SHORT COMMUNICATION

OCCURRENCE OF CITRUS HUANGLONGBING IN CUBA AND ASSOCIATION OF THE DISEASE WITH *CANDIDATUS* LIBERIBACTER ASIATICUS

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SUMMARY

Huanglongbing (HLB), the most devastating citrus disease in the world, has been recently seen in Cuba. Characteristic HLB fruit symptoms, blotchy-mottled leaves and yellow shoots were observed on trees of several citrus varieties and species in urban areas of Havana city, and in citrus groves from the central, western and eastern parts of the country. The presence of only *Ca.* Liberibacter asiaticus in symptomatic citrus plants and in adult individuals of the Asian citrus psyllid, *Diaphorina citri,* was determined by polymerase chain reaction (PCR). Transmission electron microscopy showed liberibacter-like structures to be exclusively located in the sieve tubes. DNA fragments amplified from the *rpl*KAJL-*rpo*BC operon were cloned and sequenced. Sequence analyses revealed 100% identity with the corresponding sequences of ribosomal *rpl* protein genes from *Ca*. L. asiaticus.

Key words. Citrus, greening disease, liberibacter, diagnosis, electron microscopy, PCR.

Huanglongbing (HLB) is currently the most devastating disease of citrus in the world (Bové, 2006, 2008). According to where HLB occurs, three species of Gram-negative, non-cultured, phloem-restricted bacteria belonging to the genus *Candidatus* Liberibacter (α-*Proteobacteriae*) are associated with the disease: *Ca.* L. africanus in Africa, *Ca.* L. asiaticus, in Asia, North (USA) and South America (Brazil) whereas *Ca.* L. americanus, which was first reported from Brazil (Teixeira *et al.*, 2005), has now been detected also in China (Lou *et al.*, 2008). Recently, phytoplasmas of 16Sr group IX and 16Sr group I were found associated with HLB in São Paulo state (Teixeira *et al.*, 2008) and Southern China (Chen *et al.*, 2008), respectively. The liberibacters associated with HLB are vectored by the citrus psyllids *Diaphorina citri* (Capoor *et al.*, 1967; Yamamoto *et al.,* 2006) and *Trioza erytreae* (McClean and Oberholzer, 1965) and can be transmitted by graft-inoculation and dodder (*Cuscuta campestris*) (Bové, 2006, 2008).

The Asian psyllid vector of HLB, *D. citri*, was detected in Cuba in 1999 and has already spread all over the country (González *et al.*, 2007). Since HLB-like symptoms were observed in citrus groves of western, central, and eastern Cuba in 2006, investigations were carried out for establishing the nature of the disease.

A total of 172 samples with characteristic blotchy mottle and mineral deficiency-like symptoms were collected in 2007 and 2008, from trees of 46 citrus groves in five Cuban provinces, as well as in gardens and backyards of Havana city. Each sample came from a single tree. Most of symptomatic samples came from sweet orange (*Citrus sinensis*), but some were collected from affected trees of grapefruit (*C. paradisi*), Persian lime (*C. latifolia*), mandarin (*C. reshni*), *C. macrophylla*, *C. volkameriana*, Rangpur lime (*C. limonia*), Mexican lime (*Citrus aurantifolia*), pummelo (*C. grandis*) and sour orange (*C. aurantium*). Healthy control leaves for PCR reactions were from citrus seedlings obtained *in vitro* and grown in an insect-proof greenhouse.

Thirty one batches of five adult *D. citri* were captured with a mouth aspirator from fully infected trees of sweet orange (20 trees), Mexican lime (1 tree), sour orange (1 tree), grapefruit (5 trees), and Persian lime (2 trees), in Havana city, Havana province, Cienfuegos and Ciego de Avila.

For DNA extraction, 500 mg of leaf midribs were processed by the CTAB (cetyl trimethyl ammonium bromide) method of Murray and Thompson (1980). Psyllid DNA extraction was according to Yamamoto *et al.* (2006).

