DOI: 10.1111/epp.12757

DIAGNOSTICS

PM 7/121 (2) '*Candidatus* Liberibacter africanus', '*Candidatus* Liberibacter americanus' and '*Candidatus* Liberibacter asiaticus'

Specific scope: This Standard describes a diagnostic protocol for '*Candidatus* Liberibacter africanus', '*Candidatus* Liberibacter americanus' and '*Candidatus* Liberibacter asiaticus'.[1]

This Standard should be used in conjunction with PM 7/76 (5) *Use of EPPO diagnostic protocols*.

Specific approval and amendment: First approved in 2014–09. Revised in 2021–04.

1 | INTRODUCTION

Huanglongbing (commonly referred to as HLB), also known as citrus greening or yellow-shoot-disease, was first described in Southern China in the 19th century, and has been present for many years in the main citrus growing regions of Asia and Africa and more recently in the Americas, being considered the most destructive bacterial disease of the citrus industry (Bové, 2006). Huanglongbing is present in more than 50 countries in Asia, Africa, Oceania and the Americas but the citrus-producing countries of the Mediterranean basin are still free from this disease. However, the establishment in the Iberian Peninsula of the African psyllid *Trioza erytreae*, a known vector of '*Ca*. Liberibacter africanus', has increased the risk of introduction in the region. Practically all commercial citrus species and cultivars are susceptible, regardless of rootstocks (Bové, 2006; Lopes *et al*., 2009). Although the pathogen has also been detected in orange jasmine (*Murraya paniculata*), the plant is a poor host and infections are transient (Cifuentes-Arenas *et al*., 2019). The disease has been associated with three species of phloem-restricted bacteria (Laflèche & Bové, 1970) recognized as members of '*Candidatus* Liberibacter'. Each species was named after the continent on which it was first reported: '*Ca*. Liberibacter africanus' is heat sensitive and transmitted by the psyllid *Trioza erytreae* (McClean & Oberholzer, 1965) and is mainly found in Africa, '*Ca*. Liberibacter asiaticus' is transmitted by the psyllid *Diaphorina citri* (Capoor *et al*., 1967; Yamamoto *et al*., 2006) and is mainly present in Asia and America, and '*Ca*. Liberibacter americanus' is transmitted by the psyllid *Diaphorina citri* (Yamamoto *et al*., 2006), has only been found in Brazil up to now (Teixeira *et al*., 2005a,b) and has minor epidemiological importance compared to the other species (Gasparoto *et al*., 2012). The causal agents of the disease have not been cultivated in axenic medium yet. Two reports mention the black psyllid, *Diaphorina communis*, as a vector in Bhutan (Donovan *et al*., 2012) and *Cacopsylla citrisuga* in China (Cen *et al*., 2012). In addition, '*Ca*. Liberibacter' species can be transmitted by propagative plant material and experimentally by *Cuscuta* spp. (dodder) to *Catharanthus roseus* (periwinkle) and other herbaceous plants. The presence of the bacteria has been demonstrated in citrus seeds and seedlings, but seed transmission needs to be confirmed (Graham *et al*., 2008; Hartung *et al*., 2008; Albrecht & Bowman, 2009). Historically '*Ca*. Liberibacter' species were considered non-culturable bacteria. Although four reports (Davis *et al*., 2008; Sechler *et al*., 2009; Ha *et al*., 2019; Mandadi and Ancona, 2020) refer to the cultivation of huanglongbing-related '*Ca*. Liberibacter' species, this needs more confirmation and isolation is not recommended in this diagnostic protocol. Additional information on the biology of the three species is available in the EPPO Datasheet (https://gd.eppo.int).

A flow diagram describing the diagnostic procedure for '*Ca*. Liberibacter' spp. is presented in Figure 1.

2 | IDENTITY

Name: '*Candidatus* Liberibacter africanus'.
Other scientific names: Citrus greening bacterium (heat-sensitive strain), *Liberibacter africanum*, *Liberibacter africanus*.
Taxonomic position: Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Phyllobacteriaceae.
EPPO Code: LIBEAF.
Phytosanitary categorization: EPPO A1 list no. 151, EU A1 Quarantine pest (Annex II A).
Name: '*Candidatus* Liberibacter americanus'.
Other scientific names: *Liberibacter americanus*.

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

Taxonomic position: Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Phyllobacteriaceae.
EPPO Code: LIBEAM.
Phytosanitary categorization: EPPO A1 list no. 151, EU A1 Quarantine pest (Annex II A).

Name: '*Candidatus* Liberibacter asiaticus'.
Other scientific names: Citrus greening bacterium (heat-tolerant strain), *Liberibacter asiaticum*, *Liberibacter asiaticus*.
Taxonomic position: Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Phyllobacteriaceae.
EPPO Code: LIBEAS.
Phytosanitary categorization: EPPO A1 list no. 151, EU A1 Quarantine pest (Annex II A).

3 | DETECTION

Symptoms caused by huanglongbing are not always easy to distinguish from those due to other *Citrus* diseases or abiotic factors. In addition, these bacteria have an uneven distribution and concentration in the plant, causing difficulties for a reliable diagnosis. Several molecular tests have been developed for the detection of '*Ca*. Liberibacter spp.' in plant material and vectors, and these are presented in section 3.4.

