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ANNEX 21

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DIAGNOSTIC PROTOCOLS INTERNATIONAL STANDARD FOR PHYTOSANITARY MEASURES 27

DP 21: '*Candidatus* Liberibacter solanacearum'

Produced by the Secretariat of the
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ISPM 27
Diagnostic protocols for regulated pests

DP 21: ‘*Candidatus* Liberibacter solanacearum’

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1. Pest Information

'*Candidatus* Liberibacter solanacearum' is a phloem-limited, Gram-negative, unculturable bacterium that is associated with several emerging diseases. '*Ca*. L. solanacearum' was first identified in 2008 from the psyllid *Bactericera cockerelli* by Hansen *et al*. (2008) and from potatoes, tomatoes and peppers by Liefting *et al*. (2008, 2009a, 2009b), and later from carrot and the carrot psyllid *Trioza apicalis* by Munyaneza *et al*. (2010). The bacterium has a rod-shaped morphology and is about 0.2 µm wide and 4 µm long (Liefting *et al*., 2009a; Secor *et al*., 2009).

Other '*Ca*. Liberibacter' species include those associated with citrus Huanglongbing (also known as citrus greening disease): '*Ca*. L. africanus', '*Ca*. L. americanus' and '*Ca*. L. asiaticus' (Nelson *et al*., 2013a). Several new '*Ca*. Liberibacter' species have recently been discovered such as '*Ca*. L. europaeus' (Raddadi *et al*., 2011), '*Ca*. L. caribbeanus' (Keremane *et al*., 2015) and the first cultured species from this bacterial clade, *Liberibacter crescens* (Fagen *et al*., 2014). It is unclear if these new '*Ca.* Liberibacter' species are associated with plant disease. The discovery of additional '*Ca*. Liberibacter' species is likely to continue with the application of new technologies such as next-generation sequencing.

In North and Central America and Oceania, '*Ca*. L. solanacearum' primarily infects solanaceous crops and weeds, including *Solanum tuberosum* (potato), *Solanum lycopersicum* (tomato), *Capsicum annuum* (pepper), *Solanum betaceum* (tamarillo), *Nicotiana tabacum* (tobacco), *Solanum melongena* (eggplant), *Physalis peruviana* (cape gooseberry), *Solanum elaeagnifolium* (silverleaf nightshade), *Solanum ptycanthum* (eastern black nightshade) and *Lycium barbarum* (wolfberry) (EPPO 2013; Haapalainen, 2014). In Europe and North Africa, '*Ca*. L. solanacearum' has been associated with symptoms in species of the family Apiaceae, including *Daucus carota* subsp. *sativus* (carrot), *Apium graveolens* (celery) and *Pastinaca sativa* (parsnip) (EPPO 2013; Teresani *et al*., 2014).

In solanaceous plants, '*Ca*. L. solanacearum' is primarily spread from infected to healthy plants by the tomato and potato psyllid *B. cockerelli* (Munyaneza *et al*., 2007; Munyaneza, 2012; EPPO, 2013). Horizontal transmission between plants from the family Apiaceae has been reported to occur by the psyllids *T. apicalis* (Nissinen *et al*., 2014) and *Bactericera trigonica* (Teresani *et al*., 2014, 2015). The bacterium is found in several organs and tissues of its psyllid host, including the alimentary canal, salivary glands, haemolymph and bacteriomes (Cooper *et al*., 2013), and is transmitted in a propagative, circulative and persistent manner (Sengoda *et al*., 2014). Vertical (transovarial) transmission of '*Ca*. L. solanacearum' has been reported in *B. cockerelli* (Hansen *et al*., 2008). '*Ca*. L. solanacearum' can also be transmitted by grafting and via dodder (Crosslin and Munyaneza, 2009; Secor *et al*., 2009; Munyaneza, 2012; Haapalainen, 2014; Munyaneza, 2015). Although transmitted through seed potato tubers, '*Ca*. L. solanacearum' transmission has not been shown through true potato seed or seed from other solanaceous plants (Munyaneza, 2012). It has been demonstrated that the bacterium can be disseminated with infected carrot seeds, although vertical transmission through seed has been reported only once (Bertolini *et al*., 2014).

Five haplotypes of '*Ca*. L. solanacearum' have so far been described (Nelson *et al*., 2011, 2013b; Teresani *et al*., 2014). Two haplotypes (A and B) are associated with diseases in potato and other solanaceous species in America and Oceania, whereas the other three haplotypes (C, D and E) are associated with carrot and celery crops in Europe and North Africa. The haplotypes were differentiated by single nucleotide polymorphisms (SNPs) in the 16S ribosomal (r)RNA gene, 16S-23S rRNA intergenic spacer (IGS) region, and 50S *rplJ* and *rplL* ribosomal protein genes.

Further information on '*Ca*. L. solanacearum', including its insect vectors, disease epidemiology, vector biology, and management, can be found in reviews by Secor *et al*. (2009), Munyaneza (2012, 2015), Nelson *et al*. (2013a) and Haapalainen (2014).