For transmission electron microscopy (TEM) midribs of symptomatic leaves were collected from trees PCR positive for *Ca.* L. asiaticus. Samples were fixed in 0.1 M phosphate buffer pH 7.2 containing 3% glutaraldehyde, and post-fixed in 1% osmium tetroxide. After de-

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hydration with acetone, they were embedded in SPURR resin (Spurr, 1969), and thin-sectioned with a Leica UC6 microtome. Ultra-thin sections were stained with uranyl acetate and lead citrate, and examined in a Zeiss EM 900 electron microscope.

Single, conventional PCR amplification of 16Sr DNA from putative *Ca.* L. asiaticus or *Ca.* L. africanus was carried out with forward primers OA1+OI1 and reverse primer OI2c (Jagoueix *et al.*, 1996). For duplex PCR, two primer-pairs were used in a single reaction mixture: A2/J5 for amplification of ribosomal proteins of putative *Ca.* L. asiaticus or *Ca.* L. africanus (Hocquellet *et al.*, 1999) and GB1/GB3 for amplification of 16Sr DNA of putative *Ca.* L. americanus (Teixeira *et al.*, 2005). PCR reactions were conducted in 40 µl of a reaction mixture containing 1 µM of each primer, 200 µM of each dNTPs, 2 µM $MgCl_2$, 20 µM Tris-HCl pH 8.4, 50 µM KCl, 1.5 U of Taq DNA polymerase (Invitrogen, USA) and 1 µl of DNA. The duplex PCR program consisted of 35 cycles, each at 94ºC for 30 sec, 62ºC for 30 sec and 72°C for 1 min (Teixeira *et al.,* 2008). PCR reactions were performed in a Biometra Termocycler. Following amplification, 10 µl aliquots from each reaction mixture were analyzed by electrophoresis on 1.2% agarose gels and visualized under UV light. DNAs from leaves infected with *Ca*. L. asiaticus, *Ca.* L. africanus or *Ca.* L. americanus, used as positive controls, were supplied by Fundecitrus, Brazil.

Ten µl of the 1160 bp PCR product obtained by amplification with primers (OI1+OA1)/OI2c were incubated at 37ºC overnight with 20 U of restriction enzyme *Xba* I (Promega, USA) in a final volume of 35 µl (Jagoueix *et al*., 1996). The digested DNA was further analyzed by electrophoresis in a 4% agarose gel.

Fragments amplified with primers A2/J5 from symptomatic leaf samples were cloned using the pGEM-T easy vector following the supplier's protocol (Promega, USA). Two microliters of the ligation mixture were used to transform *E. coli* DH5-α competent cells by electroporation (Dower *et al.*, 1988). The cloned DNA was sequenced at the Escola Superior de Agricultura Luiz de Queiroz, Departamento de Ciências Biológicas, Universidade São Paulo, Brazil.

DNA sequence analyses were performed using Chromas LITE version 2.01 (www.technelysium.com.au). Search for homologies in databases were carried out using the BLAST program (Altschul *et al.*, 1997). Multiple sequence alignments were performed using MULTALIN software (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html).

Characteristic leaf HLB symptoms including (i) yellow shoots, (ii) leaves with blotchy-mottle, some of which had corky veins, and (iii) small leaves showing Zn- and Mn-deficiency symptoms were observed on both young and adult trees from 46 citrus groves in the provinces of Matanzas and Havana (western region), Cienfuegos and Ciego de Ávila (central region), and Holguín (eastern region). Fruits had also characteristic symptoms of HLB. Typical blotchy mottle was observed in citrus orchards of sweet orange (cv. Valencia), mandarin (cv. Dancy), and grapefruit (cvs Marsh and Ruby). Tahiti lime leaves did not show the typical sweet orange blotchy mottle, but rather the lemon (*C. limon*) type of mottle, characterized by large, dark-green patches (Bové, 2008). While many trees of sweet orange and grapefruit were fully affected, other trees in the same orchards had still symptomless sectors, a characteristic feature of HLB. Young commercial trees, from two to seven years of age, had a much higher disease incidence than adult trees in neighboring blocks. Severely affected trees showed dieback and defoliation. In the western and the central regions most young orchards surveyed were severely affected.