3.1 | Disease symptoms

The text below is from the EPPO Datasheet. On infected plants, symptoms are expressed after a variable period of time after infection (from one to three years) which depends on several factors (e.g. initial bacterial inoculum, time of infection, environmental conditions, tree age, species/cultivar, sanitary status of the tree). Symptoms generally appear faster in young trees (Gottwald *et al*., 2007). The general aspect of citrus trees affected by huanglongbing is open growth, stunting, twig dieback, sparse yellow foliage, deformed non-marketable fruits and severe fruit drop. Certain symptoms are described as more frequently observed in some countries, such as in China, where leaf symptoms were seen initially on one limb of the tree, causing yellow branches, or in South Africa where the disease is currently called greening because of the poorly coloured fruits and the inversion of colouration when maturing. Symptoms develop relatively slowly, and

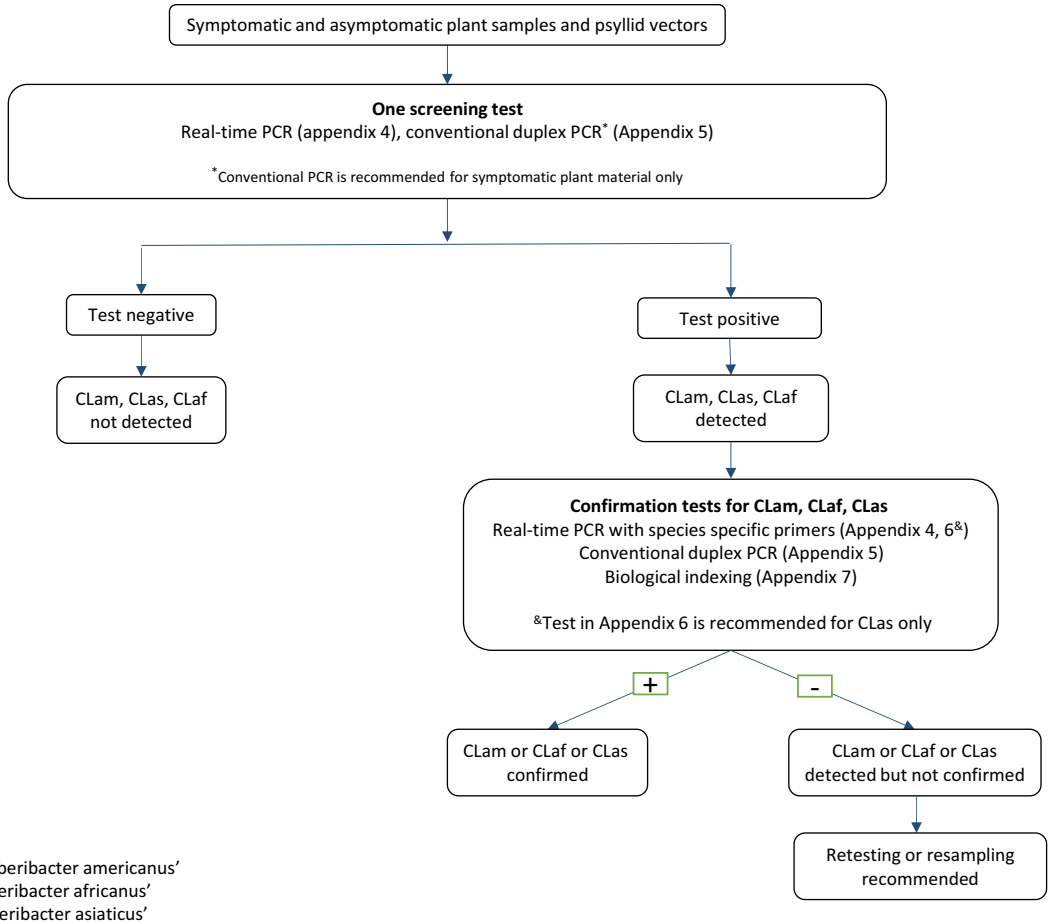

FIGURE 1 Flow diagram for the diagnosis of '*Ca*. Liberibacter' spp. in plant material and insect vectors

infected trees gradually decline in vigour and yield, and remain stunted or eventually die. The disease develops irregularly so that individual trees may show a mixture of normal and diseased sectors (Bové, 2006; Gottwald *et al*., 2007; CABI 2019; EFSA *et al*., 2019). Symptoms are generally the same for the three '*Ca*. Liberibacter' species, although the Asian form is considered to be associated with more severe symptoms because dieback can be more extensive and eventually result in tree death.

FIGURE 2 Symptoms of huanglongbing in trees. Presence of one or several yellow shoots per tree in an affected orchard (picture kindly provided by J.M. Bové)

3.1.1 | Symptoms on shoots and leaves

The text below is from the EPPO Datasheet. Symptoms usually first appear as leaf yellowing followed by mottling and chlorosis in one shoot or sector of the tree (Figure 2). Later, leaf symptoms resemble nutritional deficiencies (zinc, copper or manganese) but may vary depending on the bacterial strain. The larger leaves on the base of branches turn yellow along the main and secondary veins and later change to a blotchy-mottle (Figure 3a,b). As the discoloration spreads away from the veins, the leaves become pale to light yellow with unevenly distributed dark green areas. Leaves on weak terminal twigs are small, up-right and show a variety of chlorotic patterns. This is the most characteristic foliar symptom because the two halves of the leaf patterns of yellow and green areas are asymmetric (Figure 4a,b), in contrast with the nutrient deficiencies, which are symmetric. Mature leaves often show irregular patches between the main veins. The veins are often prominent and yellow. Frequently, there is abundant leaf drop.

