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Diagnostics

PM 7/143 (1) *'Candidatus* Liberibacter solanacearum'

Specific scope

This Standard describes a diagnostic protocol for '*Candidatus* Liberibacter solanacearum', and for its detection in the psyllid vectors *Bactericera cockerelli*, *Trioza apicalis* and *Bactericera trigonica*.[1] This Standard should be used in conjunction with PM 7/76 Use of EPPO diagnostic protocols.

Specific approval and amendment

2019–09

This Diagnostic Protocol was prepared in parallel to the IPPC Diagnostic Protocol adopted in 2017 on '*Candidatus* Liberibacter solanacearum' (Annex 21 to ISPM 27; IPPC, 2017). The EPPO Diagnostic Protocol differs in terms of format but it is consistent with the content of the IPPC Standard. With regard to molecular methods, one real-time PCR test used in the EPPO region is included, and more information on the different haplotypes is included as well as additional information on reference accessions.

1. Introduction

'*Candidatus* Liberibacter solanacearum' is a Gram-negative bacterium. It is restricted to the host plant's phloem and psyllid vector's hemolymph, alternating its life cycle between host plants and insect vectors. '*Ca*. L. solanacearum' has not been cultivated in axenic medium yet. In North and Central America and Oceania it 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* (tomatillo), *Solanum elaeagnifolium* (silverleaf nightshade), *Solanum ptychanthum* (black nightshade), *Lycium barbarum* (wolfberry) and other crops or weeds in the family *Solanaceae* (EPPO 2013; Haapalainen, 2014). In the EPPO region, '*Ca*. L. solanacearum' has been associated with symptoms in species of the *Apiaceae* family, including *Daucus carota* (carrot), *Apium graveolens* (celery), *Pastinaca sativa* (parsnip), *Petroselinum crispum* (parsley), *Anthriscus cerefolium* (chervil) and *Foeniculum vulgare* (fennel) (EPPO 2013; Alfaro-Fernández *et al*., 2014, 2017 ; Teresani *et al*., 2014; Hajri *et al*., 2017). In addition, '*Ca*. L. solanacearum' was found on *Urtica dioica* (stinging nettle) in Finland (Haapalainen *et al*., 2018a).

'*Ca*. Liberibacter solanacearum' is transmitted by different psyllids species in a propagative, circulative and persistent manner. The tomato/potato psyllid *Bactericera cockerelli* has been described as the vector of haplotypes A and B in solanaceous crops (Munyaneza *et al*., 2007; Buchman *et al*., 2011; Sengoda *et al*., 2014). Evidence of effective transovarial transmission of '*Ca*. L. solanacearum' has been provided in *B. cockerelli* (Hansen *et al*., 2008). '*Ca*. L. solanacearum' is transmitted to apiaceous species by *Trioza apicalis* (Nissinen *et al*., 2014) and *Bactericera trigonica* (Antolínez *et al*., 2016, 2017; Teresani *et al*., 2017). *T. apicalis* has been reported as a vector of haplotype C (Nissinen *et al*., 2014), and *B. trigonica* as a vector of haplotypes D and E in Spain (Nelson *et al*., 2012; Teresani *et al*., 2015, 2017; Antolínez *et al*., 2016, 2017). There are also reports of '*Ca*. L. solanacearum' detection, but not transmission, in *Bactericera tremblayi* collected from carrots (Teresani *et al*., 2015; Antolínez *et al*., 2017). '*Ca*. L. solanacearum' was also detected in *B. nigricornis*, *Trioza urticae* and *Trioza anthrisci* in Spain, Finland, Germany and the United Kingdom (Teresani *et al*., 2015; Haapalainen *et al*., 2018a; Sjölund *et al*., 2017, 2018), but no study was conducted to determine their potential as a vector of the bacterium. In addition, '*Ca*. L. solanacearum' can be transmitted by propagative plant material and, as shown in experimental setup, it can also be transmitted by *Cuscuta*

[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.

campestris (dodder) to *Catharanthus roseus* (periwinkle) and other herbaceous plants (Bertolini *et al*., 2015). Although the presence of the bacterium has been detected in seeds of chili pepper (Camacho-Tapia *et al*., 2011), parsley (Monger & Jeffries, 2016) and parsnip (Morán *et al*., unpublished data), bacterial seed transmission has only been suggested for carrot seeds and only in one study (Bertolini *et al*., 2015). Those results have never been reproduced (Loiseau *et al*., 2017a,b).

Seven haplotypes of '*Ca*. L. solanacearum' have been described (Nelson *et al*., 2011, 2012; Teresani *et al*., 2014; Haapalainen *et al*., 2018a; Swisher Grimm & Garczynski, 2018). Three haplotypes (A, B and F) are known so far to be associated with diseases caused by this bacterium in potatoes and other solanaceous crops, whereas the C, D and E haplotypes are known to be associated with apiaceous species. In 2018, a new haplotype U was described on *Urtica dioica* (stinging nettle). Haplotype A has been detected primarily from Honduras and Guatemala through Western Mexico to the USA (Arizona, California, Oregon, Washington and Idaho) and in New Zealand. Haplotype B has been detected in Mexico and the USA. Haplotype F has been detected in the USA on a single tuber (Swisher Grimm & Garczynski, 2018). Haplotype C was detected in Finland, Sweden, Norway, Germany and Austria (Haapalainen, 2014; Munyaneza *et al*., 2015; EPPO Global Database, 2019). Haplotypes D and E have been detected in the Canary Islands and in mainland Belgium, Spain, France, Greece, Tunisia, Morocco and Portugal (Haapalainen, 2014; Tahzima *et al*., 2014; Teresani *et al*., 2014; Hajri *et al*., 2017; Holeva *et al*., 2017; Ben Othmen *et al*., 2018; EPPO, 2019). Haplotype D has been detected in Israel (EPPO, 2017). An outbreak was detected in Italy (Sicily) and haplotype D was detected in one sample (Catara, pers. comm., 2019). Finally, an outbreak has been reported in Estonia (EPPO, 2018; haplotype not known). Information can also be retrieved from the EPPO Global Database (EPPO, 2019).

'*Ca*. Liberibacter solanacearum' (mainly haplotype D) was detected in old commercial seed (the earliest dating from 1973) from countries not previously reporting the presence of this bacterium in Apiaceae species: the Czech Republic, Denmark, Egypt, Japan, Netherlands, the Soviet Union, Syria, the United Kingdom and the USA (Monger & Jeffries, 2018). Haplotypes D and E of '*Ca*. Liberibacter solanacearum' have also been detected in commercial carrot seeds lots (Ilardi *et al*., 2016).

The discovery in Finland of asymptomatic potato volunteers infected with haplotype C (Haapalainen *et al*., 2018b) and symptomatic ware potato tubers infected with haplotype E in Spain (Palomo *et al*., 2014) would suggest that all haplotypes can infect potato, but transmission is limited between the different plant families because of the lack of a vector that is able to feed efficiently on plants in both families. In addition, with regards to the detection in ware tubers, Palomo *et al*. (2014) state that 'these data would indicate that this haplotype could have sporadically infected the potato and would not have any repercussion epidemiological or economic'. Similarly, the solanaceous-infecting haplotype B can infect carrot (Munyaneza *et al*., 2016).