Of the 172 leaf samples tested by duplex PCR with

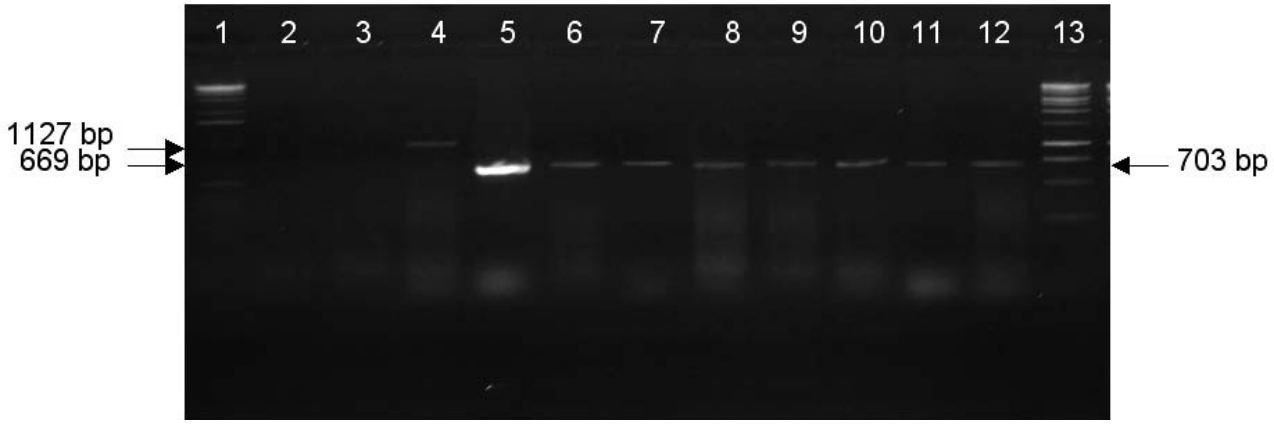

Fig. 1. Electrophoresis on 1.2 % agarose gel of duplex PCR products amplified from DNA of healthy or symptomatic leaves primed with A2/J5+GB1/GB3. Lane 1 and 13, 1Kb DNA ladder (Promega, USA); lane 2, water; lane 3, healthy plant DNA; lane 4, DNA from *Ca.* L. americanus-infected leaves; lane 5, *Ca.* L. asiaticus-infected leaves; lane 6, *Ca.* L. asiaticus-infected leaves. Symptomatic citrus samples from Havana city in lanes 7 to 12.