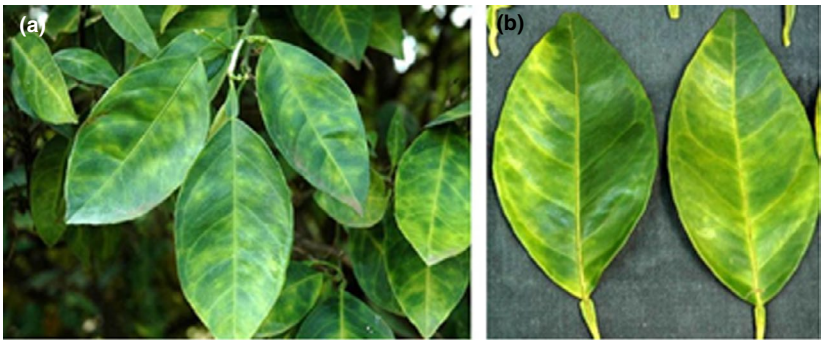

FIGURE 3 (a, b) Symptoms of huanglongbing in leaves. Typical asymmetrical blotchy mottle on both sides of sweet orange leaves (pictures kindly provided by J.M. Bové)

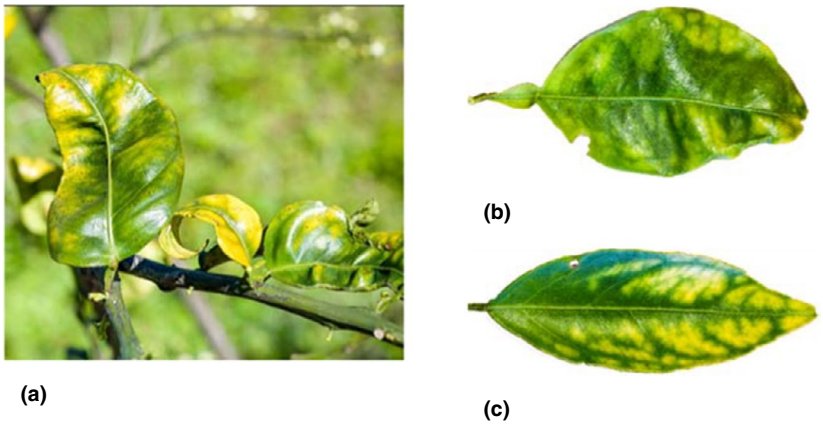

FIGURE 4 Typical huanglongbing symptoms on Citrus plants: (a, b) Blotchy mottle leaves and corky aspect of leaf due to psyllid feeding. (c) Symptoms of huanglongbing in a leaf

Additional pictures of psyllid damage are available in Global Database https://gd.eppo.int/taxon/TRIZER/photos

3.1.2 | Possible confusion

Zinc or other mineral deficiencies (Figure 5) may cause symptoms that can be confused with huanglongbing. However, as mentioned before, mineral deficiency symptoms are symmetric or show a discoloration of the veins whereas blotchy mottle is not symmetrical on both sides of the leaves.

3.1.3 | Fruit disorders

Huanglongbing induces characteristic symptoms on fruits that are produced on yellow shoots. Symptomatic fruits are small, asymmetric and lopsided, with a bent fruit axis (Figure 6a). At the time the fruit changes colour, from green to yellow/orange, those from affected shoots show colour inversion: the peduncular end of the fruit turns yellow/orange, while the stylar end is still green, as opposed to normal fruit colouration (Figure 6b). In addition, when the peduncle of a fruit with colour inversion is carefully removed, the resulting circular scar is stained orange, while on a normal fruit the scar is pale green. When fruits are cut in half, perpendicular to the fruit axis, small, brownish/black aborted seeds can be observed, but this symptom can also be present in citrus stubborn disease affected fruits. Cutting a lopsided fruit through the fruit axis reveals its asymmetry and some aborted seeds can also be found. In addition, the vascular bundles within the fruit axis at the peduncular end have a strong brownish stain. The albedo is sometimes thicker at the peduncular end than at the stylar end.

FIGURE 5 Nutrient deficiencies in *Citrus* triggered by iron, manganese and zinc deficiencies

FIGURE 6 Symptoms of huanglongbing in fruits. (a) Orange fruit with small and brownish aborted seeds and asymmetric, lopsided with a bent fruit axis sweet. (b) Colour inversion symptom. The peduncular end of the fruit turns yellow/orange, while the stylar end is still green (on the contrary to normal fruit colouration) (pictures kindly provided by J.M. Bové)

3.2 | Test sample requirements for plant material

Appropriate sample selection is critical for '*Ca*. Liberibacter' spp. detection: each host tree should be sectioned into quadrants; each quadrant should be sampled to give a total of ~15 leaves per tree to get at least ~1 g of petiole and midribs from symptomatic or asymptomatic trees to be analysed. It should be noted that detection in asymptomatic plants can be very erratic due to the patchy distribution and low concentration of bacteria in the plant (Li *et al*., 2009; Louzada *et al*., 2016). Detection on symptomatic material (e.g. leaves) is facilitated by a higher bacterial titre and conventional PCR has been demonstrated to be reliable for the detection of the bacteria (Tatineni *et al*., 2008; Teixera *et al*., 2008). Sample preparation and bacterial DNA extraction methods from plant material are presented in Appendix 2 and Appendix 3, respectively.

3.3 | Test sample requirement for vectors

Adults of *Diaphorina citri* and/or *Trioza erytreae* or other psyllids suspected to be vectors can be collected from symptomatic or asymptomatic host trees and conserved in 70% ethanol. Sample preparation and bacterial DNA extraction methods from vectors are presented in Appendix 2 and Appendix 3, respectively.

3.4 | Rapid screening tests (molecular tests)

Either of the following two screening tests is recommended for the detection of '*Candidatus* Liberibacter asiaticus', '*Candidatus* Liberibacter africanus' and '*Candidatus* Liberibacter americanus':

- Real-time PCR test using the TaqMan probe described by Li *et al*. (2006) (Appendix 4); this is the preferred screening test as it is the most rapid, sensitive and accurate test, and it is recommended for testing asymptomatic plant material.
- The conventional duplex PCR test adapted from Teixeira *et al*. (2005) and Hocquellet *et al*. (1999) (Appendix 5) can be used as a screening test on symptomatic plant material.

The performances of the real-time PCR test from Li *et al*. (2006) and the conventional duplex PCR test adapted from Teixeira *et al*. (2005) and Hocquellet *et al*. (1999) are comparable (Li *et al*., 2009; Louzada *et al*., 2016). The test from Morgan *et al*., 2012 (Appendix 6) is not recommended as a screening test as it will only detect '*Ca* Liberibacter asiaticus'. The real-time PCR test described by Bertolini *et al*. (2010, 2014) is not recommended as a screening test as it produces false-positive results and thus requires confirmation by another test.

