATUS* LIBERIBACTER ASIATICUS' A PLÁNTULAS DE NARANJO AGRIO POR *DIAPHORINA CITRI* KUWAYAMA EN CUBA

Experimental transmission of '*Candidatus* Liberibacter asiaticus' to sour orange seedlings by *Diaphorina citri* Kuwayama in Cuba

Camilo Paredes Tomás[1*], Maritza Luis Pantoja[1], Jorge Luis Rodríguez-Tapia[1], Assunta Bertaccini[2]

[1]*Instituto de Investigaciones en Fruticultura Tropical (IIFT), Miramar, La Habana, Cuba.*
[2]*Department of Agriculture and Food Sciences, Alma Mater Studiorum - University of Bologna (UNIBO), Bologna, Italy.*

ABSTRACT: "Huanglongbing" (HLB) or citrus greening, associated with three '*Candidatus* Liberibacter' species, is the most serious disease threating the citrus industry worldwide. Up to date, '*Ca*. L. asiaticus' is the only species reported in Cuba associated with HLB and its insect vector *Diaphorina citri* Kuwayama. The ability of *D. citri* to transmit the bacterium under the Cuban environmental conditions has been assumed for years, but never experimentally proven. In two trials of HLB transmission to healthy citrus plants using *D. citri,* 33 out from the 45 inoculated plants showed symptoms of the disease 6 months after inoculation, and 20 out from of the 23 insect pools used tested PCR positive for '*Ca*. L. asiaticus'. The sequences of four amplicons from the 16S gene were obtained. They showed 100% identity among them and with a number of sequences of '*Ca*. L. asiaticus' strains; therefore, one sequence per host species was submitted to GenBank. A comprehensive survey on comparable partial sequences deposited in GenBank allowed detecting nine 16Sr SNP lineages determined by the presence of 15 SNPs in at least two sequences when compared with '*Ca*. L. asiaticus' strain Las_36 (GenBank accession number MK142763) as reference. The sequences from the transmission trials, together with other 27 sequences, did not show SNPs supporting the presence in Cuba of a bacterial strain with a low genetic variability. This is the first report on experimental transmission of '*Ca*. L. asiaticus' to citrus by *D. citri* in Cuba.

Keywords: "Huanglongbing", bacterial plant disease, insect vector, transmission trials.

RESUMEN: "Huanglongbing" (HLB) o citrus greening es la enfermedad más seria que amenaza la industria citrícola asociada con tres especies de '*Candidatus* Liberibacter' mundialmente. Hasta la fecha, '*Ca*. L. asiaticus' es la única especie informada en Cuba asociada con HLB junto con su insecto vector, *Diaphorina citri* Kuwayama. Sin embargo, no existe evidencia experimental de la habilidad de *D. citri* para transmitir la bacteria en las nuestras condiciones, lo cual se ha asumido por años. En dos ensayos de transmisión a plantas citricas sanas empleando *D. citri*, 33 de las 45 plantas inoculadas mostraron síntomas de HLB despues de 6 meses de la inoculación; así como, 20 de los 23 grupos de insectos utilizados resultaron PCR positivos para la presencia de '*Ca*. L. asiaticus'. Las secuencias de los cuatro amplicones obtenidos del 16S mostraron 100 % de similitud entre sí y con un número de secuencias de referencia de cepas de '*Ca*. L. asiaticus', por lo que una sola secuencia por especie hospedante se publicó en GenBank. Un chequeo exhaustivo de secuencias parciales comparables del GenBank, permitió detectar nueve linajes SNP en el 16Sr por la presencia de 15 SNPs detectados en al menos dos secuencias al compararse con la cepa Las_36 de '*Ca*. L. asiaticus' (número de accesión de GenBank MK142763) de referencia. Las secuencias de los ensayos de transmisión, junto con otras 27 secuencias, no mostraron SNPs apoyando la presencia en Cuba de una cepa bacteriana con baja variabilidad genética. Este constituye el primer informe de la transmisión experimental de '*Ca*. L. asiaticus' por *D. citri* a cítricos en Cuba.