Consequently, although EPPO recommends regulation of *Solanaceae* haplotypes of '*Ca*. L. solanacearum' only (EPPO, 2012), testing for other hosts than *Solanaceae* is included in this Diagnostic Protocol.

Detailed information on the distribution of '*Ca*. Liberibacter solanacearum' can be found in Global Database (EPPO, 2019).

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

2. Identity

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

Synonyms: '*Candidatus* Liberibacter psyllaurous' (Hansen *et al*., 2008)

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

EPPO Code: LIBEPS

Phytosanitary categorization: EPPO A1 list no. 365 (for *Solanaceae* haplotypes)

3. Detection

The symptoms associated with '*Ca*. L. solanacearum' are not always easy to distinguish from those associated with phytoplasmas, *Spiroplasma citri* or other biotic factors as well as from those associated with abiotic factors. Several tests have been developed for the detection of '*Ca*. L. solanacearum' in plant material and vectors, and include conventional PCR (Hansen *et al*., 2008; Li *et al*., 2009; Liefting *et al*., 2009a,b; Lin *et al*., 2009, 2011; Munyaneza *et al*., 2009; Secor *et al*., 2009; Wen *et al*., 2009, 2011; Crosslin *et al*., 2011; Pitman *et al*., 2011; Ravindran *et al*., 2011) and real-time PCR (Li *et al*., 2009; Teresani *et al*., 2014).

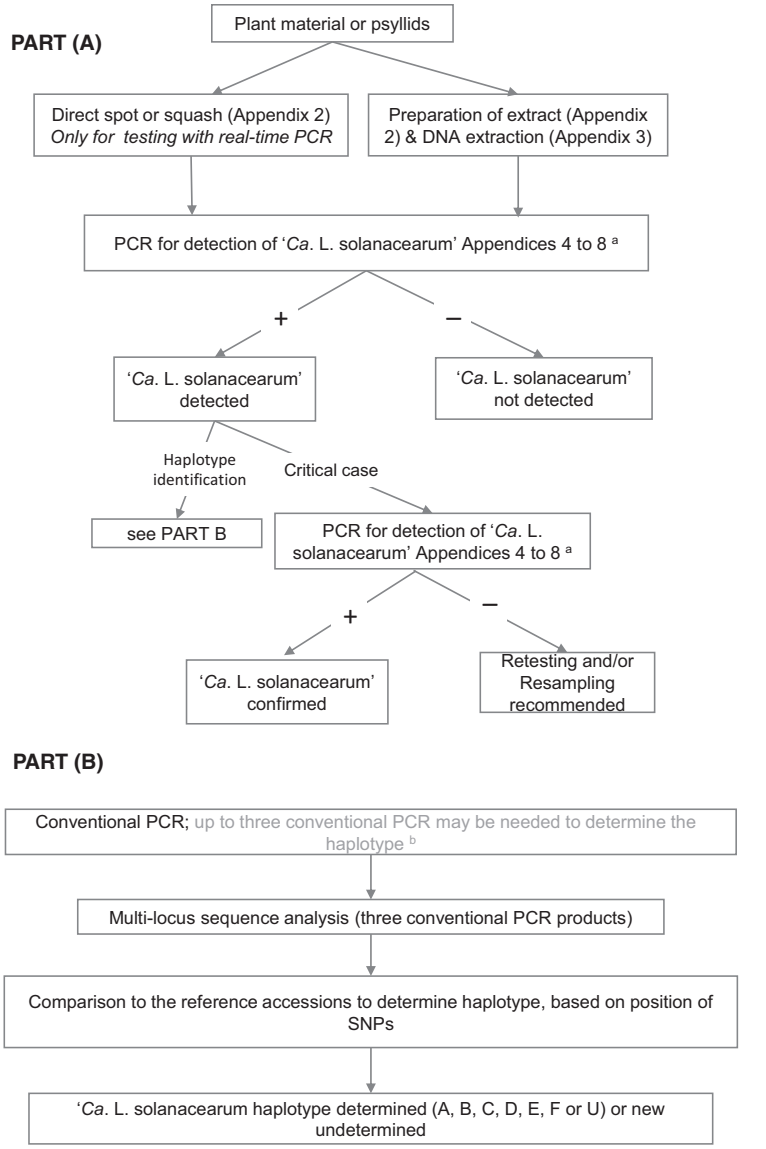
The most widely used reagents and PCR tests are presented in Appendices 4–8.

3.1. Disease symptoms

The descriptions of symptoms provided below are according to Munyaneza (2012), Haapalainen (2014) and Teresani *et al*. (2014). Symptoms (such as deformations) can be seen on all or parts of the plants, but plants may remain asymptomatic.

3.1.1. Symptoms on Solanum tuberosum *(potato): zebra chip*

A wide range of symptoms similar to potato purple top and psyllid yellows, including chlorosis, twisted stems with a zigzag appearance, proliferation of axillary buds, shortened



a) real-time PCR is recommended because of its better analytical sensitivity
b) It should be noted that it is not always possible to obtain sequences for the three genomic regions

Fig. 1 Flow diagram for the detection and identification of '*Candidatus* Liberibacter solanacearum' (Part A) and haplotype determination (Part B). [Colour figure can be viewed at wileyonlinelibrary.com]

internodes, swollen nodes, aerial tubers, vascular discoloration, and leaf scorching and wilting are associated with '*Ca*. L. solanacearum' in potato crops (Fig. 2). The specific symptoms in tubers consist of collapsed stolons, browning of vascular tissue and medullary rays throughout the entire length of the tuber (Fig. 3A). '*Ca*. L. solanacearum' has been shown to severely disrupt carbohydrate flow in potato plants, leading to zebra chip symptoms. This is usually very visible when tuber slices are fried (Fig. 3B) but can also be observed in a transversal cut of the tubers in the field or in storage. The optimum development of zebra chip symptoms was observed at a daily temperature regime of 27–32°C.

3.1.2. Symptoms on Solanum lycopersicum *(tomato): psyllid yellows*

On tomato, the symptoms associated with '*Ca*. L. solanacearum' are named psyllid yellows. The symptoms are similar to those caused by a toxin associated with feeding by the psyllid nymphal instars. Symptoms include spiky, chlorotic apical growth, general mottling of the leaves, curling of the midveins, overall stunting of the plants and in some cultivars fruit deformation (some of the symptoms are shown in Fig. 4). The severity of damage can vary between tomato cultivars and levels of disease prevalence.

Fig. 2 Potato zebra chip symptoms. Chlorosis, twisted stems with a zigzag appearance, proliferation of axillary buds, shortened internodes, swollen nodes, aerial tubers, vascular discoloration, and leaf scorching and wilting. Courtesy of G. Secor.

