

National Diagnostic Protocol for '*Candidatus Liberibacter asiaticus*', the putative causal agent of huanglongbing (HLB)



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1 INTRODUCTION

Huanglongbing (HLB) is a bacterial disease of complex symptom expression affecting Citrus species and other Rutaceae of the Aurantioideae: Aurantieae and Clauseneae and Rutoideae. The Chinese name is the official name (Moreno *et al.* 1996), other names have been yellow branch, blotchy mottle, vein phloem degeneration (Indonesia), leaf mottling (Philippines), likubin (Taiwan), citrus dieback (India) and citrus greening (van der Merwe and Anderson 1937; da Graça 1991; Halbert and Manjunath 2004). HLB is fully reviewed in the incursion management plan *Huanglongbing and its Vectors: a Pest Specific Contingency Plan for the Citrus and Nursery and Garden Industries* (Beattie and Barkley 2009).

The presumptive causal organisms of HLB are fastidious bacteria of the genus '*Candidatus Liberibacter*', within the α -subdivision of the phylum Proteobacteria. Three forms of the disease are known: an African form caused by '*Candidatus Liberibacter africanus*', an Asiatic form caused by '*Ca. L. asiaticus*' and '*Ca. L. americanus*', recorded in South America. A sub-species, '*Ca. Liberibacter africanus*' subsp. *capensis*, has been found only in Cape chestnut (*Calodendrum capense*) in South Africa. Mixed infections of the different liberibacters can occur (Bové 2006).

'*Ca. L. africanus*' is most usually vectored by the African citrus psyllid, *Trioza erytreae* (Del Guercio) (McClellan and Oberholzer 1965a, b), whilst the Asiatic citrus psyllid (ACP), *Diaphorina citri* Kuwayama commonly transmits both '*Ca. L. asiaticus*' and '*Ca. L. americanus*' (Teixeira *et al.* 2005b; Yamamoto *et al.* 2006). A diagnostic standard for the known insect vectors of HLB has been prepared (Malipatil and Semeraro 2007) and should be used as the primary source of information for those seeking to identify these insects. Experimentally, *T. erytreae* has been shown to transmit '*Ca. L. asiaticus*' (Massonié *et al.* 1976) and *D. citri* '*Ca. L. africanus*' (Lallemand *et al.* 1986). '*Ca. L. asiaticus*' has been detected in the black psyllid, *Diaphorina communis* Mathur in Bhutan (Donovan *et al.*, 2011), and in nymphs of the pomelo psyllid *Cacophylla (Psylla) citrisuga* Yang and Li in China (Cen *et al.*, 2012) but the role of either as a vector has not yet been demonstrated.

In addition to these insect vectors, HLB can also be spread by infected plants or plant parts (particularly through the use of infected budwood or by marcotting) and by dodder (*Cuscuta* spp.) (Tirtawidjaja 1981; Garnier and Bové 1993; Subandiyah 1994; Zhou *et al.* 2007; Duan *et al.* 2008) and possibly through seed (Graham *et al.* 2008; Shatters 2008; Albrecht and Bowman 2009). Stover and McCollum (2011) detected the presence of '*Ca. L. asiaticus*' in anthers by quantitative PCR, suggesting that there may also be a risk of spreading HLB through pollinations.

2 TAXONOMIC INFORMATION

The causal organisms of HLB were first assigned to the genus, '*Candidatus Liberobacter*' (Jagoueix *et al.* 1994). However, *Liberobacter* is orthographically incorrect and the generic name was amended to *Liberibacter* (Jagoueix *et al.* 1997). The term *Candidatus* is used with this genus to signify that the organism has been partially genetically characterised and that other characteristics, required by the International Code of Nomenclature of Bacteria to determine its exact phylogenetic position, are lacking (Murray and Schleifer 1994; Murray and Stackbrandt 1995).

