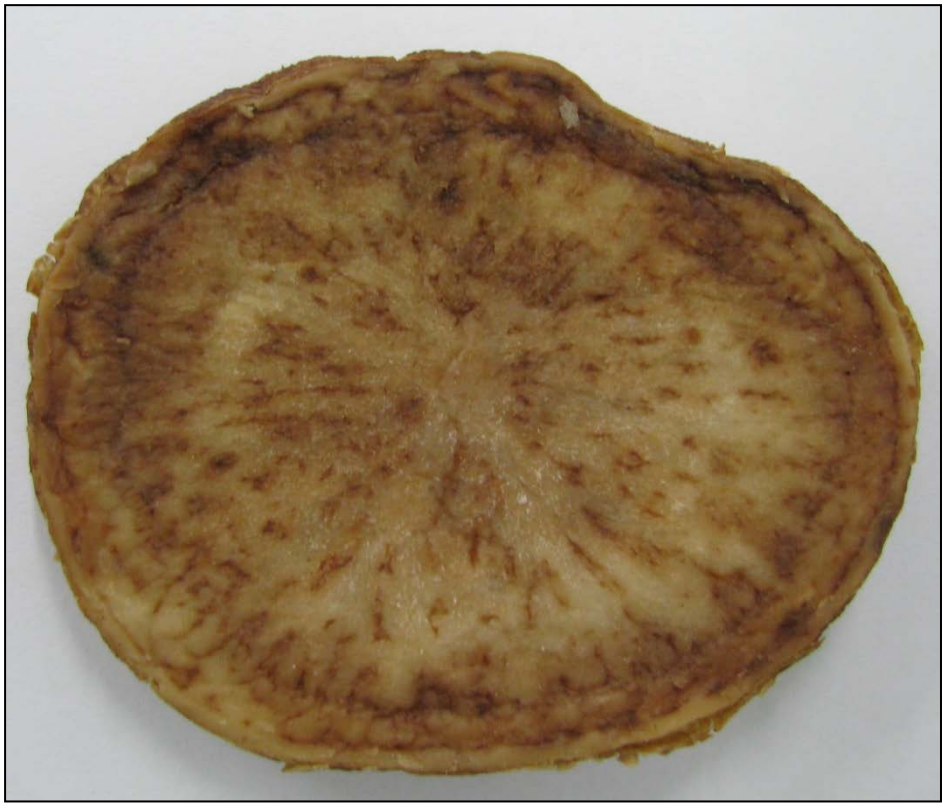
Diagnostic protocol for the identification and detection of *Candidatus* Liberibacter solanacearum, the causal agent of zebra chip of potatoes



PEST STATUS	Not present in Australia
PROTOCOL NUMBER	NDP 18
VERSION NUMBER	V1.2
PROTOCOL STATUS	Endorsed
ISSUE DATE	2012
REVIEW DATE	2017
ISSUED BY	SPHDS

Australian Government
Department of Agriculture

Prepared for the Subcommittee on Plant Health Diagnostic Standards (SPHDS)

This version of the National Diagnostic Protocol (NDP) for *Candidatus* Liberibacter solanacearum is current as at the date contained in the version control box on the front of this document.

NDPs are updated every 5 years or before this time if required (i.e. when new techniques become available).

The most current version of this document is available from the SPHDS website: http://plantbiosecuritydiagnostics.net.au/resource-hub/priority-pest-diagnostic-resources/

Contents

1. INTRODUCTION

Candidatus Liberibacter species are obligate intracellular parasites that inhabit the phloem cells of infected plant host species and are transmitted by insect vectors.

Ca. L solanacearum is a bacterium that infects several solanaceaous plant species and is transmitted by *Bactericera cockerelli* (the tomato potato psyllid). In Finland, *Ca.* L solanacearum has been detected in the carrot psyllid *Trioza apicalis* suggesting that this psyllid is also a potential vector (Munyaneza *et al.*, 2010b).

Ca. L solanacearum may also be transmitted through infected seed potato (Henne *et al.*, 2010). While plants produced from infected tubers were often weak, infected tubers could produce asymptomatic plants in which the bacterium could still be detected in the foliage (Andrew Pitman Crop and Food NZ, pers. comm.; Henne *et al.*, 2010). The rate of bacterial transmission and expression of symptoms in the tubers of these asymptomatic plants was low. However infected foliage of potato plants can act as an acquisition source for the bacterium by the psyllid vector (Henne *et al.*, 2010; Andrew Pitman, pers. comm.).

1.1 Hosts

Ca. L. solanacearum is associated with a disease complex rather than a single disease. Diseases associated with *Ca.* L. solanacearum include zebra chip in potatoes (Liefting *et al.*, 2008a; Hansen *et al.*, 2008; Secor *et al.*, 2009; Abad *et al.*, 2009; Rehman *et al.*, 2010) and psyllid yellows in tomato and capsicum (Liefting 2008a; Hansen *et al.*, 2008; Munyaneza *et al.*, 2009b; Munyaneza *et al.*, 2009c). *Ca.* L. solanacearum has also been reported causing dieback and leaf curling in tamarillo *(Solanum betaceum*) (Liefting *et al.*, 2008b; Watson, 2009) and secondary root proliferation in carrots (Munyaneza *et al.*, 2010a).

1.2 Alternative host plants

Ca. L solanacearum was detected in apparently healthy Cape gooseberry plants from a home garden in Auckland New Zealand (Liefting *et al.*, 2009b). No disease has been reported and Cape gooseberries may be a symptomless host. *Ca.* L. solanacearum has also been detected in several solanaceous weeds with psyllid yellows symptoms including *Solanum ptychanthum* (black nightshade), *Solanum elaeagnifolium* (silver leaf nightshade) and *Lycium barbarum* (wolfberry) (Wen *et al.*, 2009).

2. TAXONOMY

The bacteria are phloem-limited, non-culturable, filamentous, gram-negative which belong to the order Rhizobiales, in the class Alphaproteobacteria of the phylum Proteobacteria (Jagoueix *et al.*, 1994; Bové, 2006).