Palabras clave: "Huanglongbing", enfermedad bacteriana de planta, insecto vector, ensayos de transmisión.

INTRODUCTION

"Huanglongbing" (HLB), also known as citrus greening, is the most serious disease threating the citrus industry worldwide (1). Three phloem-limited bacteria transmitted by sap-sucking insects have been associated with its occurrence and named according to their geographic origin as '*Candidatus* Liberibacter asiaticus' (Asia and North America), '*Ca*. L. africanus' (Africa), and '*Ca*. L. americanus' (Brazil) (2). The most widespread pathogen is '*Ca*. L. asiaticus', endemic in Asia, but detected as epidemic in the other geographic areas. In Cuba, recent studies reaffirmed '*Ca*. L. asiaticus' as the only liberibacter associated with the occurrence of the disease (3, 4). The methods used to transmit these bacteria experimentally include tissue grafting and psyllid inoculation (5, 6, 7). The Asian citrus psyllid *Diaphorina citri* Kuwayama is reported as the main insect vector of '*Ca*. L. asiaticus',

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*Corresponding author: camptomas1@gmail.com

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as the transmission of the bacterium to healthy citrus plants using psyllids demonstrated (8). Low rates of successful inoculation of the bacterium with *D. citri* together with the pathogen acquisition from infected plant and the latency period needed by the pathogen to multiply and circulate within the psyllid, were reported by field observations (9). Additionally, the transmission efficiency was reported highly influenced by the environmental conditions under which the plants were maintained (10, 11, 12). Despite the lower variability of the results obtained by grafting-methods than those obtained by using *D. citri*, the insect transmission is a critical point as it provides the evidence needed to confirm the insect capability as vector (13). *D. citri* was reported in Cuba in 1999, but its ability to transmit HLB, assumed for years, has not yet been confirmed experimentally. The objective of this study was to verify and confirm the '*Ca*. L. asiaticus' transmission to healthy plants of *Citrus aurantium* by *D. citri* under Cuban conditions.

MATERIALS AND METHODS

Insect and plant material

Specimens of *D. citri* were collected from citrus orchards with high incidence and severity of HLB in Jaguey Grande, Matanzas province. The insects were caught on symptomatic plants using a manual trap consisting of a 15 mL Falcon tube coupled to another 50 mL tube (14). Two transmission trials were conducted (Fig. 1A, B and D). In the first transmission test, three seedlings of sour orange (*Citrus aurantium*) were used, and each plant was individually caged with 10-15 insects. In the second transmission test, 42 seedlings of *C. aurantium* were obtained from seeds germinated under insect-proof greenhouse and caged together with 200 insects. Before and after '*Ca*. L. asiaticus' inoculation in the seedlings, its presence was verified by nested PCR in both experiments. The insects were left on the plants until they died, at which point they were recovered and preserved in 95% ethanol for subsequent molecular analysis. All the plants were maintained under insect-proof greenhouse conditions and weekly inspected for symptom appearance for ten months.

DNA extraction and nested PCR

From each citrus plant used for transmission trials, 0.4-0.6 g of midribs and petioles were taken for DNA extraction. Pools of 10 insects were used (three pools in the first assay and 20 pools in the second assay). In the second trial, the pools were randomly selected at the time of molecular analysis. In both cases (plants and insects), the total DNA was extracted following the CTAB method described by Murray *et al*. (15). The DNA pellets were suspended in 100 µl of sterile distilled water. Amplification of ribosomal DNA was performed with the universal primer pair fD1/rP1 (16) followed by nested PCR with the internal primers OI1/OI2c (17), which targets the 16S rRNA gene from '*Ca*. Liberibacter'. DNA extracts from healthy plants maintained under greenhouse conditions and from a field collected plant tested positive for '*Ca*. L. asiaticus' (4) were used as negative and positive controls, respectively. The PCR-reaction mixture contained 0.8 µM of each primer, 50 ng of DNA, 12.5 µL of 2X TopTaq Master Mix (Qiagen), and 1.5 U of Taq DNA polymerase (Roche) for a final volume of 25 µL. PCR reactions were carried out in a Biometra Thermal Cycler (Biometra, Germany) using an initial denaturation stage at 94°C (only for the direct reaction) for 3 min; followed by 35 cycles (38 cycles for the nested reaction) of denaturation at 94°C for 1 min (40 seconds for the nested reaction), annealing at 52°C for 1 min (62°C for 40 seconds on the nested reaction) and an extension at 72°C for 1 min; and a final extension at 72°C for 5 min (only for the direct reaction). The amplified DNAs (5 µL) were separated by electrophoresis in 1% agarose gel (1X Tris-acetate/EDTA), stained with ethidium bromide and visualized under ultraviolet light in a transilluminator.