The taxonomic hierarchy of the liberibacters is given below:

Kingdom:	Bacteria
Phylum:	<u>Proteobacteria</u>
Class:	<u>Alphaproteobacteria</u>
Order:	<u>Rhizobiales</u>
Family:	<u>Rhizobiaceae</u>
Genus:	Liberibacter

Within the genus, three species infecting Rutaceae and one subspecies have been recognised:

'*Candidatus Liberibacter africanus*' Jagoueix *et al.* 1994

synonyms: *Liberobacter africanum*
 Liberibacter africanus
 '*Candidatus Liberobacter africanum*'
 '*Candidatus Liberobacter africanus*'

'*Candidatus Liberibacter africanus*' subsp. *capensis* Garnier *et al.* 2000

synonym: *Liberibacter africanus* subsp. *capensis*

'*Candidatus Liberibacter americanus*' Teixeira *et al.* 2005a

synonym: *Liberibacter americanus*

'*Candidatus Liberibacter asiaticus*' Jagoueix *et al.* 1994

synonyms: *Liberobacter asiaticum*
 Liberibacter asiaticus
 '*Candidatus Liberobacter asiaticum*'
 '*Candidatus Liberobacter asiaticus*'

In recent years, other *Liberibacter* species have been discovered in non rutaceous hosts (Hansen *et al.* 2008; Li *et al.*, 2009; Liefiting *et al.* 2008a-c; Raddadi *et al.* 2011). Different forms of liberibacters have also been found in all genera of South African native Rutaceae analysed (Pietersen & Viljoen, 2012).

Nucleotide sequence analysis of the liberibacters has been conducted for fragments of the β operon (Liefiting *et al.* 2009a) and the genes for 16S rRNA (Liefiting *et al.* 2009b), *rpIJ* (Bastaniel *et al.* 2005; Teixeira *et al.* 2005c; 2008) and *rpoB* (Liefiting *et al.*, 2009a). The topology of resulting trees is different in each case, and the precise phylogenetic relationship among these organisms requires analysis of additional gene sequences.

3 DETECTION

Introductions of liberibacters into Australia are likely to occur as a result of illegal importation of infected plants or bud wood or through the ingress of infected psyllids on plant material, in aircraft or on wind currents.

The causal organisms of HLB are found in rutaceous hosts in the Aurantieae (= Citreae) and Clauseneae within the Aurantioideae, as well as in two tribes of the Rutoideae (= Toddalioideae) (Table 1, see Beattie and Barkley (2009) for further detail). Note that some of these host records are based on symptoms only. All species of *Citrus* and *Citrus* hybrids are susceptible to the disease (Garnier and Bové 1993; Jagoueix *et al.* 1994; Halbert and Manjunath 2004). In addition, liberibacters have also been experimentally transferred using *Cuscuta* spp. to other hosts (refer to Beattie and Barkley, 2009).

The host status of orange jasmine (*Murraya exotica* L.), a widely-grown ornamental hedge or specimen plant and a relative of citrus, is unresolved (see Beattie and Barkley 2009). Street trees in Brazil show yellow leaves and shoot dieback when infected with '*Ca. L. americanus*' but no dieback when infected with '*Ca. L. asiaticus*', but neither mottled leaves or deformed fruits when infected with either bacterium (Lopes 2006; Lopes *et al.* 2010). Artificially infected plants show

symptoms similar to those of infected citrus, but liberibacters multiply less efficiently than in citrus (Lopes *et al.* 2010) and Hartung *et al.* (2010) could not find evidence for seed transmission. However, given orange jasmine (but with records widely cited as *Murraya paniculata* (L.) Jack: see Nguyen 2012) is a favoured host of the Asiatic citrus psyllid (Aubert 1985; Beattie *et al.* 2010), it potentially represents a key reservoir of HLB in Australia.

Table 1. Known or possible Rutaceous plant hosts of liberibacter species.

Subfamily: Tribe	Species
Aurantioideae: Aurantieae	<i>Aeglopsis chevalieri</i> Swingle
	<i>Atalantia</i> (syn. <i>Severinia</i>) <i>buxifolia</i> (Poir.) Oliv.
	<i>Balsamocitrus dawei</i> Stapf
	<i>Citrus</i> spp.
	<i>Limonia acidissima</i> L.
	<i>Murraya exotica</i> L.
	<i>Pamburus missionis</i> (Wight) Swingle
	<i>Swinglea glutinosa</i> (Blanco) Merr.
	<i>Triphasia trifolia</i> (Burm. f.) P. Wilson
Aurantioideae: Clauseneae	<i>Clausena anisata</i> (Willd.) Benth.
	<i>Clausena indica</i> Oliv.
	<i>Clausena lansium</i> (Lour.) Skeels
Rutoideae: Diosmeae	<i>Calodendrum capense</i> (L. f.) Thunb.
Rutoideae: Toddaliinae	<i>Vepris lanceolata</i> (Lam.) G. Don

3.1 Symptoms

It is assumed that all species of *Citrus* and *Citrus* hybrids are susceptible to HLB (Garnier and Bové 1993; Jagoueix *et al.* 1994; Halbert and Manjunath 2004). However, different levels of symptom expression are found among cultivated and commercial varieties, from relatively mild to extremely severe (Aubert 1990; Folimonova *et al.* 2009). The distribution of 'Ca. L. asiaticus' in different genotypes of citrus was found to be uneven within each plant and not correlated to disease severity (Folimonova *et al.* 2009, Stover and McCollom 2011). The strain of bacterium, the variety and flushing activity can also influence symptom expression and host range (Tsai *et al.* 2008).