2. Taxonomic Information

Name: ‘*Candidatus* Liberibacter solanacearum’ (Liefting *et al*., 2009b)

Synonym: ‘*Candidatus* Liberibacter psyllaurous’ (Hansen *et al*., 2008)

Taxonomic position: Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, ‘*Candidatus* Liberibacter’

Common names: Zebra chip, zebra complex

3. Detection

Plants infected with ‘*Ca*. L. solanacearum’ may be asymptomatic or exhibit symptoms that may be similar to those associated with other phloem-limited bacteria and physiological disorders. Specific tests are therefore required for the detection and identification of ‘*Ca*. L. solanacearum’. Because of the inability to culture ‘*Ca*. L. solanacearum’ and the overall low titre in which this bacterium occurs in its host plants, molecular tests are required for detection and identification.

3.1 Symptoms

The above-ground plant symptoms associated with ‘*Ca*. L. solanacearum’ infection in potato and other solanaceous species (Figures 1 to 3) resemble those associated with phytoplasmas and include stunting, erectness of new foliage, chlorosis and purpling of foliage, upward rolling of leaves, shortened and thickened terminal internodes resulting in plant rosetting, enlarged nodes, axillary branches or aerial tuber formation, leaf scorching, disruption of fruit-set, and production of numerous small, misshapen, poor quality fruit. In potato, the below-ground symptoms characteristic of ‘*Ca*. L. solanacearum’ include collapsed stolons and browning of vascular tissue concomitant with necrotic flecking of internal tissues and streaking of the medullary ray tissues, all of which can affect the entire tuber. Freshly cut tubers, when infected, show in minutes necrotic browning in medullary ray tissue throughout the tuber (Figure 4). Upon frying, these symptoms become more pronounced and chips or fries processed from affected tubers show very dark blotches, stripes or streaks, rendering them commercially unacceptable (Figure 4). Symptoms in carrots associated with ‘*Ca*. L. solanacearum’ infection include leaf curling, yellowish, bronze and purplish discoloration of leaves, stunting of the shoots and roots, and proliferation of secondary roots (Figure 5) (Munyaneza *et al*., 2010; Nissinen *et al*., 2014). These symptoms resemble those associated with phytoplasmas and *Spiroplasma citri* in carrots (Lee et al., 2006; Cebrián *et al*., 2010; Munyaneza *et al*., 2011). In celery, vegetative disorders associated with the pathogen include an abnormal number of shoots per plant and curled stems (Figure 6) (Teresani *et al*., 2014).

3.2 Sampling

General guidance on sampling methodologies is provided in ISPM 31 (*Methodologies for sampling of consignments*).

3.2.1 Plants

The within-plant distribution of ‘*Ca*. L. solanacearum’ is highly variable; careful sampling is therefore required to improve the accuracy of diagnosis. Sampling protocols should consider that ‘*Ca*. L. solanacearum’ may not be detectable by polymerase chain reaction (PCR) until three weeks after infective psyllids have fed on the plants (Levy *et al*., 2011). If typical foliar symptoms are present, three to five leaves and/or stems should be collected from symptomatic parts of the plant. In asymptomatic plants, leaves and/or stems from five to ten different parts of the plant should be sampled and should include newly developing leaves (Levy *et al*., 2011; Cooper *et al*., 2015). Below-ground plant parts such as tubers, roots and stolons can also be used to detect ‘*Ca*. L. solanacearum’. Potato tubers showing obvious zebra chip symptoms will result in reliable detection. Detection from asymptomatic potato tubers will be less reliable and is not recommended, even if above-ground symptoms are present, as not all tubers from an infected plant will become infected by

'*Ca.* L. solanacearum' (Buchman *et al.*, 2011). The basal end (heel) of the tuber, the end which attaches to the stolon, is the recommended tissue to sample. Before extraction, all plant material is subsampled so that the material used contains as much vascular tissue as possible (e.g. petioles, leaf midribs, cambium, and the heel end or vascular ring of potato tubers).

3.2.2 Carrot seeds

Insufficient data exist to recommend a sample size and bulking rate for seed testing. The single study of seed transmission in carrot by Bertolini *et al.* (2014) detected '*Ca.* L. solanacearum' in samples of 500 carrot seeds. The International Seed Federation (ISF) recommends testing samples of 20 000 carrot seeds composed of two subsamples of 10 000 seeds.

3.2.3 Psyllids

Crosslin *et al.* (2011) determined that '*Ca.* L. solanacearum' can be reliably detected by conventional and real-time PCR in bulks of 30 laboratory-reared adult *B. cockerelli*. However, it is best to limit bulking to ten psyllids if they are sampled from the field by either sticky traps or hand collection. If the insects are collected from sticky traps, it is not necessary to remove the glue before DNA extraction. But if desired, the glue may be removed before testing as described by Bertolini *et al.* (2014) and Teresani *et al.* (2014). '*Ca.* L. solanacearum' can be reliably detected in infected psyllids for up to ten months on sticky traps stored inside at room temperature (Crosslin *et al.*, 2011). For long-term storage before testing, psyllids are preserved in 70% ethanol.