4 | IDENTIFICATION

4.1 | Molecular tests

The following tests are recommended for the confirmation of '*Candidatus* Liberibacter asiaticus', '*Candidatus* Liberibacter africanus' and '*Candidatus* Liberibacter americanus[2]':

- Real-time PCR adapted from Li *et al*. (2006) (Appendix 4): the real-time PCR test should be adapted by using the species-specific primers in simplex.
- Conventional duplex PCR adapted from Teixeira *et al*. (2005) and Hocquellet *et al*. (1999) (Appendix 5). No validation data is available for the confirmation of '*Candidatus* Liberibacter americanus' with this test.
- Real-time PCR from Morgan *et al*., 2012 (Appendix 6) can be used for the confirmation of '*Candidatus* Liberibacter asiaticus'; cross-reaction with '*Candidatus* Liberibacter africanus' has been observed occasionally.

4.2 | Other tests

Biological indexing (Roistacher, 1991) may be used for confirmation. Detailed procedures are presented in Appendix 7.

5 | REFERENCE MATERIAL

Paper immobilized positive controls of bacterial DNA of the three '*Ca*. Liberibacter' causal agents of huanglongbing can be obtained from Plant Print Diagnostics, Faura, Valencia, ES. Note that to ensure proper conservation, the piece of paper should be removed from the tube after the positive control is reconstituted.

6 | REPORTING AND DOCUMENTATION

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 *Documentation and reporting of a diagnosis*.

[2]The TPS organized in the framework of the Euphresco project 2016-A-232 HLBValid was not performed on samples infected with '*Candidatus* Liberibacter americanus' because they were not available.

7 | PERFORMANCE CHARACTERISTICS

When performance characteristics are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc).

8 | FURTHER INFORMATION

Further information on this organism can be obtained from:

G. Cellier, Anses – Plant Health Laboratory. 7 chemin de l'Irat – Pole de Protection des Plantes (3P) 97410 Saint Pierre – Reunion Island; saint-pierre.lsv@anses.fr.

E. Marco-Noales, Instituto Valenciano de Investigaciones Agrarias (IVIA), Carretera Moncada-Naquera Km. 4.5, 46113, Moncada, Valencia (ES); emarco@ivia.es.

9 | FEEDBACK ON THIS DIAGNOSTIC PROTOCOL

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact diagnostics@eppo.int.

10 | PROTOCOL REVISION

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website. When *errata* or *corrigenda* are in press this will also be marked on the website.

ACKNOWLEDGEMENTS

The protocol was originally drafted by E. Bertolini, M. M. Lopez and M. Cambra from Instituto Valenciano de Investigaciones Agrarias (IVIA), Valencia (ES). The revision was prepared by G. Cellier from Anses – Plant Health Laboratory, La Réunion (FR).

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How to cite this article: PM 7/121 (2) '*Candidatus* Liberibacter africanus', '*Candidatus* Liberibacter americanus' and '*Candidatus* Liberibacter asiaticus'. *EPPO Bulletin* 2021;51:267–282. https://doi.org/10.1111/epp.12757

APPENDIX 1 PREPARATION OF BUFFERS

CTAB buffer **(pH = 8.0)**	
Tris-HCl (1 M autoclaved solution pH 8.0)	100 mL
NaCl	81.82 g
Ethylene diamine tetraacetic acid, EDTA (0.5 M)	50 mL
Cetrimonium bromide, CTAB	20 g
Polyvinylpyrrolidone, PVP−10	10 g
Sodium bisulfite ($NaHSO_3$)	1 g
Distilled water to	1 L

Extraction buffer#1 **Phosphate buffer saline (PBS) buffer pH = 7.2**	
NaCl	8.0 g
KCl	0.2 g
$Na_2HPO_4\ 12H_2O$	2.9 g
KH_2PO_4	0.2 g
Sodium diethyldithiocarbamate ($C_5H_{10}NS_2Na$)	2.0 g
Distilled water to	1 L

Extraction buffer#2 **Phosphate buffer (PB) pH = 7.2**	
$Na_2HPO_4\ 12H_2O$	2.15 g
$KH_2PO_4\ 2H_2O$	0.544 g
Distilled water to	1 L

Extraction buffer**#3 (pH = 8.0)**	
Tris(hydroxymethyl)aminomethane (Sigma 7–9 – 50 mM)	6.06 g
Ethylene diamine tetraacetic acid, EDTA (5 mM)	1.46 g
Sodium dodecylsulfate, SDS (1% w/v)	10.0 g
Distilled water to	1 L

APPENDIX 2 SAMPLE PREPARATION FOR TESTING

1. Plant material

1.1. Direct tissue-print

Plant samples should be immobilized on membranes (Olmos *et al*., 1996) by pressing the petioles of fresh hand detached citrus leaves on paper 3 MM (Whatman) or on nylon positively charged membranes. Several partially overlapping imprints from 5 to 10 different leaves collected in the quadrants of the tree are made on a 0.5 cm^2 membrane (Bertolini *et al*., 2008). A disk of the membrane (taken with a punch machine) harbouring the printed samples is carefully introduced with tweezers into the Eppendorf tube. Then 100 µL of distilled water is added, and the mixture is vortexed and place on ice (Bertolini *et al*., 2010; 2014). Three or 5 µL of this extract is used for real-time PCR tests. Direct tissue-print of plant material can only be used for real-time PCR detection.

1.2. Preparation of plant samples

Five to ten leaves are carefully washed under running tap water, blotted dry on filter paper, and their midribs cut into small pieces. Approximately 0.5–1.0 g of mid ribs are used for DNA extraction (see Appendix 3).