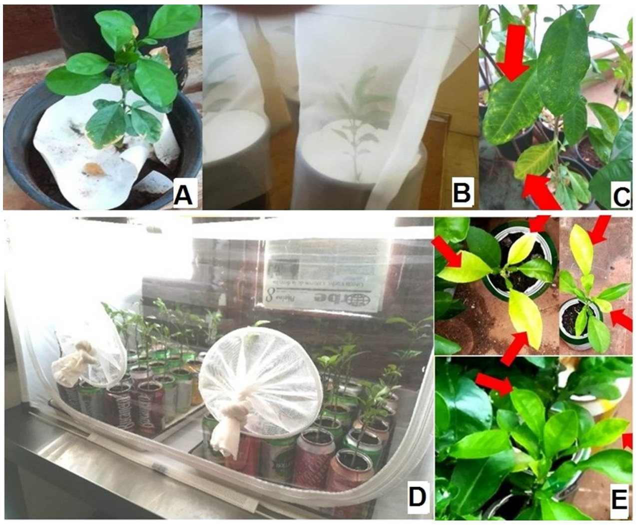

Fig. 1. *Citrus aurantium* seedlings used in transmission trials with *D. citri*: A) before insect inoculation, B and D) during incubation, C, and E) with symptoms six months after inoculation / Plántulas de *Citrus aurantium* usadas en los ensayos de transmisión con *Diaphorina citri*: A) previo a la inoculación con insectos, B y D) durante la incubación, C y E) seis meses posteriores a la inoculación, con síntomas.

Sequences and analysis

Four amplicons (two from plants and two from insects in each experiment) were purified with "High pure PCR product purification Kit" (Roche), according to the manufacturer's instructions and sequenced in both directions by the Sanger Sequencing Service (Microsynth Seqlab, Germany). The phylogenetic analysis was performed on the16S rRNA gene partial sequences of '*Ca*. L. asiaticus' from this work and of those from 54 of the 175 strains previously used (18) selected from the GenBank database for their geographical representativeness. After multiple alignment of the sequences with ClustalW, a common segment of 852 bp was trimmed and used in the subsequent analyses. The 16S ribosomal gene sequence of *Escherichia coli* (GenBank accession number V00348) was used as an outgroup to root the phylogenetic trees produced. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (19). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Bootstrap with 1,000 repetitions was used to estimate the stability of clades. Evolutionary analyses were conducted in MEGA7 (20).

To assess the presence of Single Nucleotide Polymorphisms (SNPs), 56 16S ribosomal gene sequences (including those from this study plus 54 sequences from GenBank) were aligned and trimmed to an 850 bp common fragment. Only the SNPs common to more than one strain were considered in the analysis, in order to identify the SNP-based lineages.

RESULTS AND DISCUSSION

In both transmission trials, almost all the plants showed symptoms 6 months after inoculation. The symptoms included asymmetric blotching mottle and intense chlorosis of the leaves (Fig. 1C and E). Symptomatic plants as well as most of the pools of insect tested positive for '*Ca*. L. asiaticus', only a few plants and some of the pools of insects (from the second trial) tested negative (Table 1).

