NAPPO
North American Plant Protection Organization
Organización Norteamericana de Protección a las Plantas
MEXICO - USA - CANADA

NAPPO DIAGNOSTIC PROTOCOLS

DP 02
Citrus Huanglongbing

Secretariat of the North American Plant Protection Organization
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Review

NAPPO Diagnostic Protocols are subject to periodic review and amendment. The next review date for this NAPPO Diagnostic Protocol is 2017. A review of any NAPPO Diagnostic Protocol may be initiated at any time upon the request of a NAPPO member country.

Approval

This Diagnostic Protocol was approved by the North American Plant Protection Organization (NAPPO) Executive Committee on October 15, 2012 and is effective immediately.

Signed by:

Greg Stubbings
Executive Committee Member
Canada

Rebecca A. Bech
Executive Committee Member
United States

Javier Trujillo Arriaga
Executive Committee Member
Mexico

Implementation

Not Applicable

Amendment Record

Amendments to this Standard will be dated and filed with the NAPPO Secretariat. The most recent version will be posted on the NAPPO website at: www.nappo.org/stds_e.htm

Distribution

This Standard is distributed to NAPPO member countries including Sustaining Associate Members and Industry Advisory Groups, the Secretariat of the International Plant Protection Convention, and other Regional Plant Protection Organizations (RPPOs).

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 psyllids 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) (NAPPO, n.d.). In 2009, the bacterium was detected in the state of Yucatan, Mexico, representing an imminent risk for Mexican citriculture as the psyllid vector (*Diaphorina citri* Kuwayama of the American and Asian strains) is present in all citrus-producing states of Mexico. HLB has also 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 (Texas Department of Agriculture, 2012) and in Los Angeles County, California (USA) (CDFA, 2012).

2. Taxonomic Information

During the 13th Congress of the International Organization of Citrus Virologists in Fuzhou, China, in 1995, the delegates adopted “Huanglongbing” (HLB) as the official name of the disease in honor of Professor Lin Kongxiang (Kung Hsiang) (da Graça et al, 1996). In Chinese, this means “yellow shoot disease”. It is also known as “Greening” in Africa and the United States (also translated into other European languages), “likubin” in Taiwan, “phloem vein degeneration” in Indonesia, “mottle leaf” in the Philippines, and “citrus dieback” in India (Bové, 2006).

The causal agent apparently associated consistently with the symptoms of HLB is a gram negative, phloem-limited bacterium belonging to the alpha subdivision of the proteobacteria (Jagoueix et al., 1994). 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 asiaticus’, “*Ca*. L. africanus, or *Ca*. L. americanus” in accordance with the International Journal for Systematic & Evolutionary Microbiology (IJSEM, 2012).

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

The African strain of HLB is heat sensitive and does not cause symptoms at temperatures greater than 25 – 30 ºC. The Asian strain is primarily distributed in Asia and was recently introduced into the Western Hemisphere some time in the late 1990's or early 2000's. It is heat tolerant and able to cause symptoms at temperatures greater than 30 ºC (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 (Lopes et al., 2009).

3. Detection

HLB is a disease that affects the entire plant. Symptom expression in general is delayed several months after the plant becomes infected. The initial symptom is yellowing of the leaves on some branches in contrast with the green of the rest 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 enlargement and clearing of the veins, which after a time retain a corky aspect. Different levels of defoliation may occur. 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 areas of clear green colour that contrast with the normal yellow or orange 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 diagnosis of HLB, two techniques have been used: conventional Polymerase Chain Reaction (PCR) and real time PCR (PCR-RT, also known as quantitative PCR = qPCR) that are based on the use of PCR primers that amplify DNA sequences of the Liberibacters associated with HLB. 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” of the document “New Pest Response Guidelines – Citrus Greening (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. Sequencing of the amplified fragments is also recommended.

4.2 Biological indexing

Although qPCR is currently the method of choice for diagnosis of HLB *in planta*, biological indexing techniques are also available and will be briefly summarized. Due to the sometimes low rate of graft transmission of the bacterium associated with 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 ºC for African HLB and 25 – 32 ºC 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).

5. Sampling of propagative material

Because distribution of Liberibacters may be irregular in the host plants and psyllid vector or have 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., 2009). Before and during shipment of the samples to be analyzed, they should be protected from heat to prevent the possible degradation of pathogen DNA.

The periodic exploration and sampling of the different production units of propagative material are fundamental for timely detection of HLB. It is important to indicate that these production units should be protected (under screen) and preferably removed from citrus production areas. 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

6. Records

A registry of samples analyzed for HLB diagnostics 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.

7. 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.

8. Acknowledgements

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 Martinez. Estación Nacional de Epidemiología, Cuarentena y Saneamiento Vegetal. SENASICA. Rancho G. B. Km. 21 Carretera Amazcala – Chichimequillas. El Marques, Querétaro.

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