Candidatus Liberibacter solanacearum is also referred to by the synonym *Candidatus* Liberibacter psyllaurous (Hansen *et al.*, 2008). While both scientific names occur in the literature, *Ca.* L. solanacearum should be used in reference to this bacterium as the name and a description of the bacterium was published in the "International Journal of Systematic and Evolutionary Microbiology", which is the preferred journal for descriptions of novel bacterial species (Liefting *et al.*, 2009b). Reference material of the original isolate is also available from the authors (Liefting *et al.*, 2009b). The bacteria will be referred to as *Ca.* L. solanacearum throughout this document.

Ca. Liberibacter species have a triple layered cell envelope with an outer wall membrane and an inner cytoplasmic membrane (Jagoueix *et al.*, 1996; Bové 2006). *Ca.* L. solanacearum is approximately 0.2 μm in width and 4 μm in length and has a genome size approximately 1.26 Mbp (Lin *et al.*, 2009).

3. DETECTION

Diseases associated with *Ca*. L solanacearum can be identified by the presence of symptoms, however diagnosis should be confirmed through PCR detection and sequencing of the amplified product, particularly as other organisms may cause similar symptoms. Most symptoms, particularly if they are observed on their own, may be caused by other biotic and abiotic factors.

3.1 Symptoms associated with *Candidatus* Liberibacter solanacearum

3.1.1. Potato

- Yellowing or purpling of potato leaves and shoots (Figures 1 and 2)
- Curling or rolling of leaves (Figures 1 and 2)
- Stunted shoots with shortened and swollen internodes (Figure 2)
- Aerial tuber formation (Figure 3)
- Scorched potato tops that collapse prematurely (Figure 4)
- Early senescence
- Tubers may have enlarged lenticels, necrotic flecking of the vascular tissue and streaks along the medullary rays (Figure 5) that are enhanced when slices of the potatoes are fried (Figure 6)

Figure 1. Shoots of "zebra chip" affected potato plants infected with *Candidatus* Liberibacter solanacearum. Leaves on younger shoots display purpling, and older leaves are chlorotic. The leaves are also rolled (Images from New Zealand, courtesy of Dr Lia Liefting, Ministry for Primary Industries, New Zealand (MPI)).

Figure 2. Shoots of a "zebra chip" affected potato plant infected with *Candidatus* Liberibacter solanacearum. Leaves are chlorotic, curled and rolled. Leaves on younger shoots display mild purpling. The shoots are stunted and swollen (arrows) and swelling is occurring at the nodes (Image from New Zealand, courtesy of Dr Lia Liefting, MPI).

Figure 3. Aerial tuber formation on “zebra chip” affected potato plant infected with *Candidatus* Liberibacter solanacearum (Images from New Zealand, courtesy of Dr Lia Liefting, MPI).

Figure 3. Aerial tuber formation on “zebra chip” affected potato plant that was also infected with *Candidatus* Liberibacter solanacearum. (Images courtesy of Dr Lia Liefting, Ministry of Agriculture and Forestry, New Zealand).

Figure 4. Shoots of a "zebra chip" affected potato plant infected with *Candidatus* Liberibacter solanacearum. Leaves are chlorotic and rolled and some are necrotic or scorched (Image from New Zealand, courtesy of Dr Lia Liefting, MPI).

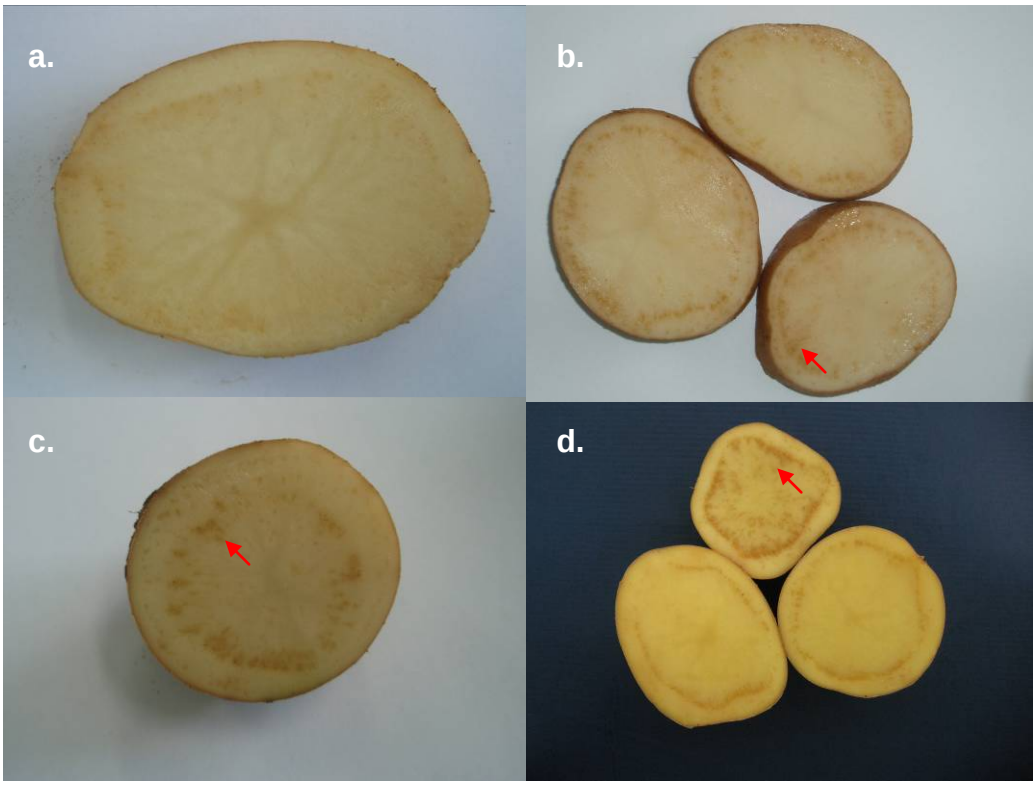


Figure 5. Fresh tuber slices of "zebra chip" affected potato plants infected with *Candidatus* Liberibacter solanacearum. The images show mild (a), moderate (b) and severe (c and d) discolouration /flecking of the vascular tissue and flecking and streaking of the medullary rays (arrows), which may be observed in fresh tubers before frying (Images from New Zealand, courtesy of Dr Lia Liefting, MPI).

Figure 6. Fried tuber slices of "zebra chip" affected potato plants infected with *Candidatus* Liberibacter solanacearum. The images show the enhanced discolouration and flecking of the vascular tissue and streaking of the medullary rays of tuber slices after frying (Images from New Zealand, courtesy of Dr Lia Liefting, MPI).