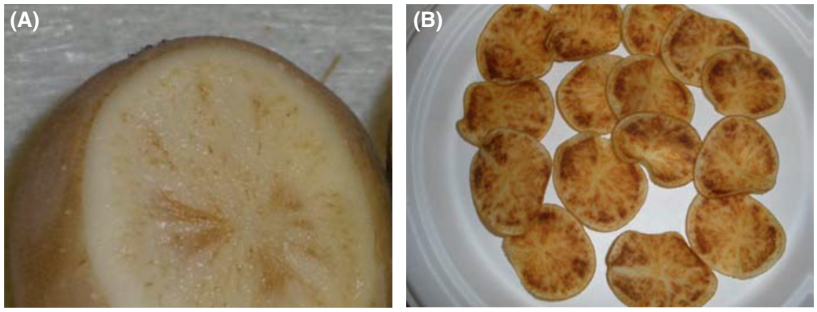


Fig. 3 Potato zebra chip symptoms. (A) Browning of vascular tissue and medullary rays throughout the entire length of the tuber. (B) Necrotic flecking and streaking of the medullary ray tissue symptoms in processed chips or fries. Courtesy of G. Secor.

3.1.3. Symptoms on Daucus carota *(carrot): yellow declines*
On carrot the symptoms associated with '*Ca*. L. solanacearum' are named yellow decline. Symptoms include leaf curling, yellowish, bronze and purplish discoloration of leaves, stunting of the carrot shoots and roots, and proliferation of secondary roots. The symptoms collectively resemble those caused by leafhopper-transmitted '*Candidatus* Phytoplasma' and *Spiroplasma citri* (Fig. 5) (Cebrián *et al*., 2010; Munyaneza *et al*., 2011).

3.1.4. Symptoms on Apium graveolens *(celery) and* Pastinaca sativa *(parsnip): vegetative disorders*
Vegetative disorders are the syndrome associated to '*Ca*. L. solanacearum' in infected celery plants. They show an abnormal number of shoots, curling of stems and yellowing (Fig. 6). Severe stunting was described on *A. graveolens*

Fig. 4 Tomato psyllid yellows symptoms. Spiky, chlorotic apical growth, general mottling of the leaves, curling of the midveins. Courtesy of J.E. Munyaneza.

Fig. 5 Carrot yellow decline symptoms. (A) Leaf curling, yellowish, bronze and purplish discoloration of leaves, (B) and (C) stunting of the carrot shoots and roots, and (C) proliferation of secondary roots. Courtesy of IVIA.

Fig. 6 Celery vegetative disorders: abnormal number of shoots, curling of stems, and yellowing. Courtesy of IVIA.

var. *rapaceum* (Mill) (Teresani *et al*., 2014). On parsnip the vegetative disorders include yellowish, leaf proliferation, root deformation and early senescence as well as proliferation of secondary roots (Alfaro-Fernández *et al*., 2017).

3.2. Sampling for laboratory testing

General guidance on sampling methodologies is provided in ISPM 31 (*Methodologies for sampling of consignments*), which provides useful information on the number of plants to be sampled[2] .

[2]ISPM 31 provides information on the numbers of units to be sampled which is considered useful to determine sample sizes for both consignments and places of production

3.2.1. Sampling of plants

The distribution of '*Ca*. L. solanacearum' in plant parts may be heterogeneous depending on the plant species and consequently appropriate sampling is required to improve detection. It should be noted that '*Ca*. L. solanacearum' may not be detectable by molecular tests until three weeks after infective psyllids have fed on the plants (Levy *et al*., 2011). In experiments conducted in Spain (under both field and greenhouse conditions) it has been shown that both symptoms and bacterial titre of haplotypes D and E can decrease at temperatures higher than 26°C (Lopez, pers. comm., 2019, unpublished results).

3.2.1.1. Plant material (except seeds). Plant material (leaves, petioles, midribs, stems, tubers and roots) is

collected from any potential host. Care should be taken to avoid cross-contamination between samples (hand collection or disinfected tools).

When typical foliar symptoms are present it is recommended to collect three to five leaves and/or stems from symptomatic parts of the plant. Experiments conducted in the framework of the POnTE project in 2017 have shown that for carrot and celery no significant differences were recorded when testing different parts of the plants, indicating that in these plants '*Ca*. L. solanacearum' is homogenously distributed (Loiseau *et al*., 2018).

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; Teresani *et al*., 2014; Cooper *et al*., 2015). Belowground plant parts such as tubers, roots and stolons can also be used to detect '*Ca*. L. solanacearum'.

Potato tubers showing obvious zebra chip symptoms should be tested individually. The tuber is cut and symptomatic tissue from the vascular area and the heel end is sampled. Detection from asymptomatic potato tubers will be less reliable and is not recommended, even if aboveground symptoms are present, as not all tubers from an infected plant will become infected by 'Ca. L. solanacearum' (Buchman *et al*., 2011).

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).

3.2.1.2. Seeds. Insufficient data exists to recommend a sample size and bulking rate for seed testing. In their study on seed transmission, Bertolini *et al*. (2015) detected '*Ca*. L. solanacearum' in samples of 500 carrot seeds. The International Seed Federation (ISF, 2015) recommends testing samples of 20 000 carrot seeds composed of two subsamples of 10 000 seeds.

3.2.2. Sampling of vectors for testing

Adults of *B. cockerelii*, *B. trigonica* and *T. apicalis* reported as vectors (see Fig. 7A–C), or of other psyllids suspected to be vectors, can be collected from symptomatic or asymptomatic plants. Some of these vectors overwinter as adults and can be collected during winter on conifers or weeds (Wenninger *et al*., 2017; Čermák & Lauterer, 2008; Kristofferson & Anderbrant, 2007). Whenever possible, psyllids should be identified before testing for '*Ca* L. solanacearum'. Morphological identification is possible based on keys published by Ossiannilsson (1992) or Ouvard (2017).

The bacterium is present in several organs and tissues of its vectors, including the alimentary canal, salivary glands, hemolymph and bacteriomes (Cooper *et al*., 2013). Crosslin *et al*. (2011) determined that '*Ca*. L. solanacearum' can be reliably detected by conventional and real-time PCR in bulk samples of 30 laboratory-reared adult *B. cockerelli*. However, experience in the EPPO region shows that 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. 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