Symptoms of the disease may occur on the whole tree or only on certain branches and can be characterised as being either primary or secondary (Schneider 1968). Primary symptoms are those that appear on leaves after they mature normally and consist of yellow blotches on dark greenish-grey leaves. Secondary symptoms occur on shoots that grow from infected branches with primary symptoms and are similar to those of mineral deficiencies (Schneider 1968). A loss of leaves showing key primary symptoms such as asymmetric blotchy mottling can occur in some citrus, especially mandarins, due to premature leaf fall. Therefore, when these trees are examined, only undersized leaves showing symptoms like nutrient deficiency can be found, and this is actually an indication of more advanced infection on any given branch.

Key HLB symptoms (Lin 1956; Schneider 1968; McClean and Schwarz 1970; Tirtawidjaja 1980; Zhao 1981; da Graça 1991; Gottwald *et al.* 2007; Bové 2006) in infected citrus plants are:

- leaves with asymmetric, sometimes dull, blotchy-mottling that crosses leaf veins (Fig. 1);
- mottled or complete yellowing of leaves and growing shoots (yellow shoots standing out from an otherwise normally green canopy) (Fig. 2);
- small upright, thickened, chlorotic leaves (sometimes resembling mineral deficiencies, particularly Zn) (Fig. 3);
- flushing of severely greened trees out of phase with healthy trees;
- yellow veins (Fig. 4);
- dieback of branches (Fig. 5);
- vein-corking associated with ultrastructural changes to phloem (but note that *Citrus tristeza Virus* and boron deficiency can also cause vein-corking (Fig. 6);
- unseasonal and heavy flowering on diseased branches;
- small, lopsided, bitter tasting fruit with small dark and aborted seeds (Fig. 7);
- unevenly coloured maturing fruit (particularly sweet oranges and mandarins in temperate and subtropical regions) on which the stylar (outer) end remains green, as the peduncle (calyx) end turns orange;
- excessive fruit drop; and
- 'silver imprints' when finger pressure is applied to the fruit (Fig. 8).



Figure 1. Leaves with asymmetric, sometimes dull, blotchy-mottling that crosses leaf veins (Photos: Pat Barkley, Brazil 2006).



Figure 2. Yellow shoots standing out from canopy (Photos: left, GAC Beattie, Florida 2007; right, Pat Barkley, Brazil 2006).



Figure 3. Leaves with HLB mottling. Left, pomelo leaves in Hong Kong (Photo: Pat Barkley). Right, sweet orange in Florida (Photos: GAC Beattie, 2007) with small upright, thickened, chlorotic leaves, some with green islands, and some resembling mineral deficiencies, particularly Zn.



Figure 4. Yellow veins left, on sweet orange leaves in Brazil (Photos: Pat Barkley, 2006) right, on sweet orange leaves in Florida (Photos: GAC Beattie, 2007).



Figure 5. Dieback of branches and small, upright leaves with Zn deficiency-like patterns (Photos: GAC Beattie, Java, Indonesia, 2003).



Figure 6. Vein-corking of pomelo leaves associated with ultrastructural changes to phloem (Photos: GAC Beattie, Viet Nam 2007).