3.1.2. Tomato

- Stunting (Figure 7)
- Apical growth can be spiky and chlorotic (Figure 7 and 8)
- Leaflets may be distorted and curled (Figure 7, 8 and 10)
- Leaves may be mottled or chlorotic (Figure 10)
- Some varieties have interveinal chlorosis and vein greening of the leaves (Figure 7)
- Purpling of the mid vein and leaves in some cultivars (Figure 11)
- Some tomato varieties may have fruit deformation (Figure 9)

Figure 7. Shoots of psyllid yellows affected tomato plants infected with *Candidatus* Liberibacter solanacearum. The images show stunting (a and b) and chlorosis of the apical growth (a, b, c, and d), which can be spiky (c). The leaflets on some affected shoots are also distorted and curled (a and c). Some varieties may have interveinal chlorosis and vein greening (d) (Images from New Zealand, courtesy of Dr Lia Liefting, MPI).

Figure 8. A psyllid yellows affected tomato plant infected with *Candidatus* Liberibacter solanacearum. The image shows chlorotic and spiky apical growth and mottling and distortion and curling of the leaves (Image from New Zealand, courtesy of Dr Lia Liefting, MPI).

Figure 9. Fruit deformation of a psyllid yellows affected tomato plant that was also infected with *Candidatus* Liberibacter solanacearum (Image from New Zealand, courtesy of Dr Lia Liefting, MPI).

Figure 10. Leaves of psyllid yellows affected tomato plants infected with *Candidatus* Liberibacter solanacearum. The images show chlorosis and mottling (a and b). The leaflets may also be distorted and curled (c) (Image from New Zealand, courtesy of Dr Lia Liefting, MPI).

Figure 11. Shoot tip of psyllid yellows affected tomato plant infected with *Candidatus* Liberibacter solanacearum showing purpling of the leaflets and petioles (Image from New Zealand, courtesy of Dr Lia Liefting, MPI).

3.1.3. Capsicum

- Stunting (Figures 12, 13 and 15)
- Shortened internodes of the stems
- Leaves can be pale green or chlorotic (Figures 12, 13, 14 and 15)
- Leaves may have shortened petioles
- Leaves may be cupped (Figures 12 and 14)
- Leaf apices may taper giving plants a spiky appearance (Figures 12 and 13)
- Flower abortion may be observed
- Tip necrosis
- Parts of the plant may die back

Figure 12. A stunted shoot of psyllid yellows affected *Capsicum annuum* plant infected with *Candidatus* Liberibacter solanacearum. The leaves are pale green and tapered and some are slightly cupped (Image from New Zealand, courtesy of Dr Lia Liefting, MPI).

Figure 13. A shoot of a psyllid yellows affected *Capsicum annuum* plant infected with *Candidatus* Liberibacter solanacearum. The leaves are pale green/chlorotic and tapered to give a spiky appearance (Image from New Zealand, courtesy of Dr Lia Liefting, MPI).

Figure 14. A stunted shoot of a psyllid yellows affected *Capsicum annuum* plant infected with *Candidatus* Liberibacter solanacearum. The leaves are pale green/chlorotic and the apical leaves are cupped (Image from New Zealand, courtesy of Dr Lia Liefting, MPI).

Figure 15. Stunted shoots of a psyllid yellows affected *Capsicum annuum* plant infected with *Candidatus* Liberibacter solanacearum. The leaves are chlorotic and curled (Image from New Zealand, courtesy of Dr Lia Liefting, MPI).

3.1.4. Tamarillo

- Poor bud-break (Figure 16)
- Stunted shoot growth (Figure 16 and 21)
- Shoot proliferation (Figure 17)
- Leaves on new shoots are small (Figure 16)
- Cupping and pink colouration of new leaves (Figure 17)
- Leaves become chlorotic and develop peripheral burning (Figure 18 and 19)
- Leaves drop
- Shoot and branch dieback (Figure 21)
- Tree death in 2-4 months
- Initially only part of the tree may display symptoms (Figure 16).

Figure 16. A Tamarillo tree infected with *Candidatus* Liberibacter solanacearum. The middle and front right pruned branches show poor bud break and shoot growth with small and yellow leaves (red arrows). The front left pruned branch shows stunted shoot growth with small leaves (blue arrow). The rear branches show normal growth (Image from New Zealand, courtesy of Craig Watson, Tamarillo Growers' Association, New Zealand).

Figure 17. A Tamarillo tree infected with *Candidatus* Liberibacter solanacearum. The new leaves show an abnormal pink colouration and shoot proliferation (Image from New Zealand, courtesy of Craig Watson, Tamarillo Growers' Association, New Zealand).

Figure 18. A Tamarillo tree infected with *Candidatus* Liberibacter solanacearum. The leaves have interveinal chlorosis, cupping and leaf scorching. Note the pink colouration of the new leaf in the centre of the image (Image from New Zealand, courtesy of Craig Watson, Tamarillo Growers' Association, New Zealand).

Figure 19. Shoot of a Tamarillo tree infected with *Candidatus* Liberibacter solanacearum. The leaves are chlorotic (Image from New Zealand, courtesy of Craig Watson, Tamarillo Growers' Association, New Zealand).

Figure 20. Shoot dieback on a Tamarillo tree infected with *Candidatus* Liberibacter solanacearum (Image from New Zealand, courtesy of Craig Watson, Tamarillo Growers' Association, New Zealand).

Figure 21. A declining Tamarillo tree infected with *Candidatus* Liberibacter solanacearum. Some branches have died and others display poor shoot growth. The "healthy" leaves are from a nearby unaffected tree (Image from New Zealand, courtesy of Craig Watson, Tamarillo Growers' Association, New Zealand).

3.1.5. Carrots

- Curling and yellowing and/or purpling of leaves (Figures 22, 23 and 24)
- Stunted shoot and root growth
- Production of secondary roots along the primary root (Figure 25)

Figure 22. Mild purpling of leaves of carrot leaves associated with psyllid (*Trioza apicalis*) damage and *Candidatus* Liberibacter solanacearum (Image from Finland, courtesy of Dr Joseph Munyaneza, USDA-ARS Yakima Agricultural Research Lab, USA, and Dr Anne Nissinen, MTT Agrifood Research, Finland).