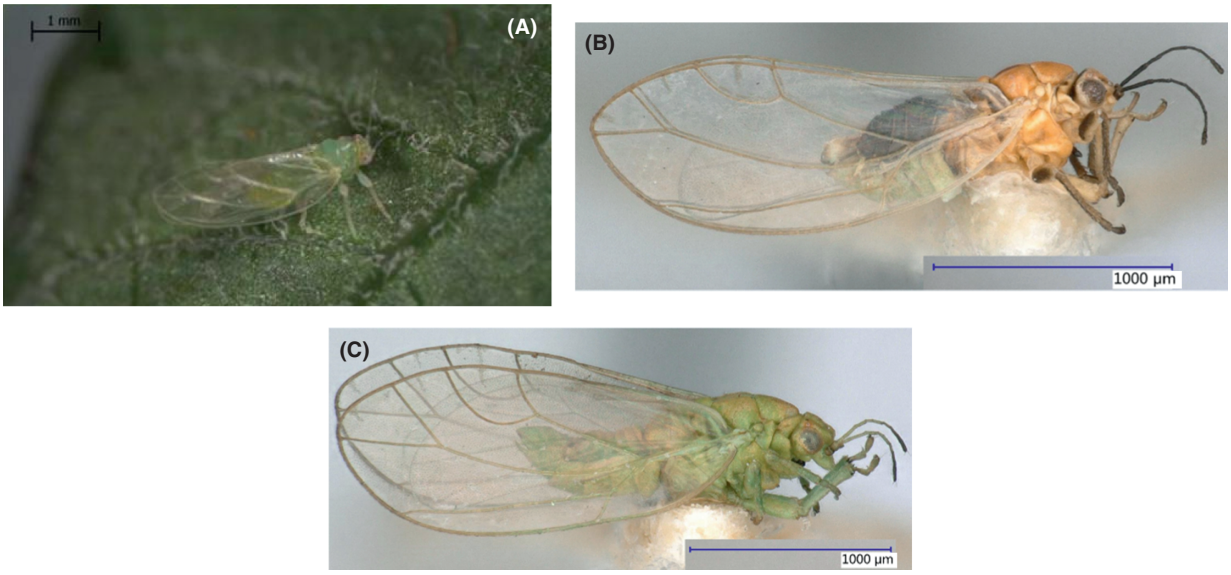


Fig. 7 (A) *Bactericera cockerelli* newly emerged adult, (B) adult female *Bactericera trigonica* and (C) adult female *Trioza apicalis*. Courtesy A: Oregon State University; B and C I Malenovský (Masaryk University).

Table 1. The performance characteristics of the different tests included in this protocol in terms of performance criteria (unpublished data)

	Real-time PCR, Teresani *et al*. (2014) (Appendix 4)	Real-time PCR adapted from Li *et al*. (2009) (see Appendix 5)	PCR, Li *et al*. (2009) and Jagoueix *et al*. (1996) (Appendix 6)	PCR, Ravindran *et al*. (2011) (Appendix 7)	PCR, Munyaneza *et al*. (2009) (Appendix 8)
Accuracy	97.3%	96.3%	93.7%	96.0%	97.3%
Diagnostic sensitivity	96.2%	100.0%	89.2%	93.4%	94.7%
Diagnostic specificity	98.5%	92.6%	98.0%	98.5%	100.0%
Analytical sensitivity*					
7.9×10^4	95%	100%	60%	88%	74%
1.7×10^4	93%	98%	45%	80%	56%
8.2×10^3	90%	100%	33%	78%	35%
1.6×10^3	17%	98%	8%	40%	6%
2.1×10^2	7%	50%	3%	8%	3%
Average repeatability	96.8%	97.5%	95.2%	97.1%	96.2%
Reproducibility	88.3%	94.3%	78.7%	84.2%	83.8%

*The probability of detection of the target at the different levels of dilution. The level is given as the concentration of bacteria for 1 g of biological material.

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. Sample preparation

Details on sample preparation are provided in Appendix 2.

3.4. Screening tests

Two real-time PCR tests are recommended for the detection of '*Ca*. Liberibacter solanacearum' in both asymptomatic and symptomatic plant material or in vectors.

- Teresani *et al*. (2014) is described in Appendix 4 and can be used for direct testing of crude extracts without DNA extraction. The test has been validated by the Instituto Valenciano de Investigaciones Agrarias (IVIA) in national and international test performance studies.
- Li *et al*. (2009) is described in Appendix 5. Validation data for this test has been generated by Anses-FR.

The three conventional PCR tests described in section 4 for haplotype identification (see Appendices 6–8) can also be used as screening tests.

3.5. Comparison of the screening tests

An international test performance study involving 26 laboratories from 14 countries (including non-EPPO countries) was organized in the framework of the following projects: POnTE (H2020), PhyLib II (Euphresco) and CaLiso (French funded project). All molecular tests included in this Diagnostic Protocol have been evaluated on DNA extracted and five different concentration levels (see table below). Five positive duplicate DNA samples including the five haplotypes known in 2017 and five negative duplicate DNA samples were provided to participants (Table 1).

A national test performance study of a diagnostic protocol for '*Ca*. L. solanacearum' in carrot seed was organized in Italy. This study was based on an adapted version of Li *et al*. (2009), different from the version recommended in this protocol (primer concentration and DNA input). The test involved 11 Italian laboratories that received both the samples (seeds and DNA). The results of this evaluation have been posted on the EPPO database on diagnostic expertise (section validation http://dc.eppo.int/validationlist.php) and published in Ilardi *et al*. (2018).

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. Confirmation is recommended for critical cases, as described in PM 7/76 (EPPO, 2018), after '*Ca*. L. solanacearum' has been detected by one rapid screening test. A conventional PCR 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. Determination of haplotypes

The haplotype can be determined by amplifying and sequencing up to three genomic regions[3] . The tests are:

(i) Li *et al*. (2009) targeting the 16S rRNA gene region (Appendix 6).

[3]The Panel on Diagnostics in Bacteriology noted that ISPM 27 Appendix 21 recommends that haplotype identification should be performed by amplifying and sequencing up to three genomic regions, but it is the experience of laboratories in the EPPO region that haplotype identification may be possible with less than three genomic regions

Table 2. Single-nucleotide polymorphism differences between haplotypes of '*Candidatus* Liberibacter solanacearum'. Source: Adapted from Nelson *et al*. (2013), Teresani *et al*. (2014), Swisher Grimm & Garczynski (2018); Haapalainen *et al*. (2018a). - = deletion

	Haplotype						
Region (gene/position of reference sequence EU812559, EU834131)	A	B	C	D	E	F	U
16S rRNA							
16S rRNA/115	A	A	A	A	G	A	A
16S rRNA/116	C	C	C	T	C	C	C
16S rRNA/151	A	A	A	A	G	G	A
16S rRNA/212	T	G	T	T	T	T	T
16S rRNA/359	A	A	A	A	A	C	A
16S rRNA/524	G	G	G	G	G	A	G
16S rRNA/581	T	C	T	T	T	C	T
16S rRNA/959	C	C	C	C	T	C	C
16S rRNA/1039	A	A	G	G	A	G	G
16S rRNA/1073	G	G	G	A	G	G	G
16S-23S IGS							
16S-23S rRNA IGS/1620	A	A	A	A	G	Unknown	Unknown
16S-23S rRNA IGS/1632	G	G	G	G	A	Unknown	G
16S-23S rRNA IGS/1648	G	G	G	G	A	Unknown	G
16S-23S rRNA IGS/1689	A	A	A	A	A	Unknown	G
16S-23S rRNA IGS/1742	A	A	A	G	A	Unknown	A
16S-23S rRNA IGS/1748	C	C	C	T	C	Unknown	C
16S-23S rRNA IGS/1858	A	G	G	A	A	Unknown	A
16S-23S rRNA IGS/1859delT	T	T	T	-	T	Unknown	T
16S-23S rRNA IGS/1867delT	T	T	-	T	T	Unknown	T
16S-23S rRNA IGS/1873	A	A	A	A	G	Unknown	A
16S-23S rRNA IGS/1920	T	T	C	T	T	Unknown	T
16S-23S rRNA IGS/1943	G	A	G	G	Unknown	Unknown	G
16S-23S rRNA IGS/2055	C	T	C	C	Unknown	Unknown	C
16S-23S rRNA IGS/2081	G	G	G	A	Unknown	Unknown	G
16S-23S rRNA IGS/2220	G	A	G	G	Unknown	Unknown	G
16S-23S rRNA IGS/2262	C	T	C	C	Unknown	Unknown	C
50S rplJ-rplL							
50S rplJ-rplL/558	T	T	T	T	T	G	T
50S *rplJ-rplL*/583	G	G	C	G	G	G	G
50S *rplJ-rplL*/622	A	A	A	G	A	A	A
50S *rplJ-rplL*/640	C	C	T	C	C	C	C
50S rplJ-rplL/669	G	C	G	G	G	G	G
50S rplJ-rplL/689	C	C	C	T	T	C	T
50S rplJ-rplL/691	G	T	T	G	G	T	G
50S rplJ-rplL/695	G	G	G	G	G	A	G
50S rplJ-rplL/697	A	A	A	A	A	G	A
50S rplJ-rplL/700	A	A	A	G	A	A	A
50S rplJ-rplL/712	G	T	G	G	G	T	G
50S rplJ-rplL/722	G	G	G	G	A	G	G
50S rplJ-rplL/749	C	C	C	A	C	C	C
50S rplJ-rplL/779_780delA	A	A	A	A	A	-	A
50S rplJ-rplL/780_781insA	-	-	A	A	A	-	A
50S rplJ-rplL/785	G	A	G	G	G	G	G
50S rplJ-rplL/849	T	T	T	C	C	T	T
50S rplJ-rplL/909	T	C	C	C	C	C	C
50S rplJ-rplL/919_920ins[C/T]TG	–	–	CTG	–	–	TTG	–
50S rplJ-rplL/938	C	C	C	C	C	C	T
50S rplJ-rplL/955	G	G	T	G	G	G	G
50S rplJ-rplL/961	G	G	G	G	G	G	T
50S rplJ-rplL/987	T	G	G	G	G	G	G
50S rplJ-rplL/993	A	A	G	A	A	A	A
50S rplJ-rplL/1005	T	T	T	T	T	C	T
50S rplJ-rplL/1041	G	A	A	G	G	A	A

