



**NAPPO**

North American Plant Protection Organization

Organización Norteamericana de Protección a las Plantas

**MEXICO - USA - CANADA**

## **NAPPO DIAGNOSTIC PROTOCOL**

### **DP 02: Citrus Huanglongbing**

Secretariat of the North American Plant Protection Organization  
1431 Merivale Rd., 3rd Floor, Room 140  
Ottawa, Ontario, K1A 0Y9 Canada

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## 1. Pest Information

Citrus huanglongbing (HLB), also known as Citrus Greening (da Graça and Korsten, 2004), is one of the most serious diseases that affect citrus worldwide (Halbert, 1998). It is associated with the bacteria *Candidatus Liberibacter* spp., a fastidious bacterium that resides in infected insects and in the elements of the phloem sieve tubes of infected plants.

It has been difficult to detect the bacteria in a consistent manner by biological assay (Roistacher, 1991), by the presence of fluorescent substances, light or electronic microscopy, or serologically, due to the low concentration and irregular distribution of the pathogen in host plants and insect vectors.

HLB was detected in 2004 in Sao Paulo, Brazil, in the Araraquara region (Coletta et al., 2004). In September of 2005, its presence was confirmed in Florida, in 2006 it was detected in Cuba, and in 2008 in Louisiana, Georgia, and South Carolina (USA)<sup>1</sup>. In 2009, the bacterium was detected in the state of Yucatan, Mexico, representing an imminent risk for Mexican citriculture as the psyllid vector of the american and asian strains (*Diaphorina citri* Kuwayama) is present in all citrus-producing states of Mexico. HLB has since been reported from the States of Quintana Roo, Nayarit, Jalisco, Campeche, Colima, Sinaloa, Michoacán, Chiapas, Hidalgo, and Baja California Sur. In 2012, HLB was detected in Texas<sup>2</sup> and in Los Angeles County, California<sup>3</sup> (USA).

## 2 Taxonomic Information

The approved common name of “Huanglongbing” (HLB), meaning “Yellow shoot disease” in Chinese, was adopted by the International Organization of Citrus Virologists in 1995 at the 12th Congress in Fuzhou, China. Previously, HLB had been referred to as “Greening” in Africa (also translated into various other European languages), “Likubin” (=“decline”) in China, “Vein phloem degeneration” in Indonesia, “Leaf mottling” in the Philippines, and “Citrus dieback” in India.

The apparent causal agent consistently associated with the symptoms of HLB is a gram negative, phloem-limited bacterium belonging to the alpha subdivision of the proteobacteria (Jagoueix et al., 1996). Electron microscopy has revealed elongated rod-like bacteria residing in the sieve tubes of infected plants. Since the bacterium has not been isolated and Koch’s postulates have not been fulfilled, the bacterium so far is called “*Candidatus*” *Liberibacter* spp (Li et al., 2009). Because this bacterium has not been cultured, it has not been well characterized on a biological basis either.

In nature, HLB exists in three forms that differ in their pathogenicity due to a combination of environmental conditions and insect vectors (Jagoueix et al., 1996). These are:

- *Candidatus Liberibacter asiaticus* Garnier (Las)

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<sup>1</sup> <http://www.pestalaert.org>

<sup>2</sup> <http://www.texasagriculture.gov/NewsEvents/NewsEventsDetails/tabid/76/Article/1802/texas-department-of-agriculture-and-usda-confirm-detection-of-plant-disease-tha.aspx>

<sup>3</sup> [http://www.cdfa.ca.gov/egov/Press\\_Releases/Press\\_Release.asp?PRnum=12-012](http://www.cdfa.ca.gov/egov/Press_Releases/Press_Release.asp?PRnum=12-012)

- *Candidatus Liberibacter africanus* Garnier (Laf)
- *Candidatus Liberibacter americanus* sp nov (Texeira et al., 2005) (Lam)

The African strain of HLB is heat sensitive and does not cause symptoms at temperatures greater than 25 – 30 °C. The Asian strain is primarily distributed in Asia but was recently reported from the Western Hemisphere. It is heat tolerant and able to cause symptoms at temperatures greater than 30 °C (Bové and Garnier, 2002; Bové, 2006). The American strain, which was reported from Brazil, appears to have heat tolerance similar to that of the African strain (Texeira et al., 2005; Bové, 2006).