Figure 23. Purpling and curling of carrot leaves associated with psyllid (*Trioza apicalis*) damage and *Candidatus* Liberibacter solanacearum (Image from Finland, courtesy of Dr, Joseph Munyaneza, USDA-ARS Yakima Agricultural Research Lab, USA, and Dr Anne Nissinen, MTT Agrifood Research, Finland).

Figure 24. Purpling, yellowing and curling of carrot leaves associated with psyllid (*Trioza apicalis*) damage and *Candidatus* Liberibacter solanacearum (Image from Finland, courtesy of Dr, Joseph Munyaneza, USDA-ARS Yakima Agricultural Research Lab, USA, and Dr Anne Nissinen, MTT Agrifood Research, Finland).

Figure 25. Carrots with production of secondary roots along the primary root associated with psyllid (*Trioza apicalis*) damage and *Candidatus* Liberibacter solanacearum. (Image from Finland, courtesy of Dr, Joseph Munyaneza, USDA-ARS Yakima Agricultural Research Lab, USA, and Dr Anne Nissinen, MTT Agrifood Research, Finland).

3.2 Diseases with similar symptoms

3.2.1. Psyllid yellows in potatoes

Psyllid yellows disease of potatoes has aerial symptoms similar to zebra chip disease and was first observed in the USA in 1927 (Richards, 1928). Psyllid yellows is associated with feeding of the tomato potato psyllid (Sengoda *et al.,* 2010). It was suggested that zebra chip and psyllid yellows diseases could be differentiated on the basis of symptomatology in the tubers: psyllid yellows affected plants do not exhibit the tuber symptoms and survive longer than zebra chip affected plants (Sengoda *et al.,* 2010). However the bacterium is not necessarily evenly distributed throughout a plant and it is possible that not all tubers of infected plants are affected. Timing of infection may also play an important role in the effect of the bacteria on tubers and some tubers may only display mild symptoms of disease. It is also possible that there is a difference in symptom expression between varieties. Consequently psyllid yellows disease of potatoes could be confused with zebra chip disease.

3.2.2. Potato purple top and other phytoplasmas in potatoes

Several phytoplasma species representing six taxonomically distinct phytoplasma groups (16SrI, 16SrII, 16SrIII, 16SrVI, 16SrXII and 16SrXIII) have also been found in association with diseases of potatoes that have similar symptoms to zebra chip disease. These diseases include haywire disease, potato purple top, or potato purple top wilt, and stolbur disease (Semancik and Peterson 1971; Harding and Teakle, 1985; Lee *et al.*, 2004; Lee *et al.,* 2006; Leyva-Lopez *et al.,* 2002; Secor, 2007; Munyaneza *et al.,* 2007; Lee *et al*., 2009). Symptoms associated with these diseases include stunting, leaf curl, chlorosis, purpling of the apical leaves, swollen nodes, proliferation of axillary buds, aerial tubers and scattered light-brown discolouration of tubers which are enhanced when tuber tissue is fried. (Munyaneza *et al.,* 2009d). The phytoplasmas and the associated diseases have been reported from Mexico (16SrI, II, III and XIII), USA (16SrIII, VI and XII), Europe (16SrI and XII), South America (16SrI and II), Asia (16SrINew Zealand (16SrXII) and Australia (16SrII)(Norris, 1954; Harding and Teakle 1985, Lee *et al.,* 2009; Liefting *et al.,* 2009c; Secor, 2007)

3.2.3. Carrots

Similar symptoms have been found on carrots in association with other organisms including a phytoplasma and an undescribed bacteria-like organism occurring in Australia (Gibb *et al.,* 2003) and stolbur phytoplasma in Spain (Font *et al.,* 1999).

4. IDENTIFICATION

The most reliable method for *Ca.* L. solanacearum detection is polymerase chain reaction (PCR), which is used to detect the DNA of the bacterium. The efficiency of this test is dependent on appropriate sampling of plant tissue, reliable nucleic acid extraction methods and species-specific primers used in the PCR test.

4.1 Summary of the recommended *Ca.* L. solanacearum identification method

- Sample vascular tissue containing phloem cells from candidate plants.
- Extract total DNA.
- Perform a housekeeping PCR with the rP1/fD2 primers. The rP1/fD2 primers amplify the 16S rRNA gene from most prokaryotes as well as from chloroplasts (Weisberg *et al.*, 1991). If this test is negative then there is no DNA present or there are DNA polymerase inhibitors co-extracted with the nucleic acid. In this situation, try cleaning the nucleic acid (Appendix I) or repeat the extraction.
- Perform PCR using the following procedure:
  Use a conventional nested PCR on the purified DNA.
  Or
  Use the quantitative PCR with a Taqman probe if there is access to a suitable real-time PCR machine.
- Analyse the conventional PCR products by agarose gel electrophoresis.
- To confirm *Ca.* L. solanacearum identity, direct sequence the PCR product. If direct sequencing is problematic, the PCR product can be cloned and then sequenced using standard cloning and sequencing procedures. Sequence data can be analysed using the Basic Local Alignment Search Tool (BLAST) available at:
  http://blast.ncbi.nlm.nih.gov/Blast.cgi.

4.2 Sample selection

Ca. L. solanacearum is phloem limited and 0.5g of vascular tissue should be sampled for successful PCR detection. Symptomatic tissue provides the best opportunity to detect *Ca.* L. solanacearum in infected plants (Lia Liefting, MPI New Zealand, pers. comm.). Higher concentrations of the bacterium were observed in root, tuber and stolon tissue compared to aerial tissue of potato plants (Li *et al.*, 2009; Wen *et al.*, 2009). Fried symptomatic potato tissue may improve the chance of detection compared to fresh symptomatic tuber tissue (Lia Liefting, MPI, pers.comm.).

Symptomatic potato tubers are most reliable and the stems of ZC symptomatic plants can also be used. For tomato, tissue from symptomatic shoots should be used. Tissue can include stems, leaf petioles, the peduncles attached to fruit and the portion of affected fruit to which the peduncle is attached.

Symptomless infections may occur and if this is suspected it is important to thoroughly sample phloem tissue from different aerial and subterranean tissue such as roots and tubers of the one plant for *Ca.* L solanacearum detection.