(continued)

Table 2 *(continued)*

Region (gene/position of reference sequence EU812559, EU834131)	Haplotype						
	A	B	C	D	E	F	U
50S rplJ-rplL/1042	A	A	A	A	A	G	A
50S rplJ-rplL/1049	A	G	A	A	A	A	A
50S rplJ-rplL/1068	C	C	C	T	C	C	C
50S rplJ-rplL/1107	G	A	G	G	G	A	G
50S rplJ-rplL/1110	C	C	C	C	C	C	T
50S rplJ-rplL/1111_1112insC	–	–	C	–	–	–	–
50S rplJ-rplL/1122	G	A	A	A	A	A	A
50S rplJ-rplL/1137	A	A	A	A	A	G	A
50S rplJ-rplL/1143	G	A	G	G	G	A	G

(ii) Ravindran *et al*. (2011) targeting a region of the 16–23S rRNA intergenic spacer (IGS) (Appendix 7). Please note that these primers will fail to amplify the 16S-23S rRNA IGS region containing the last five SNP differences between haplotypes.
(iii) Munyaneza *et al*., 2009: a region of the *rplL-rplJ* gene region (50S rRNA) (Appendix 8).

Amplicons should be sequenced to determine the species and the haplotype of the bacterium in suspect samples.

Haplotypes can be identified following the table based on data from Nelson *et al*. (2012) and Teresani *et al*.(2014). The sequence of the unknown haplotype is aligned with the reference sequences for the 16S rRNA and 16S-23S rRNA IGS region (GenBank acc. number EU812559) and for the 50S rRNA (EU834131). The haplotype is determined by comparing the sequence at each nucleotide position listed in Table 2.

There is currently no consensus in the scientific community about the delimitation of a new haplotype. Haplotypes are considered to be stable, but when 100% agreement with the SNPs of a known haplotype is not reached using the tests recommended in this protocol, it is recommended to repeat the test or to resample.

5. Reporting and documentation

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

6. Performance criteria

When performance criteria 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/validationlist.php) and it is recommended that this database is consulted as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

7. Reference material

Paper immobilized positive controls or DNA extracts can be obtained from Plant Print Diagnostics, Valencia, Spain.

Potato and tomato plant tissue infected with '*Ca*. L. solanacearum', infected plant matrix extracts (inactivated by heat treatment) or extracted DNA from those matrices can be obtained from the Bacteriology Department, The National Reference Centre (NRC), Netherlands Food and Consumer Product Safety Authority (NVWA) Wageningen, the Netherlands.

8. Further information

Further information on this organism can be obtained from E. Marco, Instituto Valenciano de Investigaciones Agrarias (IVIA), Carretera Moncada-Náquera Km. 4.5, 46113, Moncada, Valencia (ES); e-mail: emarco@ivia.es, Tjou-Tam-Sin N.N.A, National Reference Centre, NPPO-NL. P.O. Box 9102, 6700 HC. Wageningen (NL); e-mail: n.tjou-tam-sin@nvwa.nl, Loiseau M., Laboratoire de la santé des végétaux de l'ANSES (ANSES-LSV), 7 rue Jean Dixméras, 49044 Angers cedex 01 (FR); e-mail: marianne.loiseau@anses.fr.

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 and corrigenda are in press this will also be marked on the website.

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Appendix 1 – Buffers

CTAB buffer (as used in IVIA):

Tris HCl 1 M pH 8.0	0.1 L
NaCl	81.82 g
EDTA 0.5 M pH 8.0	0.1 L
CTAB	20 g
PVP-10	10 g
Optional: β-mercaptoethanol	2 mL
Distilled water to	1 L

Alternative recipes have not affected the test result, for example β-mercaptoethanol can be replaced by 30 mM ascorbic acid (Ilardi *et al*., 2018).

Phosphate buffer (PB)10 mM, pH approx. 7.2 (PB):

$Na_2HPO_4.12H_2O$	2.15 g
$KH_2PO_4.2H_2O$	0.544 g
Distilled water to	1 L

Sterilize by filtration.

Phosphate buffered saline (PBS) 10 mM, pH approx. 7.2:

NaCl	8.0 g
KCl	0.2 g
$Na_2HPO_4{\cdot}12H_2O$	2.9 g
KH_2PO_4	0.2 g
Distilled water to	1 L

Sterilize by filtration.

Extraction buffer: PBS buffer supplemented with 2 g of sodium diethyl dithiocarbamate (DIECA) and 20 g of polyvinylpyrrolidone (PVP-10) per 1 L.

Tris-HCl 1 M pH 8 (121.14 g/mol)
Tris – 60.57 g
Add water tol 400 mL
Dissolve under shaking
Add water to 500 mL
Adjust to pH 8
Store at room temperature
EDTA 0.5 M pH 8 (372.24 g/mol)
EDTA – 186.12 g
Add water to 800 mL
Dissolve under shaking
Add water to 1 L
Adjust to pH 8
Store at room temperature

Appendix 2 – Sample preparation for testing

1. Plant material

1.1 Direct spot or squash.

Direct spot of symptomatic plant material can only be used for real-time PCR detection which has a higher analytical sensitivity than conventional PCR, but it should be noted that higher sensitivity of real-time PCR is obtained when performing a DNA extraction. Samples from leaves or petioles can be immobilized on membranes (Olmos *et al*., 1996) using the spot of plant extract on 3MM Whatman paper on nylon positively charged membranes. Then 3–5 µL of plant extract are put on pieces of membrane of approximately 0.5 cm^2.