3.3 Molecular detection

PCR is the method of choice for the detection of '*Ca.* L. solanacearum'. Conventional PCR can be used, but real-time PCR is recommended because of its better sensitivity.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

3.3.1 Sample preparation

Plant material may be homogenized using one of a variety of methods. The method chosen is dependent on the nature of the plant material. Soft plant tissue can be ground using homogenizers (e.g. Bioreba HOMEX 6[1], handheld homogenizer) or bead beater machines (e.g. Roche MagNA Lyser Instrument[1], BioSpec BeadBeater[1]). Alternatively, homogenization can be carried out by hammering plant material contained in a stomacher bag with a rubber or wooden hammer. Hard plant tissue will need to be ground in a mortar with a pestle and if the tissue is very hard, the grinding will need to be aided with the addition of liquid nitrogen. Whichever grinding method is used, it is important that complete disruption of the plant vascular tissue is achieved in order to release any '*Ca.* L. solanacearum' present.

Seeds may be crushed with a pestle in a mortar, in a coffee grinder or inside a plastic bag using a hammer. The ISF protocol for carrot seed recommends bag-mixing (stomaching) rather than grinding. To remove fungicide treatments and to facilitate seed crushing, seeds are washed by shaking for 30 min in 1:10 (w/v) 0.5% Triton X-100 and, after several rinses, are left to soften in water overnight.

[1]In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

Psyllids are easily homogenized in microfuge tubes with micropestles.

3.3.2 Nucleic acid extraction

A wide range of methods are available for nucleic acid extraction. The following nucleic acid extraction kits, buffers and procedures have been used successfully for the extraction of '*Ca*. L. solanacearum' nucleic acid from plants and insects.

Samples may contain compounds that are inhibitory to PCR depending on the host species, plant tissue, age of the tissue and any treatments. It is important therefore to check the PCR competency of the DNA extractions using internal control primers that amplify a gene from the host. Inhibitory effects of the host can be overcome by further purifying the DNA through a sephacryl spin column (e.g. GE Healthcare illustra MicroSpin S-300 HR Columns[1]) or by adding bovine serum albumin to the PCR mixture at a final concentration of 0.5 mg/ml (Kreader, 1996).

3.3.2.1 CTAB extraction

DNA extraction from plant tissue is performed according to Munyaneza *et al*. (2010). In this method, 500 mg plant tissue is homogenized in 1 ml extraction buffer (100 mM Tris-HCl, pH 8.0, 50 mM ethylenediaminetetraacetic acid (EDTA), 500 mM NaCl and 10 mM 2-mercaptoethanol). The homogenate (300 µl) is mixed with 80 µl lysozyme (50 mg/ml in 10 mM Tris-HCl, pH 8.0) and incubated at 37 °C for 30 min. After incubation, 500 µl cetyl trimethylammonium bromide (CTAB) buffer (100 mM Tris, pH 8.0, 1.4 M NaCl, 50 mM EDTA, pH 8.0, 2% (w/v) CTAB, 1% (w/v) polyvinylpyrrolidone (PVP)-40 and 0.2% (v/v) 2-mercaptoethanol) is added to the homogenate and incubated at 65 °C for 30 min. The sample is allowed to cool at room temperature for 3 min before the addition of 500 µl ice-cold chloroform. Samples are mixed by vortexing and then centrifuged at 13 000 *g* for 10 min. The upper aqueous layer is transferred to a new microfuge tube, 0.6 volume of isopropanol is added and the tube is placed on ice for 20 min to precipitate the DNA. DNA is recovered by centrifugation as described above. The pellet is washed with ice-cold 75% ethanol and centrifuged at 13 000 *g* for 2 min. After removal of ethanol, the pellet is air-dried and resuspended in 100 µl sterile water.

DNA extraction from insects is described by Goodwin *et al*. (1994), where individual insects are homogenized in 125 µl CTAB extraction buffer (100 mM Tris, pH 8.0, 1.4 M NaCl, 20 mM EDTA, pH 8.0, 2% (w/v) CTAB and 1% (w/v) PVP-40). The homogenate 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 the 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 sterile water.