2. Psyllids

2.1. Direct spot or squash

Psyllids can be tested after being squashed onto nylon membranes or 3 MM Whatman paper with the rounded end of an Eppendorf tube (Bertolini *et al*. 2014). The printed and/or squashed samples immobilized on membranes are carefully cut around the samples and inserted into Eppendorf tubes containing 100 µL of distilled water, 0.5% Triton X-100 or glycine buffer (0.1 M glycine, 0.05 M NaCl, 1 mM EDTA). Samples are then incubated at room temperature for 10 min, vortexed and placed on ice until use. Three microlitres of the extract are directly used as template for the real-time PCR test. The spotted membrane/paper can only be used for real-time PCR detection.

2.2. Extraction from psyllids

Homogenize insect(s) in 100 µL of 10 mM phosphate buffer (per insect) in an Eppendorf tube, using a micro pestle. The crude extracts obtained can be immediately used after homogenization for DNA extraction or stored at approximately −20°C until use. For long storage periods preserve at approximately −80°C.

APPENDIX 3 DNA EXTRACTION

1. Plant samples

DNA extraction from plant material is performed using 0.5–1.0 g (or 0.35 g of lyophilized) of the leaf midribs, starting from and including the petiole, using the CTAB method for DNA extraction (see section 1.1) or using a DNeasy Plant Mini Kit (Qiagen) (see section 1.2).

1.1. CTAB DNA extraction

Samples are homogenized by adding 3–10 mL of CTAB buffer (Appendix 1) and 0.2% of beta-mercaptoethanol (freshly prepared and added immediately before use) in extraction bags/Bioreba bags using a Homex homogenizer device (Bioreba-Switzerland) or any manual roller. Transfer a maximum of 2 mL of extract to a 2-mL

microtube. Incubate microtubes, if possible with shaking at maximum speed, for at least 15 min at 65°C, then centrifuge at 3000*g* for 5 min in a microcentrifuge. Transfer 1 mL of supernatant in a 2-mL Eppendorf tube and add 1 mL of chloroform-isoamyl alcohol solution (24:1 v/v). Mix the two phases to obtain an emulsion. Centrifuge at 14 000*g* for 5 min. Transfer the supernatant to a new microtube and add 0.6 volume of cold isopropanol. Mix and keep at −20°C for 30 min. Centrifuge at 14 000*g* for 20 min.

Discard the supernatant. Wash with 70% ethanol twice and centrifuge at 14 000*g* for 10 min. Empty the microtubes and dry the pellet. Resuspend the pellet in 100 µL of sterile distilled water and vortex to help dissolution. The extracts can be stored at −20°C until use.

1.2. Qiagen DNA extraction

Samples are homogenized in extraction bags/Stomacher bags 1/20 (w/v) in extraction buffer #1 or 1/5 (w/v) in extraction buffer #2 (Appendix 1), then a maximum of 2 mL of extract is transferred into a 2-mL microtube and centrifuged at approximately 20 000*g* for approximately 10 min to precipitate all the target cells. Discard the supernatant and continue the subsequent steps of DNA extraction using the DNeasy Plant Mini Kit (Qiagen). Extraction is performed according to the manufacturer's instructions on the pellet from the previous step. DNA extracts can be stored for several weeks at a temperature of approximately −20°C, pending the completion of the amplification.

2. Psyllids

2.1. DNA extraction from psyllids

The crude extract is briefly vortexed and then incubated at 65°C for 5 min. The suspension is extracted once with an equal volume of chloroform:isoamyl alcohol (24:1 v/v) and centrifuged at 13 000*g* for 5 min. The supernatant is collected and DNA precipitated by adding 0.1 volume of 3 M sodium acetate, pH 5.2 and 2.5 volumes of ice-cold ethanol and incubating at −20°C for at least 1 h. After centrifuging at 13 000*g* for 15 min, the pellet is washed with 70% ice-cold ethanol, air-dried and resuspended in 15 µL of sterile water.

The suspension is extracted once with an equal volume of chloroform:isoamyl alcohol (24:1 v/v) and the DNA precipitated by adding 0.1 volume of 3 M sodium acetate, pH 5.2 and 2.5 volumes of ice-cold. Alternatively, in the framework of the projects POnTE (H2020) and CaLiso (French funded project), the TNES method (Peccoud *et al*. 2013) and the Quick-Pick TM SML Plant DNA kit (Bio-Nobile) were evaluated and provided satisfactory results. The results of their performance are available in Loiseau *et al*. (2018) or on request at ANSES. The TNES method has the advantage of being non-destructive, allowing the conservation of the specimen for further morphological identification.

APPENDIX 4 REAL-TIME PCR ADAPTED FROM LI *ET AL*. (2006)

The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1 | GENERAL INFORMATION

1.1. This PCR is suitable for the detection and confirmation of '*Ca*. Liberibacter americanus' or '*Ca*. Liberibacter asiaticus' or '*Ca*. Liberibacter africanus' on plant material and vectors.

1.2. The test is adapted from Li *et al*. (2006).

1.3. The target sequence is located on the 16S rRNA gene.

1.4. Oligonucleotides:

Primers/probe	Sequence
Forward primer HLBas	5′-TCG AGC GCG TAT GCA ATA CG-3′
Forward primer HLBAF	5′-CGA GCG CGT ATT TTA TAC GAG CG-3′
Forward primer HLBam	5′-GAG CGA GTA CGC AAG TAC TAG-3′
Reverse primer HLBr	5′-GCG TTA TCC CGT AGA AAA AGG TAG-3′
Probe HLBp	FAM - AGA CGG GTG AGT AAC GCG-BHQ1
Forward primer COXf[1]	5′-GTA TGC CAC GTC GCA TTC CAG A-3′
Reverse primer COXr[1]	5′-GCC AAA ACT GCT AAG GGC ATT C-3′
Probe COXp[1]	TET-ATC CAG ATG CTT ACG CTG G-BHQ2

[1] These primers and probe are optional.