1.2 Extraction from membranes.

Pieces of membranes harbouring the spots or the squash are carefully introduced with tweezers into Eppendorf tubes. Add 100 µL of distilled water, vortex and place on ice (Bertolini *et al*., 2014; Teresani *et al*., 2014). Use 3–5 µL of this extract for real-time PCR tests according to Teresani *et al*. (2014a) or Li *et al*. (2009).

1.3 Preparation of plant samples (except seeds)

1.3.1 Potato tubers.

After removing a small area of peel with a sterile knife from the heel (stolon) end of each tuber, small cores (e.g. 0.2–0.5 g) of the exposed vascular tissue can be removed, keeping the amount of non-vascular tissue to a minimum.

1.3.2 *Other* 'Ca. *L. solanacearum' host plant species*.

Usually 1 g of plant material is used for testing.

Plant material (leaves, stem parts or roots) is homogenized in 10 mM phosphate buffer (PB; see Appendix 1). The amount of PB supplemented should be in accordance to the volume of plant material in the ratio 1:1–5 (1 g plant material versus 1–5 mL PB). Homogenization is carried out in a disposable stomacher/Bioreba bag using Seward Stomacher 80 [LAB SYSTEM]/Homex 6 or similar equipment, by hammering or with a manual roller. Incubation for 10–30 min may be performed, followed by optional repeated homogenization, after which 1–1.5 mL crude extract is collected by squeezing the fluid from the bag into a disposable container and transferred to an Eppendorf tube (Teresani *et al*., 2014). DNA extraction and purification are performed using the methods described in Appendix 3.

1.4 Extraction from seeds.
Seeds are washed under the tap with distilled water until the water is clear, or by shaking the seeds for 30 min in washing buffer (distilled water + 0.5% Triton X-100) and several rinses, to remove any fungicide or other seed treatments. After washing, the seeds are placed in a heavy-duty plastic bag (Plant Print Diagnòstics, Bioreba or similar) with 1:10 (w/v) PBS extraction buffer supplemented (see Appendix 1) or a modified CTAB buffer (see Appendix 3) and crushed with a hammer or a mechanical homogenizer. DNA extraction and purification are performed using the methods described in Appendix 3.

2. Psyllids

2.1 Direct spot or squash.
Psyllids can be tested after being squashed onto nylon membranes or 3MM Whatman paper with the rounded end of an Eppendorf tube (Bertolini *et al*., 2014; Teresani *et al*., 2015).

2.2 Extraction from membranes.
See 1.2 above.

2.3 Extraction from psyllids.
Homogenize insect(s) in 100 µL of 10 mM PB (per insect) in an Eppendorf tube, using a tube mortar. Prior to molecular testing DNA extraction and purification should be performed (see Appendix 3) on crude extracts prepared according to this method. The crude extracts obtained can be immediately used after homogenization for DNA extraction or stored at −20°C until use. For long storage periods preserve at −80°C.

Appendix 3 – DNA extraction

1. DNA extraction for plant samples

In the framework of the projects POnTE (H2020) and CaLiso (French funded project), the protocols presented below were evaluated. The results of their performance are available in Loiseau *et al*. (2018) or on request at ANSES.

1.1 CTAB extraction.
DNA extraction from plant tissue is performed according to Munyaneza *et al*. (2010). In this method, 500 mg of plant tissue is homogenized in 1 mL of 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 of lysozyme (50 mg mL^{-1} in 10 mM Tris-HCl, pH 8.0) and incubated at 37°C for 30 min. After incubation, 500 µL of 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 of 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 of sterile water.

1.2 DNA extraction from Apiaceae seeds.
DNA extraction from *Apiaceae* seeds is performed according to Ilardi *et al*. (2018). Seeds (500 or 20 000 divided into two subsamples of 10 000 seeds each) are washed by shaking them for 30 min in 0.5% Triton X-100 and, after several rinses, they are left to soften in water overnight. The seeds are crushed with a mechanical homogenizer in heavy plastic bags (Bioreba) in 1:10 (w/v) of a modified trimethylammonium bromide (CTAB) buffer (2.5% CTAB, NaCl 1.4 M, Tris-HCl 100 mM pH 8.0, EDTA 20 mM pH 8.0, 1% PVP-40, 30 mM ascorbic acid). 400 µg of RNase A is added to 500 µL of homogenate, and after incubation at 65°C for 30 min total genomic DNA is extracted using a DNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's instructions. DNA is eluted in 100 µL of AE buffer provided by the kit.

1.3 Alternative methods.
Commercial kits: NucleoSpin® Food kit, NucleoMag® Plant kit with MC1 buffer (Macherey-Nagel) or DNeasy® Plant Mini kit (Qiagen) can also be used for DNA extraction. These are performed according to manufacturer's instructions, using the samples prepared as described in Appendix 2.

2. DNA extraction for 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 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 of sterile water.Alternatively, in the framework of the projects POnTE (H2020) and CaLiso (French funded project), TNES method (Peccoud *et al*., 2013) and QuickPick™ 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.

3. DNA extraction for crude extracts both from plants and psyllids

DNA extraction using the QuickPick Plant DNA Kit (Bio-Nobile).

DNA extraction and purification are performed using crude sample extract from plants or psyllids following the manufacturer's instructions.

Appendix 4 – Real-time PCR according to Teresani *et al.* (2014)

'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 Real-time PCR using '*Ca*. Liberibacter' universal primers and '*Ca*. L. solanacearum' specific TaqMan probe for detection and diagnostic of '*Ca*. L. solanacearum'.

1.2 The test can be performed on plant material and psyllids (for details see Appendix 2).

1.3 Amplicon sequence location: 16S rRNA gene.

1.4 Oligonucleotides:

CaLsppF	5′-GCA GGC CTA ACA CAT GCA AGT-3′
CaLsppR	5′-GCA CAC GTT TCC ATG CGT TAT-3′
CaLsolP	5′-FAM- AGC GCT TAT TTT TAA TAG GAG CGG CAG ACG -TAMRA-′3

1.5 Amplicon size in base pairs: 111 bp.

1.6 Real-time PCR system: StepOne Plus (Applied Biosystems) using Path-ID qPCR master mix kit (Ambion) or Light Cycler 480 (Roche) using Quantimix-Easy-Probes-kit master mix (Biotools).

A commercial real-time PCR kit for '*Ca*. Liberibacter solanacearum' detection on plant material and psyllid vectors, based on immobilized targets on membranes, is available (Plant Print Diagnòstics). This kit is based on the described real-time PCR protocol (Teresani *et al*., 2014).

2. Methods

2.1 Nucleic acid extraction is not needed for spotted or squashed samples. For other samples see Appendix 3.

2.2 Polymerase chain reaction

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	–	1.62	–
Path-ID qPCR master mix (Ambion)	2×	6.00	1×
CaLsppF	10 µM	0.60	0.5 µM
CaLsppR	10 µM	0.60	0.5 µM
CaLsolP	10 µM	0.18	150 nM
Subtotal	–	9.00	–
DNA sample	–	3.00	–
Total PCR volume	–	12.00	–

*Molecular grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

2.3 PCR cycling conditions: An initial step at 95°C for 10 min followed by 45 cycles (95°C for 15 s, and 60°C for 1 min).