3.3.2.2 Commercial kits

Commercial kits based on silica spin columns (e.g. Qiagen DNeasy Plant Mini Kit[1] for plants, Qiagen DNeasy Blood and Tissue Kit[1] for insects) (Li *et al*., 2009) or magnetic beads (e.g. InviMag Plant DNA Mini Kit[1]) are used according to the manufacturer's instructions. The advantage of using magnetic beads is that the extractions can be performed on an automated workstation (e.g. Thermo Scientific KingFisher Magnetic Particle Processors[1]). For plant tissue that contains high levels of polyphenolic compounds (e.g. *S. betaceum*, *S. elaeagnifolium* and *S. ptycanthum*) a modified lysis step as described by Green *et al*. (1999) is recommended. The plant material is homogenized in CTAB extraction buffer (100 mM Tris, pH 8.0, 1.4 M NaCl, 50 mM EDTA, pH 8.0, 2.5% (w/v) CTAB, 1% (w/v) PVP-40 and 0.2% (v/v) 2-mercaptoethanol added just before use). The homogenate (0.5 ml) is transferred to a microcentrifuge tube, mixed by inversion with 22 µl ribonuclease (RNase) A (20 mg/ml) and incubated at 65 °C with intermittent shaking for 25–35 min. The homogenate is then processed according to the manufacturer's instructions from the commercial kit being used.

3.3.2.3 Tissue print or squash

For high-throughput screening of plant or psyllid samples, the tissue print or squash method described by Bertolini *et al*. (2014) and Teresani *et al*. (2014) may be used instead of DNA extraction. Fresh or frozen plant material or psyllids are immobilized by spotting 5 µl crude extract onto small pieces of positively charged nylon membranes or Whatman 3MM[1] filter paper held inside microfuge tubes. Spotted extracts are left to dry for 5 min and then stored at room temperature in the dark until required. The DNA is released by adding 100 µl distilled water, vortexing and placing on ice, and 3 µl is used as the template in PCR. This method is less sensitive than testing DNA extracts; these samples can therefore be tested only by real-time PCR, and the method is not recommended when a reliable result is critical.

3.3.3 Real-time PCR

Real-time PCR is performed using the assay of Li *et al*. (2009) or Teresani *et al*. (2014). Both assays are designed to target the same region of the16S rRNA gene. The assay of Li *et al*. (2009) is based on the real-time PCR of Li *et al*. (2006) designed to detect the three citrus-infecting '*Ca.* Liberibacter' species (Huanglongbing). All liberibacter species use the same reverse primer and probe, whereas the forward primer is specific to each liberibacter species. The assay was specific as no cross-reactivity was observed with phytoplasmas, viruses, *Xylella fastidiosa*, the citrus-infecting liberibacters and 64 DNA extracts from healthy potato plants both when run as a simplex reaction and when multiplexed with internal control primers that target the *cytochrome oxidase* (COX) gene (Li *et al*., 2009). The detection limit of the real-time PCR when multiplexed with the COX internal control primers was about 20 copies of the 16S rDNA templates of '*Ca*. L. solanacearum' for field-collected potato samples, and it was about tenfold more sensitive than conventional PCR with the LsoF/OI2c primer pair (Li *et al*., 2009).

The primers and probe for the '*Ca*. L. solanacearum' real-time PCR are:

LsoF (forward primer): 5′-GTC GAG CGC TTA TTT TTA ATA GGA-3′ (Li *et al*., 2009)

HLBr (reverse primer): 5′-GCG TTA TCC CGT AGA AAA AGG TAG-3′ (Li *et al*., 2006)

HLBp (TaqMan probe): 5′-FAM-AGA CGG GTG AGT AAC GCG-BHQ-3′ (Li *et al*., 2006)

The 25 µl reaction mixture consists of a final concentration of 1× TaqMan real-time PCR master mix, 250 nM of each primer, 120 nM probe and 2 µl DNA template. Depending on the master mix used, additional $MgCl_2$ may need to be added to ensure that the final concentration is 6.0 mM. All samples are tested in duplicate. The amplification conditions are an initial denaturation step of 95 °C for 20 s followed by 40 cycles of 95 °C for 1 s and 58 °C for 40 s. Cycling conditions may vary depending on the type of master mix and machine used (e.g. some mixes require a polymerase activation step of 95 °C for 10 min, mixes that contain uracil-DNA glycosylase (UDG) require an initial hold at 50 °C for 2 min, and the cycling may require longer than 1 s at 95 °C). Real-time PCR results are analysed with the manufacturer's software.

The presence of amplifiable DNA in the plant extracts can be confirmed using the COX primers and probe of Weller *et al*. (2000):

COX-F (forward primer): 5′-CGT CGC ATT CCA GAT TAT CCA-3′

COX-R (reverse primer): 5′-CAA CTA CGG ATA TAT AAG AGC CAA AAC TG-3′

COX-P (TaqMan probe): 5′-FAM-TGC TTA CGC TGG ATG GAA TGC CCT-BHQ-3′

The 25 µl reaction mixture consists of a final concentration of 1× TaqMan real-time PCR master mix, 100 nM of each primer, 50 nM probe and 2 µl DNA template. The amplification conditions are an initial hold step at 50 °C for 2 min and an initial polymerase activation step of 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min. Cycling conditions may vary depending on the type of master mix and machine used (e.g. some mixes do not require the UDG hold or polymerase activation steps described).