There is very little information available on the best time for detection. The translocation and titre of *Ca.* L. solanacearum and symptom development in any host may be dependent on the time of infection, the abundance of the insect vector and on environmental factors that affect replication of the bacterium and symptom expression.

4.3 DNA extraction procedure

This method uses the QIAGEN DNeasy® Plant mini kit (Green *et al.*, 1999). DNA extraction methods using other column-based or magnetic-bead based protocols can be used if they have been validated for use by the laboratory performing the diagnostic test.

Materials and equipment

1. QIAGEN DNeasy® Plant mini kit
2. 1.5 ml centrifuge tubes
3. 20-200 µl and 200-1000 µl pipettes
4. 20-200 µl and 200-1000 µl sterile filter pipette tips
5. Autoclave
6. Balance
7. Bench top centrifuge
8. Distilled water
9. Ice machine
10. Freezer
11. Sterile mortars and pestles or "Homex" grinder (Bioreba) and grinding bags (Agdia or Bioreba) or hammer and grinding bags (Agdia or Bioreba)

If using mortar and pestles, ensure they are thoroughly cleaned prior to use to prevent cross-contamination from previous extractions. To clean thoroughly, soak mortars and pestles in 2% bleach for 1 hour. Rinse with tap water then soak in 0.2 M HCl or 0.4 M NaOH for 1 hour. Rinse thoroughly with distilled water.

12. Scalpel handle
13. Sterile scalpel blades
14. Vortex
15. Water bath or heating block at 55-65°C
16. Latex or nitrile gloves
17. Buffers:
    - CTAB grinding buffer (Table 1)
    - Absolute ethanol

The 2% cetyltrimethylammonium bromide (CTAB) buffer (Table 1) is required for all extraction procedures:

Table 1. 2.5% cetyltrimethylammonium bromide (CTAB) extraction buffer for DNA purification.

Reagent*	Final	Amount needed for 1 L
CTAB (cetyltrimethylammonium bromide)	2.5%	25 g
Sodium chloride	1.4 M	56 g
1 M Tris-HCl, pH 8.0 (sterile)	100 mM	100 ml
0.5 M EDTA, pH8.0 (sterile)	20 mM	40 ml
Polyvinylpyrrolidone (PVP-40)	1%	10 g

*Make up to volume with sterile distilled water. Store at room temperature. Just before use, add 0.2% 2-mercaptoethanol (v/v) to the required volume of buffer.

If a fume hood is unavailable β – mercaptoethanol can be omitted but the quality of the extract from some plant species may be affected.

Method

1. Grind 0.5 g of tissue in 5 ml of CTAB extraction buffer (room temperature) containing 0.2% β – mercaptoethanol.
2. Transfer 500 µl of extract to a 1.5 ml microfuge tube and add 4 µl of RNase A (Supplied with the DNeasy kit), cap tube and incubate at 65°C for 25-35 min, mixing gently several times.
3. Add 130 µl of QIAGEN buffer AP2 to extract. Invert 3 times to mix and place on ice for 5 minutes.
4. Apply lysate onto a QIAshredder™ column and centrifuge at 20,000 x g (14,000 rpm or maximum speed) for 2 minutes.
5. Transfer 450 µl of flowthrough from the QIAshredder™ column to a 1.5 ml centrifuge tube containing 675 µl QIAGEN buffer AP3/E. Mix by pipetting.
6. Transfer 650 µl of extract onto a DNeasy column and spin at 6,000 × g (8000 rpm) for 1 minute.
7. Discard flow-through and add the rest of the sample to the column and spin at 10000 rpm for 1 minute.
8. Place DNeasy column in a new 2 ml collection tube and add 500 µl of QIAGEN buffer AW (wash buffer) and spin at 10000 rpm for one minute.
9. Discard flowthrough and add another 500 µl of QIAGEN buffer AW and spin at maximum speed for 2 minutes.
10. Discard flowthrough and collection tube. Ensure that the base of the column is dry (blot on tissue if it is not) and place in an appropriately labelled microfuge tube. Add 100 µl of pre-warmed 65°C AE buffer directly to the filter (don't apply down the side of the tube) and spin at 10000 rpm for 1 minute. Discard column and store DNA in Freezer.

Table 2. PCR primers used for phytoplasma detection and internal control primers.

PCR test[†]	Primer name (direction)	Primer sequence (5´-3´)	Tm	Product size (bp)	Reference
Candidatus Liberibacter solanacearum					
Nested PCR - First stage	OA2	GCGCTTATTTTTAATAGGAGCGGCA	60°C	1,160 bp	Jagoueix *et al.,* 1996 Liefting *et al.,* 2009a Liefting *et al.,* 2009b
	OI2c	GCCTCGCGACTTCGCAACCCAT	(use 66°C for capsicum samples)		
Nested PCR – second stage	Lib16S01F	TTCTACGGGATAACGCACGG	58°C	580 bp	Liefting *et al.,* 2009b
	Lib16S01R	CGTCAGTATCAGGCCAGTGAG			
Real Time PCR	LsoF	GTCGAGCGCTTATTTTTAATAGGA	58°C	68 bp	Li *et al.,* 2009
	HLBr	GCGTTATCCCGTAGAAAAAGGTAG			
	HLBp (hydrolysis probe)*	FAM-AGACGGGTGAGTAACGCG-BHQ1			
Internal control					
16S bacterial and plant chromosomal	FD2	AGAGTTTGATCATGGCTCAG	55ºC	approx. 1400-1500 bp.	Weisberg *et al.,* 1991
	RP1	ACG GTT ACC TTG TTA CGA CTT			

* Hydrolysis probes are often referred to as Taqman; "Taqman" is the proprietary name held by Applied Biosystems. Hydrolysis probes can be purchased from a number of biotechnology companies. FAM= 6-carboxy-fluorescein reporter; BHQ = Black hole quencher

4.4 Polymerase Chain Reaction

To reduce the risk of contamination and possible false positive results, particularly when nested PCR is used for *Ca.* L solanacearum detection, it is desirable to set up PCR reactions in a different lab to where nucleic acid extractions have been done. It is also desirable to handle PCR reagent stocks and to set up PCR reactions in a clean room or bio-safety cabinet with dedicated pipettes, PCR tubes and tips that have not been exposed to nucleic acid extracts and PCR products. Use a separate pipette for the addition of nucleic acids to the PCR reactions. Do not add nucleic acid to reactions in the same clean room or bio-safety cabinet in which PCR stocks are handled.