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 isolation and amplification of the target organism and target nucleic acid, respectively.

Negative isolation control (NIC) to monitor cross-reactions with the host tissue and/or contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or 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 sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with DNA of the target organism).

Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of 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 or a synthetic control (e.g. cloned PCR product).

3.2 Interpretation of results:

Verification of the controls:

- The PIC and PAC 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.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

A. Validation data generated in the framework of the EU-funded project POnTE.Validation was performed according to PM 7/98. A statistical analysis will be performed and data reviewed after this analysis.

Analytical sensitivity*	7.9×10^4	95%
	1.7×10^4	93%
	8.2×10^3	90%
	1.6×10^3	17%
	2.1×10^2	7%

Accuracy 97.3% (determined with DNA from healthy host plants)

Diagnostic sensitivity 96.2%

Diagnostic specificity 98.5%

Average Repeatability 96.8%

Reproducibility 88.3%

B. Intra-laboratory performance evaluation (IVIA).

An intra-laboratory performance study of a complete real-time PCR kit (Plant Print Diagnòstics) for '*Ca*. Liberibacter solanacearum' detection was done in three different IVIA laboratories using three different thermocyclers.

Ten blind samples immobilized on paper were used: 5 positive and 5 negative. The positive samples consisted of crude extracts spotted (5 µL/spot) from '*Ca*. L. solanacearum' infected celery and carrot plants. The negative samples consisted of spots of extracts from healthy periwinkle, carrot, tobacco and potato plants. There was no DNA extraction. Results are reported in Teresani *et al*. (2014).

Diagnostic sensitivity: 100%

Diagnostic specificity: 100%

Repeatability of 100%

Reproducibility of 100%

C. Test performance study involving 27 laboratories from 15 countries.

A test performance study of a real-time PCR kit (Plant Print Diagnòstics) for '*Ca*. Liberibacter solanacearum' detection in several hosts was done.

Five positive and 5 negative samples as above were provided (from the same original samples). After preparation the spotted samples were kept for 1 month in the laboratory at room temperature before being distributed to the different participants. There was no DNA extraction. Results are available in 'Detection of '*Candidatus* Liberibacter solanacearum' by real-time PCR in different types of plant material using Plant Print diagnòstics kit' (http://dc.eppo.int/validationlist.php).

Diagnostic sensitivity 87%

Diagnostic specificity 99%

D. Test performance study involving 10 laboratories from Spain.

A performance study of a real-time PCR kit (Plant Print Diagnòstics) for '*Ca*. Liberibacter solanacearum' detection in carrot seeds was performed. Positive and negative spotted carrot seed samples were sent for direct analysis without DNA extraction. Results are available in 'Detection of '*Candidatus* Liberibacter solanacearum' by real-time PCR in carrot seeds using Plant Print diagnòstics kit' (http://dc.eppo.int/validationlist.php).

Diagnostic sensitivity 100%

Diagnostic specificity 100%

Appendix 5 – Real-time PCR for the specific detection of '*Candidatus* Liberibacter solanacearum' adapted by Anses, FR from Li *et al.* (2009)

'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 The following real-time PCR protocol is performed for the specific detection of '*Ca*. Liberibacter solanacearum'.

1.2 The test was developed by Li *et al*. (2009).

1.3 The test can be performed on plant material and psyllids (for details see Appendix 2).

1.4 Primers and probe were designed within a 16S rDNA.

1.5 Oligonucleotides

LsoF	5′-GTC GAG CGC TTA TTT TTA ATA GGA-3′
HLBr	5′-GCG TTA TCC CGT AGA AAA AGG TAG-3′
HLBp	5′-FAM-AGA CGG GTG AGT AAC GCG-3′-BHQ-1

1.6 Amplicon size in base pairs: 78 bp for 'Ca. Liberibacter solanacearum'.

1.7 Real-time PCR system: StepOne Plus thermal cycler (Applied Biosystems) using Quantimix-Easy-Probes-kit master mix (Biotools)/Mx3005P (Stratagene) or C 1000 Touch Thermal Cycler / Bloc CFX96 (Biorad) using TaqMan Universal Master Mix, No Amperase UNG (Applied Biosystems) chemistry and given concentrations of oligonucleotides here beneath.

2. Methods

Nucleic acid extraction and purification: DNA extraction methods that are described in Appendix 3 may be used.

2.1 Real-time PCR

2.1.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	-	7.35	-
TaqMan Universal PCR Master Mix, No amperase UNG (Applied Biosystems)	2×	12.5	1×
LsoF	100 µM	0.06	0.24 µM
HLBr	100 µM	0.06	0.24 µM
HLBp	100 µM	0.03	0.12 µM
Subtotal	-	20.0	-
DNA**	-	5.0	-
Total	-	25.0	-

*Molecular grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.
**As the DNA extracts can contain lot of inhibitors of PCR, it is advisable to amplify the DNA extract in two tubes, one without dilution and the other with a ten-fold dilution in DNase-free water.

2.1.2 Real-time PCR conditions: initial denaturation at 95°C for 10 min; 45 cycles consisting of 15 s at 95°C and 1 min at 60°C (temperatures adapted).

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 a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with DNA of target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of 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 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.

Annex 21 to ISPM 27 (IPPC, 2017) recommends the use of an internal positive control (IPC) to monitor each individual sample separately. Internal positive controls can be genes either 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 include:
- 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:

Verification of the controls:

- The PIC and PAC (as well as IC and IPC) 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 produces no exponential amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

Data from the POnTE project (validation was performed according to PM 7/98) A statistical analysis will be performed, and data reviewed after this analysis.

Analytical sensitivity*	7.9×10^4	100%
	1.7×10^4	98%
	8.2×10^3	100%
	1.6×10^3	98%
	2.1×10^2	50%

Accuracy 96.3% (determined with DNA from healthy host plants)

Diagnostic sensitivity 100 %

Diagnostic specificity 92.6 %

Average Repeatability 97.5%

Reproducibility 94.3%

Appendix 6 – Conventional PCR according to Li *et al.* (2009) and Jagoueix *et al.* (1996)

'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

This conventional PCR amplifies a region of 16S rRNA gene using the forward primer of the real-time PCR designed by Li *et al*. (2009) in a region specific for '*Ca*. L. solanacearum'. The forward primer is used in combination with the universal Liberibacter reverse primer of Jagoueix *et al*. (1996).

1.1 This test can be used for detection or to produce an amplicon for screening for haplotype identification of '*Ca*. Liberibacter solanacearum' (in combination with the conventional PCR tests described in Appendices 7 and 8).