### 3. Detection

HLB is a disease that affects the entire plant. Symptom expression is delayed at least six months after the plant becomes infected. The initial symptom is yellowing of the leaves in contrast with the green of the plant. This is most evident during fall and winter, when intense yellowing and mottling is observed (Bové, 2006).

**In leaves**, a pale yellow colouration with irregular (asymmetrical) areas of green colour (mottling) is observed (Figure 1), with defoliation, enlargement, and clearing of the veins which after a time retain a corky aspect. Diffusion of colors in the veins and blades is observed, which can be confused with mineral deficiencies (zinc and copper) (Colletta-Filho et al., 2004).

**In branches**, when the disease has evolved, there is an intense defoliation. The symptoms can appear in the entire crown and the trees can dry out and die. **In fruit**, deformation and asymmetry are observed, along with reduced size, and the appearance of clear green circular spots that contrast with the normal green of the fruit. Internally, there are differences in maturation and seed abortion (Figure 2), misalignment of the axis and in some cases, the white portion of the skin (albedo) shows a larger than normal thickness (Bové, 2006).



Figure 1. Asymmetrical mottling, typical symptoms of HLB. Photo: Iobana Alanís



Figure 2. Deformation of fruit and seed abortion. Photo: Pedro Robles

## 4 Identification

### 4.1 Molecular diagnostics

For the detection of HLB, two techniques have been used: conventional Polymerase Chain Reaction (PCR) and real time PCR (PCR-RT, also known as quantitative PCR = qPCR). Conventional PCR methods use specific primers that amplify the sequences of the rDNA 16s genes and primers based on the proteinaceous genes (operon-B) (Jagoueix et al., 1996; Tian et al., 1996; Hocquellet et al., 1999, Teixeira et al., 2005). The low concentration and irregular distribution of the pathogen in host plants, along with the inhibitors of PCR present in citrus extracts, have made detection of the pathogen difficult. Although conventional PCR and qPCR are accepted techniques for the confirmation of trees symptomatic for HLB in Brazil and the United States, qPCR is much more sensitive and robust than conventional PCR and the technique has been validated with DNA extracts from different species of citrus and different tissues from diverse geographic regions. The currently accepted qPCR technique was developed by Li and associates (Li et al., 2006, 2007).

The NAPPO member countries approve for use the following protocols developed by USDA-APHIS-PPQ-CPHST:

- 1) “Plant sample extraction for use in citrus greening or huanglongbing molecular diagnostic assays”, and
- 2) “Real-time PCR for diagnostic detection of citrus greening or huanglongbing from plant samples”.

These protocols are available in the chapter “DNA extraction and PCR detection in citrus” del documento “New Pest Response Guidelines – Citrus Greening ([http://www.aphis.usda.gov/plant\\_health/plant\\_pest\\_info/citrus\\_greening/downloads/pdf\\_files/cg-nprg.pdf](http://www.aphis.usda.gov/plant_health/plant_pest_info/citrus_greening/downloads/pdf_files/cg-nprg.pdf)). Additional to the protocols mentioned, confirmation of HLB by conventional PCR using the primers OI1-OI2c (Jagoueix et al., 1996), A2-J5 (Hocquellet et al., 1999) for the Asian and African strains and primers GB1-GB3 (Teixeira et al., 2005) for the American strain is recommended.