Table 1. Transmission trials of '*Ca.* L. asiaticus' to *Citrus aurantium* seedlings by pools of *D. citri* (each pool consisted of 10 insects)/ Ensayos de transmisión de '*Ca*. L. asiaticus' a plántulas de *Citrus aurantium* mediante grupos de *D. citri* (cada grupo consistió en 10 insectos).

	'*Ca*. L. asiaticus' PCR detection	
	Number of positive plants/Total	**Positive pools of insects /Total**
Trial 1	1/1	1/1
	1/1	1/1
	1/1	1/1
Trial 2	30/42	17/20

As the partial sequences obtained from plants were identical to each other and nucleotide identity was observed between both pools of insects, one sequence per host was deposited in GenBank (accession numbers OQ892131 and OQ892130, respectively). These sequences shared 100% identity to each other in the 1,090 bp fragment overlapping and with the sequence of the strain Las_36 (GenBank accession number MK142763). In the phylogenetic tree, they clustered with strains from several regions in the American and Asian continents, including the Cuban strains previously reported (Fig. 2).

Following multiple alignment of 56 16S ribosomal gene sequences (including those from this study plus 54 sequences from GenBank), an 850 bp common fragment was used to assess SNPs numbers and their distribution across the selected accessions. The comparison of this portion of the 16S rRNA gene sequences of '*Ca*. L. asiaticus' strains showed that 29 accessions were identical (including all Cuban sequences and others from American and Asian areas). A total of 50 SNPs was observed in the remaining 27 sequences, mostly occurring in a single accession (35 SNPs, 70%). However, 15 SNPs (30%) were observed common to more than one strain, suggesting the presence of '*Ca*. L. asiaticus' populations with 16S rRNA gene variability; in particular nine SNP-based lineages were identified (Table 2).

The epidemiology of vector-borne plant diseases directly depends on the insect vector (21), and the precise identification of the insect vector species is a critical step. Despite the accepted hypothesis of the role of *D. citri* in the spread of '*Ca*. L. asiaticus' in the Cuban national context (22), there is not experimental confirmation to support this assumption. The experimental trials carried out with naturally infected *D. citri* adults collected from citrus orchards with high incidence of HLB confirmed the ability of this insect to transmit '*Ca*. L. asiaticus' to citrus seedlings in Cuban environments too. In all cases in which field-collected infected insects were used on experimental plants, they were able to transmit the bacterium, and the plants showed HLB symptoms after six to eight months, a time span that corresponds with the incubation period reported for '*Ca*. L. asiaticus' (23, 24, 25). These results were confirmed by PCR detection and sequence identification of the bacterium. In the second trial, despite the collective manner of being inoculated, almost all the plants showed symptoms. The positive tests for most of the insect pools are consistent with the infectivity ability reported for *D. citri* (8). The adults used in the transmission study were collected from high-infected orchards; thus, it is very likely that they emerged from nymphs under infected conditions, a fact that could explain the high transmission ability observed (26).

The four 16S rRNA amplicons obtained from two plants and two pools of insects sharing 100% nucleotide identity each other and clustering together with '*Ca*. L. asiaticus' strains in a phylogenetic subclade, and the identity detected between the Cuban strains and those previously reported (3, 4), suggest the presence of low genetic variability in the Cuban strains. This was further confirmed by the alignment of the 16S ribosomal gene partial sequences of a number of '*Ca*. L. asiaticus' strains, including those obtained in this work. Moreover, the identification of nine 16Sr SNP lineages is consistent with what Adkar-Purushothama *et al*. reported (27). Our analysis suggested the presence of genetic variability in the strains from some areas of China and USA. However, the Cuban sequences (together with other 27 sequences) belonged to a lineage without SNPs, which supports the presence of low genetic variability for bacteria infecting citrus in this country. This could imply the presence of a stable bacterial population and epidemic in severe stable conditions. Despite the presence of molecular variability in the 16S ribosomal gene sequence, this region has a high degree of conserved information confirmed also by other authors (18, 27); therefore, additional molecular markers, such as prophage regions or numbers of simple tandem repeats, should be developed to conduct more focused epidemiological studies (28). This is the first experimental confirmation of the ability of *D. citri* to transmit '*Ca*. L. asiaticus' to sour orange (*Citrus aurantium*) seedlings in Cuba. More epidemiological data should be developed to provide information that allows a better understanding of the HLB pathosystem in this and other neighboring countries.