3.3.4 Conventional PCR

Conventional PCR is performed using the primers of Ravindran *et al*. (2011) that amplify the 16S-23S rRNA IGS region. These primers are specific to '*Ca*. L. solanacearum' and are more sensitive than the LsoF/OI2c primers (section 4.1.1). DNA extracted from a symptomatic potato plant was detected down to a dilution of 0.65 ng by Ravindran *et al*. (2011).

The primers for the '*Ca*. L. solanacearum' conventional PCR are:

Lso TX 16/23F (forward primer): 5′-AAT TTT AGC AAG TTC TAA GGG-3′

Lso TX 16/23R (reverse primer): 5′-GGT ACC TCC CAT ATC GC-3′

The 25 µl reaction mixture consists of a final concentration of 1× Taq DNA polymerase buffer containing 2 mM $MgCl_2$, 500 nM of each primer, 200 µM dNTPs, 0.5 U Taq DNA polymerase and 2 µl DNA template. The amplification conditions are an initial denaturation step of 98 °C for 30 s followed by 35 cycles of 98 °C for 10 s, 55 °C for 20 s and 72 °C for 30 s, and a final extension step of 72 °C for 7 min. Cycling conditions may vary depending on the type of master mix and machine used. The amplicon size is 383 base pairs (bp).

The presence of amplifiable DNA in the extracts can be confirmed using the general eukaryotic 28S rRNA gene primers of Werren *et al*. (1995):

28Sf (forward primer): 5′-CCC TGT TGA GCT TGA CTC TAG TCT GGC-3′

28Sr (reverse primer): 5′-AAG AGC CGA CAT CGA AGG ATC-3′

The reaction mixture for the 28S rRNA assay has the same components and is cycled under the same conditions as the '*Ca*. L. solanacearum' conventional PCR so the two assays can be run simultaneously in separate tubes. The 28Sf/28Sr primer pair produces a 500–600 bp amplicon: the size of the amplicon will vary depending on the presence of expansion domains.

3.3.5 Controls for molecular tests

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the target pest or target nucleic acid. For PCR a positive nucleic acid control, an internal control and a negative amplification control (no template control) are the minimum controls that should be used.

Positive nucleic acid control. This control is used to monitor the efficiency of the test method (apart from the extraction), and specifically the amplification. Pre-prepared (stored) DNA extracted from an infected host or a synthetic control (e.g. cloned PCR product) may be used.

Internal control. For conventional and real-time PCR, plant internal controls such as the general eukaryotic 28S rRNA gene (Werren *et al*., 1995) or the COX gene (Weller *et al*., 2000) should be incorporated into the protocol to eliminate the possibility of PCR false negatives due to nucleic acid extraction failure or degradation or the presence of PCR inhibitors.

Negative amplification control (no template control). This control is necessary for conventional and real-time PCR to rule out false positives due to contamination during preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

Additional controls that could be considered for each series of nucleic acid extractions from the test samples are described below.

Negative extraction control. This control is used to monitor contamination during nucleic acid extraction and/or cross-reaction with the host tissue. The control comprises nucleic acid that is extracted from uninfected host tissue and subsequently amplified. It is recommended that multiple controls be included when large numbers of positive samples are expected.

3.3.6 Interpretation of results from PCR

3.3.6.1 Real-time PCR

The real-time PCR will be considered valid only if the following criteria are met:

- the positive control produces an exponential amplification curve with the pathogen-specific primers
- the negative extraction control and the negative amplification control do not produce an amplification curve with the pathogen-specific primers.

For the COX internal control assay, the negative extraction control (if used), positive control and each of the test samples must produce an amplification curve. Failure of the samples to produce an amplification curve with the internal control primers suggests, for example, that the DNA extraction has failed, the DNA has not been included in the reaction mixture, compounds inhibitory to PCR are present in the DNA extract or the DNA has degraded.

A sample will be considered positive if it produces an exponential amplification curve. The cycle threshold (Ct) cut-off value needs to be verified in each laboratory when implementing the test for the first time.

3.3.6.2 Conventional PCR

The conventional PCR will be considered valid only if the following criteria are met:

- the positive control produces the correct size amplicon with the pathogen-specific primers
- the negative extraction control (if used) and the negative amplification control do not produce amplicons of the correct size with the pathogen-specific primers.

For the 28S rRNA internal control assay, the negative extraction control (if used), positive control and each of the test samples must produce an amplicon of the correct size. Note that synthetic and plasmid positive controls will not produce an amplicon. Failure of the samples to amplify with the internal control primers suggests, for example, that the DNA extraction has failed, the nucleic acid has not been included in the reaction mixture, compounds inhibitory to PCR are present in the DNA extract or the DNA has degraded.

A sample will be considered positive if it produces an amplicon of the correct size.