### 4.2 Biological indexing

Although qPCR is currently the method of choice for detection of HLB *in planta*, biological indexing techniques are also available and will be briefly summarized for completeness. Due to the sometimes low rate of graft transmission of HLB, the success rate for biological indexing or HLB is variable. The appropriate indicator plants are sweet orange or Orlando tangelo for African HLB and sweet orange or Ponkan mandarin for Asian HLB. Presence of the citrus tristeza virus can interfere with HLB symptom expression and if CTV is present, grapefruit may be used as an indicator. The preferred inoculation technique is the side graft, with leaf grafts being the alternative. The seedling indicators are trained to single leaders and held at 20 – 25 °C for African HLB and 25 – 32 °C for Asian HLB. Symptom expression is the typical mottle and chlorosis. The shoots are distinctly smaller,

more chlorotic, and with smaller leaves than the uninoculated controls. Symptoms should appear 8 to 12 weeks after inoculation. For more information on biological indexing in general and for HLB in particular, see Roistacher (1991) or Roistacher (1998).

### 4.3 Sampling of propagative material

Because distribution may be irregular in the host plants and psyllid vector or of a very low incidence and titer, the sampling method is critical for the detection, identification and quantification of *Liberibacter*. In symptomatic trees, samples are taken from 1 – 4 branches with symptomatic leaves or fruit. If symptoms are not present in a suspect tree, samples are taken from one year-old branches with 5 – 10 leaves from the upper portion of each of the four quadrants of the tree. If branches are not present, as in the case of small nursery trees, 1 – 12 mature leaves are taken from each tree (Li et al, 2009b).

The periodic exploration and sampling of the different production units of propagative material are fundamental for timely detection of HLB. The following visual and molecular diagnostic activities are recommended.

Unit	Molecular diagnostic (qPCR)	Visual diagnostic
Germplasm bank	100 % individual annual	100 % every 3 months
Foundation block	100 % individual annual	100 % every 3 months
Increase block	10 % annual, composite sample from 5 plants	100 % every month
Seed producing block	25 % individual annual, rotational to cover 100 % of the block in 4 years	100 % every month
Nursery	2 % of the total of all plants to be moved	100% every month

## 5. Records

A registry of samples analyzed should be maintained, which should contain:

- Code or reference number of the sample.
- Variety and origin of the sample.
- Description of symptoms (including photographs, if pertinent) or absence of these.
- Methods used in the diagnostic and the results obtained.
- Name of the laboratory and when available, name of the persons responsible for the diagnostics.

The registry and the evidence of the results of the diagnostics should be retained for at least four years with the goal of tracing the results in the different production units such as the germplasm bank and the seed producing orchard.

## 6. Points of contact for additional information

Centro Nacional de Referencia Fitosanitaria. Dirección General de Sanidad Vegetal. Guillermo Pérez Valenzuela No. 127 Col. Del Carmen, Coyoacán, Del. Coyoacán, México, DF 04100.

Citrus Clonal Protection Program, Department of Plant Pathology, University of California, Riverside, CA 92521, USA.

USDA-ARS National Clonal Germplasm Repository for Citrus & Dates, 1060 Martin Luther King Blvd., Riverside, CA 92507, USA.

## 7. Acknowledgements

The experts who wrote the first draft of the diagnostic protocol are:

**Wenbin Li and Laurene Levy.** National Plant Germplasm and Biotechnology Laboratory USDA-APHIS-PPQ-CPHST, Beltsville, MD 20705, United States.

**Robert Krueger.** USDA-ARS National Clonal Germplasm Repository for Citrus & Dates, 1060 Martin Luther King Blvd., Riverside, CA 92507, USA.

**Georgios Vidalakis,** Citrus Clonal Protection Program, Department of Plant Pathology, University of California, Riverside, CA 92521, USA.

**Elena Iobana Alanís Martínez,** Estación Nacional de Epidemiología, cuarentena y saneamiento vegetal. SENASICA. Rancho G. B. Km. 21 Carretera Amazcala – Chichimequillas. El Marques, Querétaro.

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