1.2 The test can be applied to any kind of plant material after a DNA extraction.

1.3 Amplicon sequence location: 16S rRNA gene.

1.4 Oligonucleotides:

LsoF	5′-GTC GAG CGC TTA TTT TTA ATA GGA-3′
OI2c:	5′-GCC TCG CGA CTT CGC AAC CCA T-3′

1.5 Amplicon size in base pairs: 1163 bp.

1.6 Enzyme: Taq polymerase, 5 U μL^{-1} (Invitrogen).

2. Methods

2.1 Nucleic acid extraction and purification: see Appendix 3.

2.2 Polymerase chain reaction

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	-	16.30	-
PCR buffer	10×	2.50	1×
$MgCl_2$	25 mM	2.50	2.5 mM
dNTPs	10 mM	0.50	0.2 mM
LsoF	10 µM	0.50	0.2 µM
OI2c	10 µM	0.50	0.2 µM
Taq polymerase	5 U μL^{-1}	0.20	1 U
Subtotal	-	23.00	-
DNA	-	2.00	-
Total PCR volume	-	25.00	-

*Molecular grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

2.3 PCR cycling conditions: An initial step at 94°C for 2 min followed by 35 cycles (94°C for 30 s, 62°C for 30 s and 72°C for 60 s) and one final step at 72°C for 10 min before cooling at 4°C.

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 isolation and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor cross-reactions with the host tissue and/or contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or 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 sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with DNA of the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of 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).

3.2 Interpretation of results: the following criteria should be followed:

Verification of the controls:

- NIC and NAC should produce no amplicons.
- PIC and PAC should produce amplicons of the 1163 bp.

When these conditions are met:

- A test will be considered positive if amplicons of 1163 bp are 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 criteria available

Data from the POnTE project (validation was performed according to PM 7/98). A statistical analysis will be performed, and data reviewed after this analysis.

Analytical sensitivity*	7.9×10^4	60%
	1.7×10^4	45%
	8.2×10^3	33%
	1.6×10^3	8%
	2.1×10^2	3%

Accuracy 93.7% (determined with DNA from healthy host plants)

Diagnostic sensitivity 89.2%

Diagnostic specificity 98.0 %

Average repeatability 95.2%

Reproducibility 78.7%

Appendix 7 – Conventional end-point PCR according to Ravindran *et al.* (2011)

'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 test can be used for detection of '*Ca*. Liberibacter solanacearum' or to produce an amplicon for screening for haplotype identification in combination with the conventional PCR tests described in Appendices 6 and 8.

1.2 The test can be performed on plant material after DNA extraction.

1.3 Amplicon sequence location: 16–23S rRNA intergenic spacer.

1.4 Oligonucleotides:

Lso TX 16/23 F	5′-AAT TTT AGC AAG TTC TAA GGG-3′
Lso TX 16/23 R	5′-GGT ACC TCC CAT ATC GC-3′

1.5 Amplicon size in base pairs: 383 bp.

1.6 Enzyme: Taq polymerase 5 U μL^{-1} (Biotools).[2]

2. Methods

2.1 Nucleic acid extraction and purification: see Appendix 3

2.2 Polymerase chain reaction

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	–	15.40	–
PCR buffer	10×	2.50	1×
$MgCl_2$	25 mM	2.00	2 mM
dNTPs	10 mM	0.50	0.2 mM
Lso TX 16/23 F	10 µM	1.25	0.5 µM
Lso TX 16/23 R	10 µM	1.25	0.5 µM
Taq polymerase	5 U μL^{-1}	0.10	0.5 U
Subtotal	–	23.00	–
DNA	–	2.00	–
Total PCR volume	–	25.00	–

*Molecular grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

2.3 PCR cycling conditions. An initial step at 98°C for 30 s followed by 35 cycles (98°C for 10 s, 55°C for 20 s and 72°C for 30 s) and one final step at 72°C for 7 min before cooling at 4°C.

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 isolation and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor cross-reactions with the host tissue and/or contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or 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 sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of 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).

3.2 Interpretation of results: the following criteria should be followed:

Verification of the controls:

- NIC and NAC should produce no amplicons.
- PIC and PAC should produce amplicons of the 383 bp.

When these conditions are met:

- A test will be considered positive if amplicons of 383 bp are 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 criteria available

A Data from the POnTE project (validation was performed according to PM 7/98). A statistical analysis will be performed and data reviewed after this analysis.

Analytical sensitivity*	7.9×10^4	88%
	1.7×10^4	80%
	8.2×10^3	78%
	1.6×10^3	10%
	2.1×10^2	8%

Accuracy 96.0% (determined with DNA from healthy host plants)

Diagnostic sensitivity 93.4%

Diagnostic specificity 95.5 %

Average repeatability 97.1%

Reproducibility 84.2%

B Data from the ASPROPI project specifically performed for seed by Ilardi *et al*. (2018).

Test performance study involving 10 laboratories from Italy that received both the samples (seed DNA) and the material necessary to carry out the experiments.

Diagnostic sensitivity	100.0%
Diagnostic specificity	81.5%
Relative accuracy	88.9%
Accordance	82.2%
Concordance	80.0%
Concordance odds ratio (COR)	1.15

Appendix 8 – Conventional PCR according to Munyaneza *et al.* (2009)

'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 test can be used for detection of '*Ca*. Liberibacter solanacearum' or to produce an amplicon for screening for haplotype identification in combination with the conventional PCR tests described in Appendices 6 and 7.

1.2 The test can be performed on plant material after DNA extraction.

1.3 Amplicon sequence location: *rplj* gene (50S rRNA).

1.4 Oligonucleotides:

Primer CL514 F:	5′-CTC TAA GAT TTC GGT TGG TT-3′
Primer CL514 R:	5′-TAT ATC TAT CGT TGC ACC AG-3′

1.5 Amplicon size in base pairs: 669 bp.

1.6 Enzyme: Taq polymerase 5 U μL^{-1} (Invitrogen).

2. Methods

2.1 Nucleic acid extraction and purification: see Appendix 3

2.2 Polymerase chain reaction

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	–	14.30	–
PCR buffer	10×	2.50	1×
$MgCl_2$	25 mM	2.00	2 mM
dNTPs	10 mM	1.00	0.4 mM
CL514 F	10 µM	1.00	0.4 µM
CL514 R	10 µM	1.00	0.4 µM
Taq polymerase	5 U μL^{-1}	0.20	1.0 U
Subtotal	–	22.00	–
DNA	–	3.00	–
Total PCR volume	–	25.00	–

*Molecular grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

2.3 PCR cycling conditions. An initial step at 94°C for 30 s followed by 40 cycles (94°C for 30 s, 53°C for 30 s and 72°C for 30 s) and one final step at 72°C for 7 min before cooling at 4°C.

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 isolation and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor cross-reactions with the host tissue and/or contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or 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 sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with DNA of the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of 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).

3.2 Interpretation of results: the following criteria should be followed:

Verification of the controls:

- NIC and NAC should produce no amplicons.
- PIC and PAC should produce amplicons of the 669 bp.

When these conditions are met:

- A test will be considered positive if amplicons of 669 bp are 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 criteria available

Data generated in the framework of the EU-funded project POnTE (validation was performed according to PM 7/98). A statistical analysis will be performed, and data reviewed after this analysis.

Analytical sensitivity*	7.9×10^4	74%
	1.7×10^4	56%
	8.2×10^3	35%
	1.6×10^3	6%
	2.1×10^2	3%

Accuracy 97.3% (determined with DNA from healthy host plants)

Diagnostic sensitivity 94.7%

Diagnostic specificity 100 %

Average Repeatability 96.2%

Reproducibility 83.8%