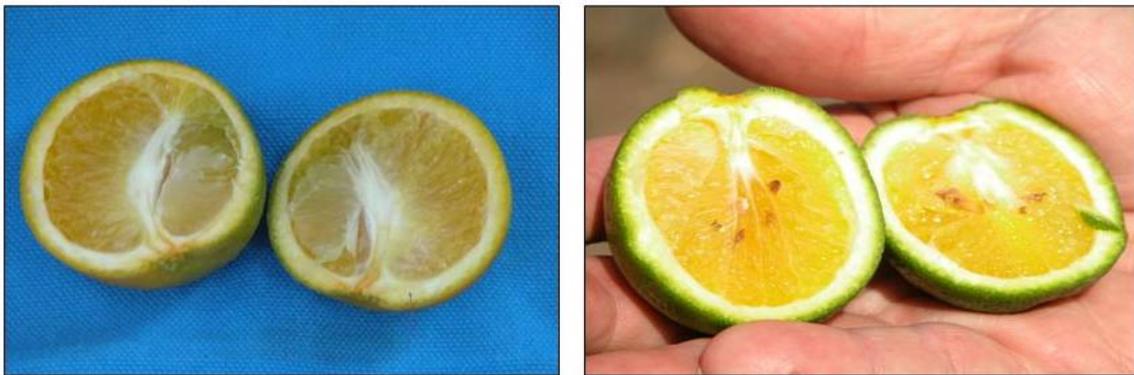


Figure 7. Small, lopsided fruit (left and right) with small dark and aborted seeds (right) (Photos: Pat Barkley, Brazil 2006).



Figure 8. 'Silver imprint' when thumb pressure is applied to immature fruit from diseased trees (left) and unevenly sized fruit, one with an abnormal red-brown button (right) (Photos: Pat Barkley, Brazil 2006).

Symptom development may be delayed for several months or as long as 2 to 3 years after infection (Lin 1956; Capoor *et al.* 1974; Zhao 1981; Hung *et al.* 2001; Gottwald *et al.* 2007). Gottwald (2010) reports that disease incidence may reach high, asymptomatic levels in 3 to 13 years from the onset of first symptoms and that disease progress is influenced by the inoculum reservoir, vector population, age of grove on infection and environmental factors. Nutrition also plays a part in disease progression (Spann and Schumann 2009). As a result, disease incidence in young plantings (<3 years old) can reach 50% within 3-5 years whilst older orchards take 5 years or longer to reach this level. Field studies showed that the extent to which HLB affects a tree depends on the age of the tree at the time of infection and the population of insect vectors. The rapid spread of the disease within older trees may be dependant not so much on internal movement of the pathogen but more on infection of healthy branches by multiple feeding of infective vectors. Severely infected trees are either infected during propagation or by exposure to high populations of infective vectors after planting in the orchard (Gonzales and Viñas 1981). Bassanezi and Bassanezi (2008) observed that in younger blocks disease incidence and severity increases faster than in older blocks, so younger tissues may be more suitable for sampling.

3.1.1 Sources of potential confusion - dual infections, other diseases, nutrient disorders

The effects of dual infection of plants with both *Citrus tristeza virus* (CTV) and liberibacters have been recorded in a number of studies. Chen *et al.* (1972) reported that the pathogens were found singly in both widely separated and adjacent cells but were rarely found together within the same cell in dual-infected plants. Martinez (1972) working with mandarin, calamondin and rough lemon, Bhagabati and Nariani (1980) working with kagzi lime and Prommintara (1990) working with mandarin all reported that dual infection produced more severe symptoms and/or faster declines than individual infection by either pathogen. In contrast, Davis *et al.* (2005) found little effect of dual infection with CTV and HLB on leaf symptoms (Figs. 9, 10, 11); however, chlorotic blotching and corky veins were more prominent in the dual infections. In South Africa, an isolate of CTV containing a mixture of strains kept the percentage of fruit showing symptoms of HLB to a 'low level' whereas, on some control trees not infected with CTV, the proportion of fruit showing symptoms of greening reached 100% (van Vuuren and da Graca, 2000).



Figure 9. Citrus from north-west Papua New Guinea infected with *Citrus tristeza virus* only (Photos: Richard Davis).



Figure 10. Citrus from north-west Papua New Guinea infected with '*Ca. L. asiaticus*' only (Photos: Richard Davis).



Figure 11. Citrus from north-west Papua New Guinea infected with '*Ca. L. asiaticus*' and *Citrus tristeza Virus* (Photos: Richard Davis).

Distinguishing between the symptoms of HLB and those of nutrient deficiency or excess can be difficult, especially as infection with HLB causes mineral imbalances including changes in levels of nitrogen, potassium, calcium, magnesium, iron, zinc, manganese, sodium and chloride (Tirtawidjaja *et al.* 1965; Aubert 1979; Koen and Langenegger 1978; Manicom and van Vuuren 1990; Pustika *et al.* 2008). One key difference is that the chlorotic blotch or mottle of HLB is usually asymmetrical, whereas that of nutrient deficiencies are typically symmetrical across the mid vein (Fig.12, Carlos *et al.* 2006).