4. Identification

The minimum identification requirement for '*Ca*. L. solanacearum' is a positive result from one of the PCR tests described in this diagnostic protocol. Both tests are specific to '*Ca*. L. solanacearum', but if the outcome is critical (e.g. post-entry quarantine sample, new host record, new distribution), the conventional PCR (section 3.3.4) should be performed and the product should be sequenced. For the sequence to be considered as the same species as '*Ca*. L. solanacearum', it should be ≥98% identical to the sequence from the reference isolate (GenBank accession number EU834130).

4.1 Haplotype identification

The known haplotype can be determined by amplifying and sequencing three genomic regions, as described in the sections below.

4.1.1 16S rRNA gene

A 1 163 bp region of the 16S rRNA gene is amplified using the same forward primer as for the real-time PCR designed by Li *et al*. (2009) to a region of the 16S rRNA gene that is unique to '*Ca*. L. solanacearum'. The forward primer is used in combination with the universal liberibacter reverse primer of Jagoueix *et al*. (1996).

The primers for the '*Ca*. L. solanacearum' conventional PCR are:

LsoF (forward primer): 5′-GTC GAG CGC TTA TTT TTA ATA GGA-3′ (Li *et al*., 2009)

OI2c (reverse primer): 5′-GCC TCG CGA CTT CGC AAC CCA T-3′ (Jagoueix *et al*., 1996)

The 25 µl reaction mixture consists of a final concentration of 1× Taq DNA polymerase buffer containing 2.5 mM $MgCl_2$, 200 nM of each primer, 200 µM dNTPs, 1 U Taq DNA polymerase and 2 µl DNA template. The amplification conditions are an initial denaturation step of 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 1 min, and a final extension step of 72 °C for 10 min. Cycling conditions may vary depending on the type of master mix and machine used.

4.1.2 16S-23S rRNA IGS region

The 16S-23S rRNA IGS region is amplified using the Lso TX 16/23F / Lso TX 16/23R primer pair as described in section 3.3.4. These primers will fail to amplify the 16S-23S rRNA IGS region containing the last five SNP differences between haplotypes.

4.1.3 *rplJ-rplL* ribosomal protein genes

The partial 50S *rplJ* and *rplL* ribosomal protein genes are amplified using the primers of Munyaneza *et al*. (2009):

CL514F (forward primer): 5′-CTC TAA GAT TTC GGT TGG TT-3′

CL514R (reverse primer): 5′-TAT ATC TAT CGT TGC ACC AG-3′

The 25 µl reaction mixture consists of a final concentration of 1× Taq DNA polymerase buffer containing 2 mM $MgCl_2$, 400 nM of each primer, 400 µM dNTPs, 1 U Taq DNA polymerase and 2 µl DNA template. The amplification conditions are an initial denaturation step of 94 °C for 30 s followed by 40 cycles of 94 °C for 30 s, 53 °C for 30 s and 72 °C for 30 s, and a final extension step of 72 °C for 7 min. Cycling conditions may vary depending on the type of master mix and machine used. The amplicon size is 669 bp.

4.1.4 Haplotype sequence analysis

The sequence from the unknown haplotype is aligned with the reference sequences for the 16S rRNA gene and the 16S-23S rRNA IGS region (GenBank accession number EU812559) and the 50S *rplJ* and *rplL* ribosomal protein genes (GenBank accession number EU834131). The haplotype is determined by comparing the sequence at each of the nucleotide positions listed in Table 1.

Table 1. Single-nucleotide polymorphism differences between haplotypes of '*Candidatus* Liberibacter solanacearum'

Region (gene / position)	**Haplotype†**				
	A	B	C	D	E
16S rRNA / 116	C	C	C	T	C
16S rRNA / 151	A	A	A	A	G
16S rRNA / 212	T	G	T	T	T
16S rRNA / 581	T	C	T	T	T
16S rRNA / 959	C	C	C	C	T
16S rRNA / 1049	A	A	G	G	A
16S rRNA / 1073	G	G	G	A	G
16S-23S rRNA IGS / 1620	A	A	A	A	G
16S-23S rRNA IGS / 1632	A	A	A	A	G
16S-23S rRNA IGS / 1648	G	G	G	G	A