PCR materials and equipment

1. PCR reagents of choice
2. Primers (Table 4)
3. PCR grade water
4. 0-2 µl, 2-20 µl, 20-200 µl and 200-1000 µl pipettes
5. 0-2 µl, 2-20 µl, 20-200 µl and 200-1000 µl sterile filter pipette tips
6. 1.5 ml centrifuge tubes to store reagents
7. PCR tubes (volume depends on thermal cycler)
8. Bench top centrifuge – with adapters for small tubes
9. Freezer
10. Ice machine
11. Latex or nitrile gloves
12. Conventional thermal cycler
13. Real time thermal cycler
14. DNA molecular weight marker

4.4.1. Housekeeping PCR

The housekeeping PCR should be conducted to determine if the nucleic extract is of sufficient quality for *Ca.* L. solanacearum detection. For the house keeping PCR the primers and the expected size of the PCR product are listed in Table 2, the components and concentrations are listed in Table 3 and the cycling times are listed in Table 4. Run the PCR products on a gel as described below. The house keeping PCR is successful if a product of the expected size is observed, indicating the presence of quality DNA in the nucleic acid extract. If the housekeeping PCR is successful continue with the nested PCR assay for *Ca.* L solanacearum detection. The housekeeping PCR is not successful if no PCR product is observed. If this occurs, the nucleic acid extract should be cleaned up or the sample should be re-extracted and a housekeeping PCR conducted on these extracts. It may also be useful to dilute some of the original DNA extract 1:5 or 1:10 and repeat the housekeeping PCR. Should this work the diluted sample could be used for the nested PCR for *Ca*. L solanacearum detection.

Controls

Positive control:	DNA of known good quality
No template control:	Sterile distilled water

4.4.2. Conventional PCR

For *Ca.* L. solanacearum detection the primers and the expected size of the PCR product are listed in Table 2, the components and concentrations for the first stage PCR are listed in Table 3 and the cycling conditions are given in Table 4. The 16S rRNA gene, to which all the primers and probe were designed is highly conserved amongst the four known *Candidatus* Liberibacter species, of the primers used for detection in the nested PCR and real-time PCR only OA2 and LsoF are specific for *Ca.* L. solanacearum.

For nested PCR, the first-stage PCR products are diluted 1:25 (v/v) in water prior to re-amplification using the second-stage PCR primers.

If a positive result is obtained the PCR product should be sequenced to confirm the identity of the organism that is detected.

When establishing the test initially, it is advised that a negative control (DNA extracted from healthy plant tissue) is included. The PCR will only be considered valid if no amplicons are produced in this negative control.

Controls

Positive control:	DNA extracted from *Ca.* L. solanacearum infected tissue or plasmid containing *Ca.* L. solanacearum first stage PCR product
Negative control:	DNA extracted from plant tissue not known to be infected with *Ca.* L. solanacearum
No template control:	Sterile distilled water

Table 3. Conventional PCR reaction master mix – housekeeping gene PCR and first stage and second stages of the *Ca.* L. solanacearum nested PCR

Reagent	**First stage PCR for *Ca.* L. solanacearum and House keeping PCR**	**Second Stage for *Ca.* L. solanacearum**
	Volume per reaction	**Volume per reaction**
Sterile (RNase, DNase free) water	13.9 µl	14.9 µl
10 × reaction buffer	2 µl	2 µl
50 mM $MgCl_2$	0.6 µl	0.6 µl
10 mM dNTP mixture	0.4 µl	0.4 µl
10 µM Forward primer	0.5 µl	0.5 µl
10 µM Reverse primer	0.5 µl	0.5 µl
5 units/µl Platinum® *Taq* DNA polymerase (Invitrogen 10966-026)	0.1 µl	0.1 µl
DNA template or control	2 µl	1 µl
Total reaction volume	**20 µl**	**20 µl**

For the house keeping PCR and first stage of the nested PCR for *Ca.* L. solanacearum pipette 18 µl of reaction mix into each PCR tube then add 2 µl of DNA template.

For the second stage of the nested PCR for *Ca.* L. solanacearum pipette 19 µl of reaction mix into each PCR tube then add 1 µl of first stage PCR product as the template.

Table 4. PCR cycling conditions for the housekeeping PCR, and the first and second stages of the nested PCR for detection of *Ca*. L. solanacearum.

Step	Housekeeping PCR assay			*Ca.* L. solanacearum – Nested PCR assay					
				First stage			Second stage		
	Temp.	Time	No. of cycles	Temp.	Time	No. of cycles	Temp.	Time	No. of cycles
Initial denaturation	94°C	5 min	1	94°C	5 min	1	94°C	5 min	1
Denaturation	94°C	45 s		94°C	45 s		94°C	30 s	
Annealing	60ºC	45 s	40	60ºC 66ºC for Capsicum)	45 s	40	58ºC	30 s	40
Elongation	72°C	1 min		72°C	1 min		72°C	45 s	
Final elongation	72°C	7 min	1	72°C	7 min	1	72°C	7 min	1

4.4.3. Quantitative PCR

Quantitative PCR can be used to confirm detection of *Ca.* L solanacearum in samples with high titres of the bacterium. The primers and probe for detection of *Ca.* L. solanacearum are given in Table 2, the components and their concentrations for the real time PCR are given in Table 5 and the cycling conditions are given in Table 6. Like the conventional nested assay this protocol is based on the 16S rRNA gene; however the use of a probe in addition to the primers increases the specificity of the assay. The production of a smaller amplicon over 50 cycles should increase the sensitivity of the assay compared to single (first) stage PCR but is not as sensitive as the nested PCR.

Table 5. Quantitative PCR reaction master mix for detection of *Ca.* L. solanacearum.

Reagent*	**Volume per reaction**
Sterile (RNase, DNase free) water	6.75 µl
Platinum® Quantitative PCR SuperMix-UDG	10 µl
10 µM LsoF primer	0.5 µl
10 µM HLBr primer	0.5 µl
10 µM HLBp hydrolysis probe	0.25 µl
DNA template or control	2 µl
Total reaction volume	**20** µl

*0.8 µl of 10 mg/ml BSA can be added to the reaction mix and may reduce the risk of inhibition of the DNA polymerase – if BSA is used, adjust the volume of water so that the final reaction volume remains at 20 µl.