CONCLUSIONS

The ability of *D. citri* to transmit strains of '*Ca*. L. asiaticus' to sour orange (*Citrus aurantium*) seedlings under our conditions was successfully confirmed for the first time. This is the first report of experimental evidence supporting the role of D. citri as a vector of HLB in Cuba.

Fig. 2. Phylogenetic tree derived from analysis of 16S rRNA gene sequences from 54 strains of '*Ca*. L. asiaticus' from several geographic origins and those identified in this work constructed by the Maximum-likelihood method using MEGA 7. The strain sequences obtained in this study are indicated in red; in bold, the sequences with SNPs to the Cuban and '*Ca*. L. asiaticus' strain Las_36 (GenBank accession number MK142763) sequences as reference (not included on the tree). In green background, the strains composing the nine SNP lineages described in Table 2. Bootstrap values are shown on the branches / Árbol filogenético derivado del análisis de las secuencias del gen 16Sr de 54 cepas de 'Ca. L. asiaticus' de distintos orígenes geográficos y aquellas identificadas en este trabajo, construido por el método de Máxima Verosimilitud usando MEGA 7. Las secuencias de cepas obtenidas en este estudio se indican en rojo, se marcan en negrita las secuencias con SNPs respecto a las cepas cubanas y la de *Ca*. L. asiaticus Las_36 (Número de Accesión de GenBank MK142763) de referencia (no incluida en el árbol). Las cepas con fondo verde son las que conforman los nueve linajes SNP descritos en la Tabla 2. Los valores de bootstrap se muestran en las ramas.

Table 2. '*Ca.* L. asiaticus' single nucleotide polymorphisms (SNPs) in 16S rRNA gene sequences considered in the present work (a common background color means identical SNP lineage; white background means different lineages; in bold, the strains present also in Figure 2 representing the various lineages) / Polimorfismos de un solo nucleótido en las secuencias 16S rRNA de 'Ca. L. asiaticus' consideradas en el presente trabajo (el mismo color de fondo significa mismo linaje SNP, fondo blanco significa linajes diferentes, en negrita las cepas que representan los diferentes linajes presentes también en la Figura 2).

Position of the nucleotide in the trimmed sequence	SNP	GenBank accession number of sequences (Country/State)	Lineage number
40	A/T	**FJ750456; FJ750457** (USA: Florida)	1
163	T/C	DQ432005; DQ432002; **DQ431999** (China: Guangdong)	2
186	A/T	DQ432005; DQ432002; **DQ431999** (China: Guangdong)	2
210	A/G	**DQ432004**; **DQ431997** (China: Guangdong)	3
234	C/T	FJ750456; **FJ750457** (USA: Florida)	1
508	C/T	FJ750456; **FJ750457** (USA: Florida)	1
596	C/T	DQ778016 (China: Jianxi); **FJ263702** (USA: Florida)	4
597	T/G	DQ778016 (China: Jianxi); **FJ263702** (USA: Florida)	4
632	A/G	FJ263701; **FJ263700** (USA: Florida)	5
669	T/A	**DQ432003** (China: Jianxi); **GQ369792** (India: Saktara-Tripura)	6
675	T/C	**DQ303210**; **DQ157275** (China: Guangdong)	7
682	A/C	FJ750459; **FJ750458** (USA: Louisiana)	8
	A/-	FJ263704; **FJ263699** (USA: Florida)	9
705	-/C	**FJ750459;** FJ750458 (USA: Louisiana)	8
809	A/T	DQ432005; DQ432002; **DQ431999** (China: Guangdong)	2

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