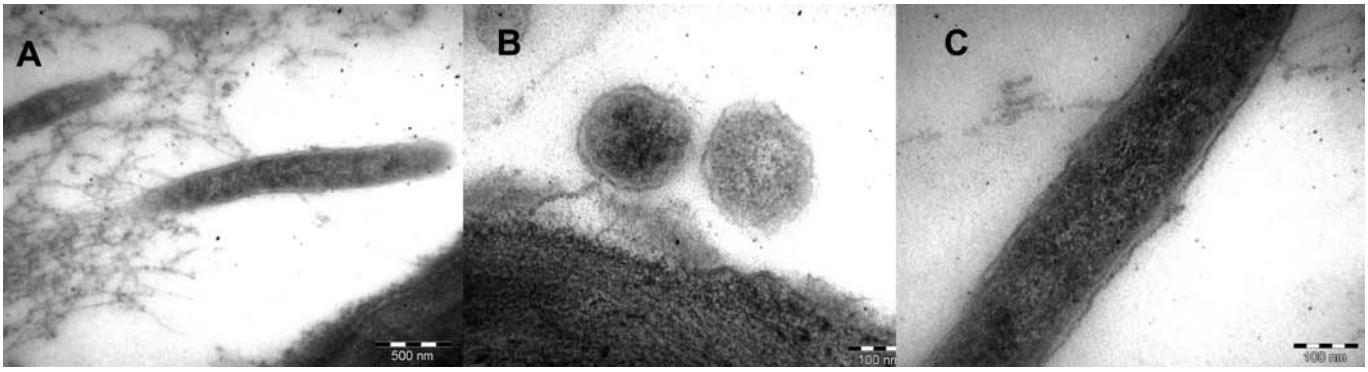

Fig. 2. Electron micrograph of ultrathin sections through sieve tubes of leaf midribs from *C. grandis* (a, b and c). Filamentous (a and c) and circular (b) liberibacter-like structures. The liberibacter wall is seen in (c) as an outer, electron-dense layer.

primers A2/J5, 168 yielded amplicons of 703 bp, characteristic of *Ca*. L. asiaticus, of which 61 were sweet orange, 37 Persian lime, 33 grapefruit, 15 Mexican lime, 8 mandarin (cvs Cleopatra and Dancy), 8 sour orange, 3 *C. volkameriana,* 1 Rangpur lime, 1 Carrizo citrange, 3 *C. macrophylla* and 2 *C. grandis* (Fig. 1). Amplicons obtained with conventional PCRs using primers (OI1+OA1)/OI2c which had a size of 1160 bp, characteristic of either *Ca*. L. africanus or *Ca*. L. asiaticus were subsequently digested with restriction enzyme *Xba* I to determine which species was present in the samples. The digestion yielded two bands, 520 bp and 640 bp in size, a pattern typical of *Ca*. L. asiaticus.

PCR products obtained from amplifications with primers A2/J5 were used for cloning and sequencing. Three clones from Havana city, three from Jagüey Grande (Matanzas), and one from Los Quemados (Ciego de Ávila) were obtained. All sequences shared 100% identity. The unique sequence (accession No. FJ394022) was 703 bp long and shared 100% identity with the corresponding sequence of *Ca.* L. asiaticus from Brazil (accession No. DQ4719041).

Liberibacter-like structures (Fig. 2a and b), surrounded by an electron-dense, outer wall-like layer (Fig. 2c), were observed only in the sieve tubes of samples PCR-positive for *Ca*. L. asiaticus,

During surveys in citrus orchards from Matanzas, Cienfuegos, Ciego de Avila and Havana provinces, *D. citri* were collected from 31 trees that showed characteristic HLB symptoms and were PCR-positive for *Ca.* L. asiaticus. Twenty eight of 29 psyllid samples collected in citrus orchards as well as two samples collected from citrus trees in residential Havana city gave positive PCR reactions for *Ca.* L. asiaticus with primer pairs A2/J5 and GB1/GB3 for duplex PCR. Amplification with (OA1+OI1)/OI2c followed by digestion of the 1160 bp amplicon with *Xba* I also detected *Ca*. L. asiaticus. No evidence was obtained for the presence of *Ca.* L. africanus or *Ca.* L. americanus in any of the leaves or insects tested.

Symptoms characteristic of HLB were observed on citrus trees all throughout the country, from west to east, even in remote areas where only a few citrus trees were present. The incidence of the disease was much higher in young orchards of sweet orange and grapefruit than in adult ones, most of which were over 30 years of age and in bad conditions because of poor mangement. This situation as well as the lack of platforms to examine tree tops made HLB diagnosis more difficult in adult trees than in young ones. Nevertheless, the wide distribution of PCR of *Ca.* L. asiaticus was amply confirmed throughout Cuba, whereas no evidence was obtained for the presence of *Ca*. L. africanus or *Ca*. L. americanus.

It is difficult to determine the precise date of introduction of the HLB in the Cuba because of the poor sanitary and nutritional conditions of most of the citrus orchards. However, based on the high incidence and the wide distribution of HLB throughout Cuba as observed in 2007 and 2008, it seem plausible to conclude that probably the disease had been present in the island well before 2006, when it was first seen on backyard trees of Havana city. Should this be so, the situation agrees with what reported from São Paulo state and Florida, where the HLB was first recognized in 2004 and 2005, respectively, but is thought to have been present several years before its identification.