Table 6. PCR cycling conditions for the quantitative PCR detection of Ca. L. solanacearum.

Step	Temperature	Time	No. of cycles
Initial Hold	50°C	2 min	1
Initial Denaturation	95°C	2 min	1
Denaturation	95°C	10 s	50
Annealing and Elongation	58°C	45 s	

4.5 Electrophoresis for conventional PCR

Separate the PCR products (5-10 µl) on a 1% agarose gel containing ethidium bromide or SybR-Safe and visualise using an UV transilluminator (ethidium bromide staining) or blue light box (SybR-Safe staining). Use a DNA molecular weight marker to determine the size of the products. Table 4 lists the expected PCR product size for each primer pair.

4.6 Interpretation of results

Failure of the samples to amplify with the housekeeping primers suggests that the DNA extraction has failed, compounds inhibitory to PCR are present in the DNA extract or the DNA has degraded.

The *Ca.* L. solanacearum conventional PCR tests will only be considered valid if:

(a) the positive control produces the correct size product as indicated in Table 4; and

(b) no bands are produced in the negative control (if used) and the no template control.

The *Ca.* L. solanacearum quantitative PCR tests will only be considered valid if a Ct value above the threshold automatically set by the real time PCR instrument occurs.

5. CONTACTS

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6. ACKNOWLEDGEMENTS

This protocol was developed and compiled by Dr Fiona Constable, Department of Primary Industries, Victoria.

The following people provided training, information, advice and images for this protocol:

Dr Lia Liefting MPI, New Zealand
Dr Nadine Berry, Crop and Food, New Zealand
Dr Andrew Pitman, Crop and Food, New Zealand
Dr Ian Scott, Crop and Food, New Zealand
Craig Watson, Tamarillo Grower's Association, New Zealand
Dr Joe Munyaneza, USDA-ARS, Yakima Agricultural Research Laboratory, USA

This protocol was reviewed and verified by Dr Lia Liefting, (Senior Scientist, Virology & Post-Entry Quarantine), Plant Health and Environment Laboratory, Ministry for Primary industries (MPI), New Zealand.

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8. APPENDIX

8.1 Nucleic acid cleanup

Materials and equipment

1. 1.5 ml centrifuge tubes
2. 20-200 µl and 200-1000 µl pipettes
3. 20-200 µl and 200-1000 µl sterile filter pipette tips
4. Autoclave
5. Balance
6. Bench top centrifuge
7. Distilled water
8. Freezer
9. Vortex
10. Latex or nitrile gloves
11. Reference:
12. Buffers/solutions:
    - Chloroform:iso-amyl alcohol (24:1 v/v)
    - Ice-cold isopropanol
    - 70% (v/v) ethanol
    - Sterile distilled water
    - TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5 or 8.0)

Method

1. Add an additional 100-200 µl of sterile water or TE to the nucleic extract to assist ease of handling.
2. Add an equal volume of chloroform:isoamyl alcohol (24:1) and mix thoroughly by vortexing. Centrifuge in a microfuge at room temperature for 15 minutes at 13000 rpm.
3. Transfer the epiphase into a new 1.5ml microcentrifuge tube and add an equal volume of isopropanol (stored at -20°C). Mix immediately by inversion. Centrifuge for 15 minutes at 13000rpm.
4. Discard the supernatant and wash the pellet once with 70% ethanol.
5. Air dry the pellet and resuspend in 20-50 µl of water.

Alternatively the DNA may be purified through a MicroSpin™ S-300 HR column (GE Healthcare Cat. No 27-5130-01) according to the manufacturer's instructions.

8.2 Additional information

Candidatus Liberibacter species are obligate intracellular parasites that inhabit the phloem cells of infected plant host species and are transmitted by insect vectors. They were first described in 1994 and were associated with citrus greening (Huanglongbing) disease (Jagoueix *et al.*,, 1994).

Since their discovery, four *Candidatus* Liberibacter species have been described, including three which are reported from citrus, "*Candidatus* Liberibacter asiaticus" (Jagoueix *et al.,* 1994, Jagoueix *et al.,* 1996), "*Candidatus* Liberibacter africanus" (Jagoueix *et al.,* 1994; Planet *et al.,* 1995; Jagoueix *et al.,* 1996; Garnier *et al.,* 2000), and "*Candidatus* Liberibacter americanus" (Teixeira *et al.,* 2005) and one species, *Candidatus* Liberibacter solanacearum (syn. *Ca.* L. psyllaurous), associated with diseases of solanaceaous plants and carrots (Liefting 2008; Liefting *et al.,* 2008a; Liefting *et al.,* 2008b; Liefting *et al.,* 2009a; Hansen *et al.,* 2008; Munyaneza *et al.,* 2010a).

The association between *Ca.* L. solanacearum and diseases of solanaceaous hosts, including *Solanum lycopersicum* (tomato), *Solanum tuberosum* (potato) and *Capsicum annuum* (pepper and chilli) was reported independently by two research groups in New Zealand and the USA in 2008 (Liefting *et al.,* 2008a; Liefting *et al.,* 2008b; Hansen *et al.,* 2008). Preliminary microscopy studies indicated the presence of a bacteria-like organism in the phloem of affected plants (Liefting *et al.,* 2009). Prior to this discovery the cause of the associated diseases, including zebra chip in potatoes and psyllid yellows of tomatoes and capsicums, was unknown. A phytoplasma aetiology was suspected for zebra chip disease and phytoplasmas have been associated with similar symptoms (Munyaneza *et al.,* 2009d). In tomatoes and capsicums no potential pathogen had been identified.

Although Koch's postulates for cause of disease have not been fulfilled, *Ca.* L. solanacearum and the associated diseases have been successfully transmitted to potatoes and tomatoes by grafting infected material onto unaffected plants (Crosslin and Munyaneza, 2009; Secor *et al.,* 2009) The bacterium and diseases have also been transmitted to healthy plants by *B cockerelli* (Hansen *et al.,* 2008; Secor *et al.,* 2009). Both experiments provide further evidence for an association between the bacterium and disease (Secor *et al.,* 2009).