1.5. Real-time PCR system: SmartCycler II (Cepheid) or QuantStudio 5 (ThermoFisher Scientific)

2 | METHODS

2.1. Nucleic acid extraction and purification.

2.1.1. Matrices: naturally infected symptomatic and asymptomatic plant material and insect vectors.

2.1.2. 1 g of fresh material (or 0.35 g of lyophilized material) is ground in 5 mL of NaOH 2% (w/v). The debris are pelleted using a bench top centrifuge for 5 s. The supernatant is collected and diluted 1/50 and can be used for the PCR test. Another protocol for DNA extraction is presented in Appendix 3.

2.1.3. DNA should preferably be stored at approximately −20°C.

2.2. Real-time polymerase chain reaction (real-time PCR).

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		2.056	
GoTaq® qPCR Master Mix (Promega)	2×	6.500	1×
Forward primer HLBas	10 µM	0.325	0.25 µM
Forward primer HLBaf	10 µM	0.325	0.25 µM
Forward primer HLBam	10 µM	0.325	0.25 µM
Reverse primer HLBr	10 µM	0.325	0.25 µM
Probe HLBp	10 µM	0.169	0.13 µM
Forward primer COXf[1]	10 µM	0.390	0.30 µM
Reverse primer COXr[1]	10 µM	0.390	0.30 µM
Probe COXp[1]	10 µM	0.195	0.15 µM
Subtotal		11.000	
DNA sample		2.000	
Total PCR volume		13.000	

[1] As COX primers and probe are optional, their volume can be replaced by molecular-grade water.

2.3. The real-time PCR can be performed with TaqMan or SYBRGreen (no validation data available for SYBRGreen) chemistries. For SYBRGreen real-time PCR, the volume of the probe can be replaced by molecular-grade water.

2.4. PCR cycling conditions: an initial step at 95ºC for 10 min followed by 40 cycles (95ºC for 15 s and 58ºC for 1 min). Melting temperatures are as follows: T_m for CLam = 83.2°C, T_m for CLaf = 81.4°C, T_m for CLam = 83.2°C. Melting curve from 75°C to 95°C with 0.10°C/s increments.

3 | ESSENTIAL PROCEDURAL INFORMATION

3.1 | Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA).
- Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2 | Interpretation of results: to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.

- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Additionally, for SYBRGreen-based real-time PCR tests: the *T*m value should be as expected.
- Tests should be repeated if any contradictory or unclear results are obtained.

4 | PERFORMANCE CHARACTERISTICS AVAILABLE

Validation data from the Euphresco project 2016-A-232 HLBVALID: '*Candidatus* Liberibacter africanus'.

Validation was carried out in accordance with PM7/98.

4.1 Analytical sensitivity data

5.5×10^3

4.2 Analytical specificity data

Inclusivity: 100%, evaluated on five strains of '*Candidatus* Liberibacter africanus'.

Exclusivity: 100%, evaluated on samples infected by '*Candidatus* Liberibacter solanacearum', *Xanthomonas citri* pv. *citri* and several non-target DNA samples corresponding to different non-infected matrices.

4.3 Diagnostic sensitivity

1

4.4 Diagnostic specificity

0.959

4.5 Repeatability

Detection level (DL100): 91.7%, accordance score (DA): 0.988.

4.6 Reproducibility

Concordance score (CO): 0.976.

Validation data from the Euphresco project 2016-A-232 HLBVALID: '*Candidatus* Liberibacter asiaticus'.

Validation was carried out in accordance with PM7/98.

4.1 Analytical sensitivity data

4.4×10^4

4.2 Analytical specificity data

Inclusivity: 94.4%, evaluated on 12 strains.

Exclusivity: 100%, evaluated on samples infected by '*Candidatus* Liberibacter solanacearum', *Xanthomonas citri* pv. *citri* and several non-target DNA samples corresponding to different non-infected matrices.

4.3 Diagnostic sensitivity

1

4.4 Diagnostic specificity

0.959

4.5 Repeatability

Detection level (DL100): 92.2%, accordance score (DA): 0.988.

4.6 Reproducibility

Concordance score (CO): 0.976.

APPENDIX 5 DUPLEX CONVENTIONAL PCR ADAPTED FROM TEIXEIRA *ET AL.* (2005) AND HOCQUELLET *ET AL.* (1999)

The test below is described as it was carried out to generate the validation data provided in section 4. *Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.*

1 | GENERAL INFORMATION

1.1. This PCR is suitable for the detection and confirmation of '*Ca.* Liberibacter spp.' on plant material and insect vectors.

1.2. The test is adapted from Teixeira *et al.* (2005) and Hocquellet *et al.* (1999).

1.3. The target sequence is located on the 16S rRNA gene and rplKAJL-rpoBC operon gene.

1.4. Oligonucleotides:

Primers	Sequence	Amplicon size
Forward primer GB1	5′-AAG TCG AGC GAG TAC GCA AGT ACT-3′	1027 bp (CLam) 703 bp (CLas) 669 bp (CLaf)
Reverse primer GB3	5′-CCA ACT TAA TGA TGG CAA ATA TAG-3′	
Forward primer A2	5′-TAT AAA GGT TGA CCT TTC GAG TTT-3′	
Reverse primer J5	5′-ACA AAA GCA GAA ATA GCA CGA ACA A-3′	

2 | METHODS

2.1. Nucleic acid extraction and purification.
  2.1.1. Matrices: naturally infected symptomatic and asymptomatic plant material and insect vectors.
  2.1.2. The procedure for DNA extraction is presented in Appendix 3.
  2.1.3. DNA should preferably be stored at approximately −20°C.
2.2. Conventional polymerase chain reaction (PCR).