REFERENCES

Altschul S.F., Madden T.L., Schaffer A.A., Zhang J., Miller Z.W., Lipman D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25:** 3389-3402.

Bovè J.M., 2006. Huanglongbing: a destructive, newly emerging, centrury old disease of citrus. *Journal of Plant Pathology* **88:** 7-37.

Bové J.M., 2008. Huanglongbing. IOCV Citrus Diseases, http://www.ivia.es/iocv/

Capoor S.P., Rao D.G., Viwanath S.M., 1967. *Diaphorina citri* Kuwayama, a vector of the Greening disease of citrus in India. *Indian Journal of Agricultural Science* **37:** 572-576.

Chen J., Deng X., Liu S., Pu X., Li H., Civerolo E., 2008. Detection of phytoplasma *Candidatus* Liberibacter asiaticus in citrus showing Huanglongbing (yellow shoot disease) symptoms in Guangdong, P.R. China. *Phytopathology* **98:** S35.

Dower W.J., Miller J.F., Ragsdale C.W., 1988. High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Research* **16:** 6127-6145.

González C., Gómez M., Fernández M.D., Hernández, Tapia J-L., Batista L. 2007. *Diaphorina citri* Kuw. (Hemipthera: Psillidae) Behavior and natural enemies in Cuban citriculture. *Programs and Abstracts of the 17th Congress of IOCV, Adana*: 180.

Hocquellet A., Toorawa P., Bové J.M., Garnier M., 1999. Detection and identification of the two ´*Candidatus* Liberibacter´ species associated with the citrus huanglongbing by PCR amplification of ribosomal protein genes of the β-operon. *Molecular and Cellular Probes* **13:** 373-379.

Jagoueix S., Bové J.M., Garnier M., 1996. PCR detection of the two *Candidatus* Liberobacter species associated with greening disease of citrus. *Molecular and Cellular Probes* **10:** 43-50.

Lou B.H., Zhou C.Y., Zhao C.Y., Li Z.G., Xu M., Liu J.X., Zhou Y., Tang K.Z., 2008. Primary study on species and intraspecific differentiations of HLB pathogens in Eight provinces of China. *Proceedings of 11th International Citrus Congress. Wuhan, China*: 232.

McClean A.P.D., Oberholzer P.C.J., 1965. Citrus psylla, a vector of the greening disease of sweet orange. *South Africa Journal of Agricultural Science* **8**: 297-298.

Murray M.G., Thompson W.F., 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* **8:** 4321-4325.

Spurr A.R., 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *Journal Ultrastructural Research* **26:** 31-43.

Teixeira D.C., Saillard C., Eveillard S., Danet J.L., Ayres A.J., Bové J.M., 2005. "Candidatus Liberibacter americanus", associated with citrus huanglongbing (greening disease) in Sao Paulo state, Brazil. *International Journal of Systematic Evolutionary Microbiology* **55:** 1857-1862.

Teixeira D.C., Wulff N.A., Martins E.C., Kitajima E.W., Bassanezi R., Ayres J., Eveillard S., Saillard C., Bové J.M., 2008. A phytoplasma closely related to the Pigeon pea witches' broom phytoplasma (16Sr IX) is associated with huanglongbing disease of citrus in the State of São Paulo, Brazil. *Phytopathology* **98:** 977-984.

Yamamoto P.T., Teixeira D.C., Martins E.C., Santos M.A., Felippe M.R., Garbim L.F., Carmo A.U., Abrahao D.P., Souza M.C., Bové J.M., 2006. Detection of *Candidatus* Liberibacter americanus and asiaticus in *Diaphorina citri* Kuwayama (Hemiptera: Psillidae). *Proceedings of Huanglongbing-Greening International Workshop, Ribeirão Preto 2006*: 87.

Received February 10, 2009
Accepted March 16, 2009