Region (gene / position)	**Haplotype†**				
16S-23S rRNA IGS / 1742	A	A	A	G	A
16S-23S rRNA IGS / 1748	C	C	C	T	C
16S-23S rRNA IGS / 1858	–	G	G	–	–
16S-23S rRNA IGS / 1859	–	T	–	–	–
16S-23S rRNA IGS / 1860	T	T	T	–	T
16S-23S rRNA IGS / 1873	A	A	A	A	G
16S-23S rRNA IGS / 1920	T	T	C	T	T
16S-23S rRNA IGS / 1943	G	A	G	G	Unknown
16S-23S rRNA IGS / 2055	C	T	C	C	Unknown
16S-23S rRNA IGS / 2081	G	G	G	A	Unknown
16S-23S rRNA IGS / 2218	G	A	G	G	Unknown
16S-23S rRNA IGS / 2260	C	T	C	C	Unknown
50S *rplJ* and *rplL* / 583	G	G	C	G	G
50S *rplJ* and *rplL* / 622	A	A	A	G	A
50S *rplJ* and *rplL* / 640	C	C	T	C	C
50S *rplJ* and *rplL* / 669	G	C	G	G	G
50S *rplJ* and *rplL* / 689	C	C	C	T	T
50S *rplJ* and *rplL* / 691	G	T	T	G	G
50S *rplJ* and *rplL* / 700	A	A	A	G	A
50S *rplJ* and *rplL* / 712	G	T	G	G	G
50S *rplJ* and *rplL* / 722	G	G	G	G	A
50S *rplJ* and *rplL* / 749	C	C	C	A	C
50S *rplJ* and *rplL* / 780	–	–	A	A	A
50S *rplJ* and *rplL* / 786	G	A	G	G	G
50S *rplJ* and *rplL* / 850	T	T	T	C	C
50S *rplJ* and *rplL* / 909	T	C	C	C	C
50S *rplJ* and *rplL* / 920	T	C	C	T	T
50S *rplJ* and *rplL* / 922	–	–	TGT	–	–
50S *rplJ* and *rplL* / 955	G	G	T	G	G
50S *rplJ* and *rplL* / 987	T	G	G	G	G
50S *rplJ* and *rplL* / 993	A	A	G	A	A
50S *rplJ* and *rplL* / 1041	G	A	A	G	G
50S *rplJ* and *rplL* / 1049	A	G	A	A	A
50S *rplJ* and *rplL* / 1072	C	C	C	T	C
50S *rplJ* and *rplL* / 1107	G	A	G	G	G
50S *rplJ* and *rplL* / 1110	–	–	C	–	–
50S *rplJ* and *rplL* / 1122	G	A	A	A	A
50S *rplJ* and *rplL* / 1143	G	A	G	G	G

Source: Adapted from Nelson *et al.* (2013b) and Teresani *et al.* (2014).

IGS, intergenic spacer (region); rRNA, ribosomal RNA.
[†] Dashes represent a deletion at that position.

5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

In cases where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance (ISPM 13 (*Guidelines for the notification of non-compliance and emergency action*)) and where '*Ca.* L. solanacearum' is found in an area for the first time, the following records and evidence and additional material should be kept for at least one year in a manner that ensures traceability:

- the original sample should be kept frozen at −80 °C or freeze-dried, or dried over calcium chloride and kept at 4 °C
- if relevant, DNA extractions should be kept at −20 °C or at −80 °C, and plant extracts spotted on membranes should be kept at room temperature
- if relevant, PCR amplification products should be kept at −20 °C or at −80 °C.

6. Contact Points for Further Information

Further information on this protocol can be obtained from:

Plant Health and Environment Laboratory, Ministry for Primary Industries, PO Box 2095, Auckland 1140, New Zealand (Lia W. Liefting; e-mail: lia.liefting@mpi.govt.nz; tel.: +64 9 909 5726; fax: +64 9 909 5739).

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United States Department of Agriculture, Agricultural Research Service (USDA-ARS), Yakima Agricultural Research Laboratory, 5230 Konnowac Pass Road, Wapato, WA 98901, United States of America (Joseph E. Munyaneza; e-mail: joseph.munyaneza@ars.usda.gov; tel.: +1 509 454 6564; fax: +1 509 454 5646).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

7. Acknowledgements

The first draft of this protocol was written by L.W. Liefting (Plant Health and Environment Laboratory, Ministry for Primary Industries, New Zealand (see preceding section)), M.M. López (Centro de Protección Vegetal y Biotecnología, IVIA, Spain (see preceding section)) and J.E. Munyaneza (USDA-ARS, United States of America (see preceding section)).

8. References

The present annex refers to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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9. Figures

Figure 1. Early infection of '*Candidatus* Liberibacter solanacearum' in *Solanum tuberosum* (potato).
Photo courtesy J.E. Munyaneza, United States Department of Agriculture, Agricultural Research Service, Wapato, WA, United States of America.

Figure 2. '*Candidatus* Liberibacter solanacearum' infection in *Solanum lycopersicum* (tomato).
Photo courtesy L.W. Liefting, Plant Health and Environment Laboratory, Ministry for Primary Industries, Auckland, New Zealand.

Figure 3. '*Candidatus* Liberibacter solanacearum' infection in *Capsicum annuum* (pepper).
Photo courtesy J.E. Munyaneza, United States Department of Agriculture, Agricultural Research Service, Wapato, WA, United States of America.