Ca. L. solanacearum was also reported in symptomless *Solanum betaceum* (tamarillo) and *Physalis peruviana* (Cape gooseberry) (Liefting *et al.,* 2008b). Subsequent observations indicate that infection by the bacterium may lead to death of affected tamarillo trees (Watson, 2009). Symptoms in Cape gooseberry have not been reported. *Ca.* L solanacearum was detected in carrots with symptoms associated with carrot psyllid (*Trioza apicalis)* damage in Europe (Munyaneza *et al.,* 2010a). The bacterium has also been detected in the carrot psyllid (Munyaneza *et al.,* 2010b).

8.3 Diseases associated with *Candidatus* Liberibacter solanacearum

8.3.1. Zebra chip in potatoes

Zebra chip disease in potato was first reported in Mexico in 1994, where it was called "papa manchada" (Secor and Rivera-Varas, 2004; Secor *et al.,* 2009). The disease has since been found in the USA, Guatemala, where it is known as "papa rayada",

Honduras and New Zealand and is associated with *Ca.* L. solanacearum (Liefting *et al.,* 2008a; Hansen *et al.,* 2008; Secor *et al.,* 2009; Abad *et al.,* 2009; Rehman *et al.,* 2010). Zebra chip disease has caused substantial economic damage in the regions where it is found. The disease is named for the darkened streaks that formed along the medullary rays of the potato tuber slices after frying.

Symptoms in the aerial parts of Zebra chip affected potato plants include: yellowing or purpling of potato leaves and shoots, interveinal chlorosis, vein-greening and downward curling of leaves. Stunted shoots are associated with shortened and thickened internodes and potato tops can appear scorched and collapse prematurely. Affected plants may form aerial tubers. Early senescence may also be observed (Liefting *et al.,* 2008a; Liefting *et al.,* 2009a; Liefting *et al.,* 2009b; Hansen *et al.,* 2008; Li *et al.,* 2009; Rehman *et al.,* 2010).

Tubers of Zebra chip affected plants may have necrotic flecking and streaks that are enhanced when slices of the potatoes are fried (Munyaneza *et al.,* 2007). Affected fried potato slices are darker than unaffected potatoes and have a burnt taste which makes them unsaleable and the presence of zebra chip symptoms can result in crops being rejected for use by processors. There may be significant yield loss and affected tubers may have less dry matter (13%) than unaffected potatoes (Munyaneza *et al.,* 2007; Liefting *et al.,* 2008a).

8.3.2. Psyllid yellows in tomatoes and Capsicum annuum

Tomatoes and *Capsicum annuum* (peppers and chilli) have long been known to be affected by psyllid yellows (Binkley, 1929; Blood *et al.,* 1933) but no pathogen had been associated with the disease in either crop. In 2008 *Ca.* L solanacearum was first reported on green house grown tomatoes and capsicums (bell peppers) with psyllid yellows symptoms from New Zealand and independently from tomatoes with psyllid yellows symptoms in the USA (Liefting 2008; Hansen *et al.,* 2008). It has subsequently been reported in *C. annuum* (Bell pepper and jalapeno chilli) and in tomatoes in Mexico (Munyaneza *et al.,* 2009b; Munyaneza *et al.,* 2009c) and chillies and in field grown tomatoes in Texas, USA (Wen *et al.,* 2009; French-Monar *et al.,* 2010). Infection results in loss of quality and yield in *C. annuum* and tomatoes (Liefting *et al.,* 2009b).

Psyllid yellows affected tomato plants may appear stunted and the apical growth can be spiky and chlorotic. Leaves may be mottled or chlorotic and leaves of some varieties have interveinal chlorosis and vein greening (Liefting *et al.,* 2009c; Brown *et al.,* 2009). Some cultivars have purpling of the mid-vein and the leaflets may be curled. Some tomato varieties may have fruit deformation resulting in a strawberry-shaped appearance and some varieties may have no fruit (Liefting *et al.,* 2009c; Brown *et al.,* 2009).

The stems of *C. annuum* plants may have shortened internodes and plants may be stunted. Leaves can be pale green or chlorotic leaves with shortened petioles, the leaves may be cupped and the leaf apices may taper giving plants a spiky appearance. Flower abortion may be observed. Tip necrosis can occur and parts of the plant may die back. Disease severity is dependent on cultivar (Liefting *et al.,* 2009c).

8.3.3. *Ca.* L. solanacearum in tamarillo

Ca. L. solanacearum was first reported in symptomless Tamarillo plants from a home garden in Auckland, New Zealand (Liefting *et al.,* 2009a). Subsequent observations indicate that the bacterium is associated with a significant disease of Tamarillo and may lead to tree death in 2-4 months (Watson, 2009). Early symptoms on Tamarillo include cupping and pink colouration of new leaves. As the disease progresses leaves become yellow and develop peripheral burning. Eventually the leaves drop, branches dieback and the tree dies. Early in the season there is poor bud-break and the shoots and leaves that develop are small. Proliferation of shoots may be observed from a single bud. Fruit may not be produced on affected shoots. It is possible that initially only part of the tree may display symptoms. The disease and the aerial symptoms are similar to those caused by phytophthora, however no root rot is observed in *Ca.* L. solanacearum infected trees and the trees remain stable in the ground (Watson, 2009).

8.3.4. *Ca.* L. solanacearum and an associated disease of carrots

In Europe a disease of *Daucus carota* (carrot) thought to be caused by feeding of the carrot psyllid (*Trioza apicalis*) was first reported in 1977 (Markkula *et al.,* 1976, Nissinen 2007). In 2009, *Ca.* L. solanacearum was detected, by two PCR assays for the 16S rRNA gene and rplJ/rplL ribosomal protein genes respectively, in similarly diseased carrot plants and some asymptomatic carrot plants that were infested with the carrot psyllid in Finland (Munyaneza *et al.,* 2010a). Symptoms of the disease include curling and yellowing and/or purpling of leaves. Affected carrots also have stunted shoot and root growth and production of secondary roots along the primary root. Up to 100% yield loss has been observed in psyllid infested and diseased crops (Nissinen *et al.,* 2007). Like potatoes similar symptoms have been found in association with other organisms including a phytoplasma and an undescribed bacteria-like organism occurring in Australia (Gibb *et al.,* 2003) and stolbur phytoplasma in Spain (Font *et al.,* 1999).