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	–	10.375	–
GoTaq® Flexi/ HotStart reaction buffer (Promega)	5×	5.000	1×
$MgCl_2$	25 mM	2.000	2 mM
dNTPs	10 mM	0.500	0.2 mM of each dNTP
Primer GB1	20 µM	1.250	1 µM
Primer GB3	20 µM	1.250	1 µM
Primer A2	20 µM	1.250	1 µM
Primer J5	20 µM	1.250	1 µM
GoTaq® Flexi/ HotStart polymerase (Promega)	5 U/µL	0.125	0.625 U
Subtotal	–	23.000	–
DNA	–	2.000	–
Total PCR volume	–	25.000	–

2.3. PCR cycling conditions. An initial step at 96ºC for 5 min followed by 35 cycles (94ºC for 30 s, 62ºC for 30 s and 72ºC for 1 min) and one final step at 72ºC for 10 min before cooling at 4ºC.

3 | ESSENTIAL PROCEDURAL INFORMATION

3.1 | Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA).
- Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2 | Interpretation of results: to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- NIC and NAC: no band is visualized.
- PIC, PAC (and if relevant IC): a band of the expected size is visualized. This is 1027 bp for '*Ca*. Liberibacter americanus', 703 bp for '*Ca*. Liberibacter asiaticus' and 669 bp for '*Ca*. Liberibacter africanus'.

When these conditions are met

- A test will be considered positive if a band of 1027 or 703 or 669 is produced.

- A test will be considered negative if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

4 | PERFORMANCE CHARACTERISTICS AVAILABLE

Validation data from the Euphresco project 2016-A-232 HLBVALID: '*Candidatus* Liberibacter africanus'.

Validation was carried out in accordance with PM7/98.

4.1 Analytical sensitivity data

5.5×10^2

4.2 Analytical specificity data

Inclusivity: 80.0%, evaluated on five strains.

Exclusivity: 100%, evaluated on samples infected by '*Candidatus* Liberibacter solanacearum', *Xanthomonas citri* pv. *citri* and several non-target DNA samples corresponding to different non-infected matrices.

4.3 Diagnostic sensitivity

0.868

4.4 Diagnostic specificity

1.000

4.5 Repeatability

Detection level (DL) 100: 94.4%, accordance score (DA): 1.

4.6 Reproducibility

Concordance score (CO): 0.952.

Validation data from the Euphresco project 2016-A-232 HLBVALID: '*Candidatus* Liberibacter asiaticus'.

Validation was carried out in accordance with PM7/98.

4.1 Analytical sensitivity data

2.60×10^3

4.2 Analytical specificity data

Inclusivity: 80.60%, evaluated on 12 strains.

Exclusivity: 100% evaluated on samples infected by '*Candidatus* Liberibacter solanacearum', *Xanthomonas citri* pv. *citri* and several non-target DNA samples corresponding to different non-infected matrices.

4.3 Diagnostic sensitivity

0.868

4.4 Diagnostic specificity

1.000

4.5 Repeatability

Detection level (DL)100: 96.70%, accordance score (DA): 1.

4.5 Reproducibility

Concordance score (CO): 0.952.

APPENDIX 6 REAL-TIME PCR ADAPTED FROM MORGAN *ET AL*. (2012)

The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1 | GENERAL INFORMATION

1.1. This PCR is suitable for the confirmation of '*Ca.* Liberibacter asiaticus' on plant material and insect vectors.

1.2. The test is adapted from Morgan *et al.* (2012).

1.3. The target sequence is located on the hyvI/hyvII gene.

1.4. Oligonucleotides:

Primers/probe	Sequence
Forward primer LJ900f_f	5′-GCC GTT TTA ACA CAA AAG ATG AAT ATC-3′
Reverse primer LJ900f_r	5′-ATA AAT CAA TTT GTT CTA GTT TAC GAC-3′
Probe LJ900 p_p	FAM-ACA TCT TTC GTT TGA GTA GCT AGA TCA TTG A-BHQ1

1.5. Real-time PCR system: 7500 Fast real-time PCR system (Applied Biosystems) or QuantStudio 5 (ThermoFisher Scientific).

2 | METHODS

2.1. Nucleic acid extraction and purification.

2.1.1. Matrices: naturally infected symptomatic and asymptomatic plant material and insect vectors.

2.1.2. The protocol for DNA extraction is presented in Appendix 3.

2.1.3. DNA should preferably be stored at approximately −20°C.

2.2. Real-time Polymerase chain reaction (real-time PCR)

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		2.50	
GoTaq® Probe qPCR Master Mix (Promega)	2×	7.50	1×
Forward primer LJ900f_f	10 µM	0.90	0.60 µM
Reverse primer LJ900f_r	10 µM	1.35	0.90 µM
Probe LJ900 p_p	10 µM	0.75	0.50 µM
Subtotal		13.00	
DNA sample		2.00	
Total PCR volume		15.00	

2.3. The real-time PCR can be performed with TaqMan or SYBRGreen chemistries. For SYBRGreen real-time PCR, the volume of the probe can be replaced by molecular-grade water and the Mastermix to be used would be the SYBRGreen PCR Master Mix (ThermoFisher Scientific).

2.4. PCR cycling conditions: An initial step at 95ºC for 10 min followed by 40 cycles (95ºC for 10 s and 62ºC for 1 min). Melting temperatures are as follows: T_m for CLam = 83.2°C, T_m for CLaf = 81.4°C, T_m for CLam = 83.2°C. Melting curve from 75°C to 95°C with 0.10°C/s increments.

3 | ESSENTIAL PROCEDURAL INFORMATION

3.1 | Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA).
- Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2 | Interpretation of results: to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Additionally, for SYBRGreen-based real-time PCR tests: the melting temperature value should be as expected.

- Tests should be repeated if any contradictory or unclear results are obtained.

4 | PERFORMANCE CHARACTERISTICS AVAILABLE

Validation data from the Euphresco project 2016-A-232 HLBVALID: '*Candidatus* Liberibacter asiaticus' (Taqman).

Validation was carried out in accordance with PM7/98.