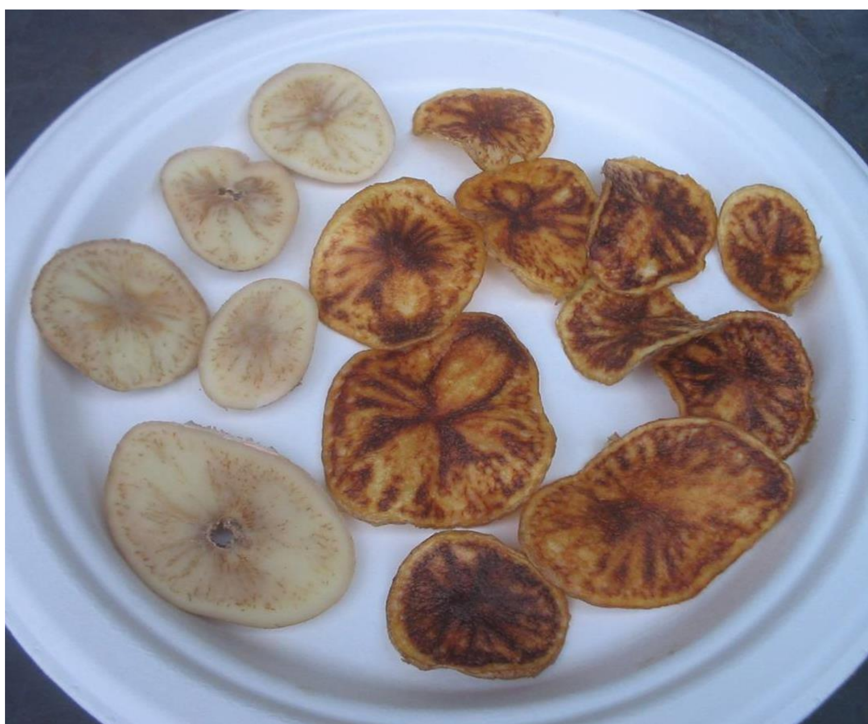

Figure 4. Slices of raw (left) and fried (right) tubers of *Solanum tuberosum* (potato) infected with '*Candidatus* Liberibacter solanacearum'.
Photo courtesy J.E. Munyaneza, United States Department of Agriculture, Agricultural Research Service, Wapato, WA, United States of America.

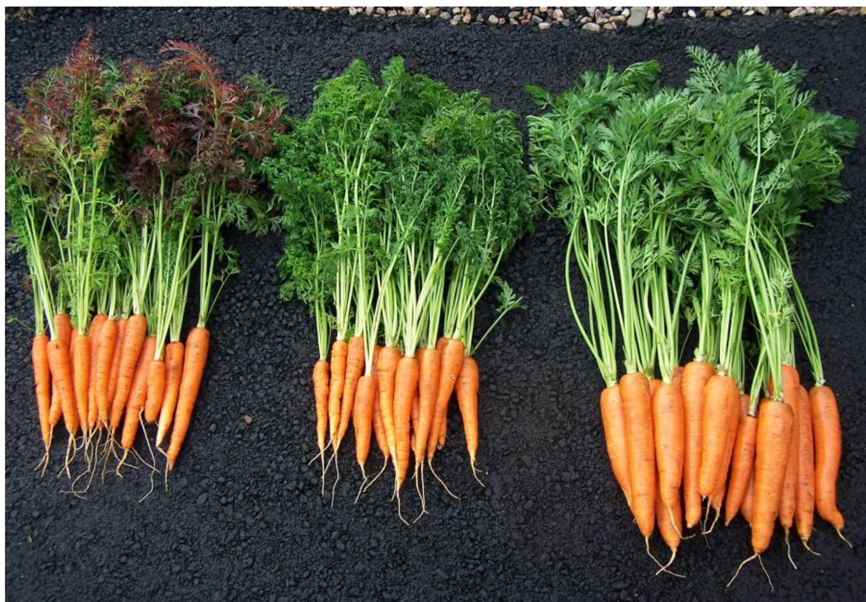

Figure 5. '*Candidatus* Liberibacter solanacearum' infection in *Daucus carota* subsp. *sativus* (carrot), showing leaf discoloration, leaf curling and reduced root size (left and middle), compared with uninfected control plants (right).
Photo courtesy J.E. Munyaneza, United States Department of Agriculture, Agricultural Research service, Wapato, WA, United States of America.

Figure 6. '*Candidatus* Liberibacter solanacearum' infection in *Apium graveolens* (celery), showing an abnormal number of shoots and curling of stems.
Photo courtesy M.M. López, Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain.

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IPPC

The International Plant Protection Convention (IPPC) is an international plant health agreement that aims to protect cultivated and wild plants by preventing the introduction and spread of pests. International travel and trade are greater than ever before. As people and commodities move around the world, organisms that present risks to plants travel with them.

Organization

- There are over 180 contracting parties to the IPPC.
- Each contracting party has a national plant protection organization (NPPO) and an Official IPPC contact point.
- Nine regional plant protection organizations (RPPOs) work to facilitate the implementation of the IPPC in countries.
- IPPC liaises with relevant international organizations to help build regional and national capacities.
- The Secretariat is provided by the Food and Agriculture Organization of the United Nations (FAO).

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