4.1 Analytical sensitivity data

7.8×10^5

4.2 Analytical specificity data

Inclusivity: 88.9%, evaluated on 12 strains.

Exclusivity: 100%, evaluated on samples infected by '*Candidatus* Liberibacter solanacearum', *Xanthomonas citri* pv. *citri* and several non-target DNA samples corresponding to different non-infected matrices. Cross-reaction with '*Candidatus* Liberibacter africanus' has been observed occasionally.

4.3 Diagnostic sensitivity

0.626

4.4 Diagnostic specificity

0.980

4.5 Repeatability

Detection level (DL100): 92.2%, accordance score (DA): 0.976.

4.6 Reproducibility

Concordance score (CO): 0.952.

Validation data from the Euphresco project 2016-A-232 HLBVALID: '*Candidatus* Liberibacter africanus' (Taqman).

Validation was carried out in accordance with PM7/98.

4.1 Analytical sensitivity data

No possible calculation (NPC).

4.2 Analytical specificity data

Inclusivity: 60.0%, evaluated on five strains.

Exclusivity: 100%, evaluated on samples infected by '*Candidatus* Liberibacter solanacearum', *Xanthomonas citri* pv. *citri* and several non-target DNA samples corresponding to different non-infected matrices.

4.3 Diagnostic sensitivity

0.626

4.4 Diagnostic specificity

0.980

4.5 Repeatability

Detection level (DL100): 100%, accordance score (DA): 0.976.

4.6 Reproducibility

Concordance score (CO): 0.952.

Validation data from the Euphresco project 2016-A-232 HLBVALID: '*Candidatus* Liberibacter asiaticus'. (SYBR green).

Validation was carried out in accordance with PM7/98.

4.7 Analytical sensitivity data

2.8×10^3

4.8 Analytical specificity data

Inclusivity: 97.20%, evaluated on 12 strains.

Exclusivity: 100%, evaluated on samples infected by '*Candidatus* Liberibacter solanacearum', *Xanthomonas citri* pv. *citri* and several non-target DNA samples corresponding to different non-infected matrices. Cross-reaction with '*Candidatus* Liberibacter africanus' has been observed occasionally.

4.9 Diagnostic sensitivity

0.626

4.10 Diagnostic specificity

0.980

4.11 Repeatability

Detection level (DL100): 93.3%, accordance score (DA): 0.976.

4.12 Reproducibility

Concordance score (CO): 0.952.

Validation data from the Euphresco project 2016-A-232 HLBVALID: '*Candidatus* Liberibacter africanus' (SYBR green).

Validation was carried out in accordance with PM7/98.

4.1 Analytical sensitivity data

No possible calculation (NPC).

4.2 Analytical specificity data

Inclusivity: 66.7%, evaluated on five strains.

Exclusivity: 100%, evaluated on samples infected by '*Candidatus* Liberibacter solanacearum', *Xanthomonas citri* pv. *citri* and several non-target DNA samples corresponding to different non-infected matrices.

4.3 Diagnostic sensitivity

0.626

4.4 Diagnostic specificity

0.980

4.5 Repeatability

Detection level (DL100): 97.2%, accordance score (DA): 0.976.

4.5 Reproducibility

Concordance score (CO): 0.952.

APPENDIX 7 BIOLOGICAL INDEXING (ACCORDING TO ROISTACHER, 1991)

Graft transmission of '*Ca*. Liberibacter' species is possible but is very variable and can be affected by several factors such as the type of tissue used, the age of the tissue, the indicator plants used and the amount of bacteria in the tree as well as the season of the year that the inoculum is collected.

The availability of healthy, vigorous indicator test plants, free of micronutrient deficiency or other symptoms, is very important for huanglongbing diagnosis. Seedlings of sweet orange and Orlando tangelo for '*Ca*. Liberibacter africanus', sweet orange or Ponkan mandarin for '*Ca*. Liberibacter asiaticus' and sweet orange and Murcott tangor for '*Ca*. Liberibacter americanus' are recommended. Positive and negative control plants are essential in any indexing procedure.

Two inoculation techniques are recommended: side grafts and leaf-piece grafts. Young shoots with small emerging leaves are collected around of the canopy of each test tree.

Side grafting

Two side grafts are put into each seedling. Tissues consist of a part of a branch approximately 4–5 mm thick and 3–5 cm long. A wedge cut is made at one end of the budstick and a cut made in the seedling, and the wedged fitted into the cut. The side grafts are then wrapped with polythene budding tape and a sleeve cut from a polythene bag is placed over the area above and below the grafts.

Leaf grafting

A small rectangular section of leaf about 3 × 12 mm is cut from the midrib area of a young succulent leaf and placed into a T-cut in the bark of the seedling, as for standard bud-grafting. The area is then wrapped with polythene tape in the same manner as with buds. Two to three leaf grafts per plant are recommended.

For side grafts, after 10 days to 2 weeks post inoculation, the bottom ends of the polythene sleeves are partially opened. After 3 weeks the polythene sleeves are removed and grafts observed for survival. The plants are then cut back to about 25 cm from the soil surface. One terminal shoot is then permitted to grow and is trained and staked to grow as a single leader. For leaf grafts, the wrapping tape surrounding the leaf grafted is cut 2–3 weeks after inoculation and inoculum survival recorded. Plants are then cut back and new growth trained to a single shoot as for side grafting. Keep indicator plants at 20–25ºC for '*Ca*. Liberibacter africanus' and '*Ca*. Liberibacter americanus', and 25–32ºC for '*Ca*. Liberibacter asiaticus'.

Symptoms can appear after 3 months to 1 year and in sweet orange, tangelo or mandarin will be a typical leaf blotchy mottle and chlorosis. The shoots of the huanglongbing affected symptomatic plants will be distinctly smaller, chlorotic and with smaller leaves when compared with those of the non-inoculated control and can be tested by PCR-based methods.