Figure 12. Symptoms of deficiency of (A) zinc, (B) iron, (C) manganese and (D) magnesium in citrus (Photos: DPI NSW).

In certain areas of Australia, many symptoms similar to those of HLB are caused by a syndrome referred to as Australian citrus dieback (Fig. 13, Broadbent *et al.* 1976; Broadbent 2000), which has been associated with a phytoplasma in a strain group that is widespread in Australia (Davis *et al.* 1997, Monique Garnier, pers. comm. 1998; Fiona Constable pers. comm.. 2011). One of the few differences is that branches affected by Australian citrus dieback often droop down, whereas HLB dieback occurs on upright / vertical branches (A. Beattie pers. com.).

Some symptoms similar to one or more HLB symptoms can also be caused by blight (Fig. 14, Broadbent *et al.* 1996), starch accumulation associated with boron deficiency (Haas and Klotz 1931a, b) or winter yellows (Broadbent and Fraser 1979), severe stem pitting strains of *Citrus tristeza Virus* in grapefruit and *Phytophthora* root rot (Beattie and Barkley 2009).

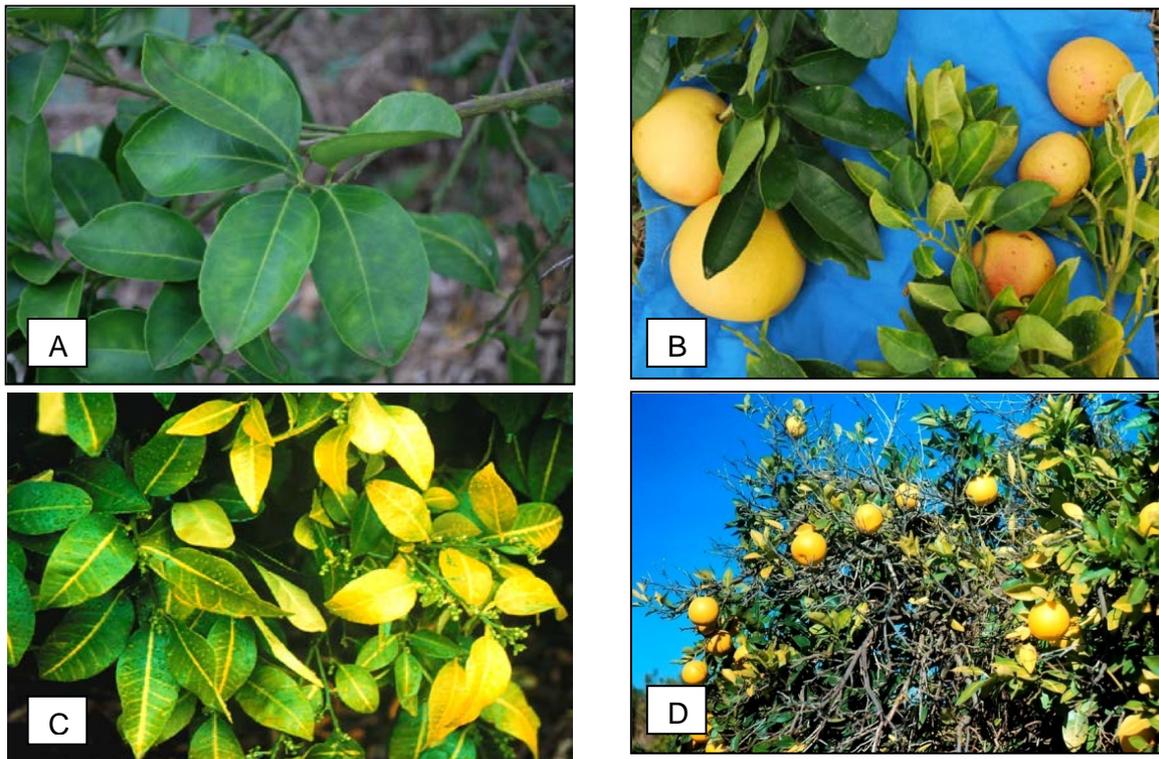


Figure 13. Australian citrus dieback. (A) leaf symptom - dull, asymmetric, blotchy-mottling crossing leaf veins. (B) small, rounded fruit, leaves with yellow veins. (C) yellow veins, and severe chlorosis. (D) Severe dieback on grapefruit trees (Photos: Pat Barkley).



Figure 14. Blight on orange at Nangiloc, Victoria (Photos: Pat Barkley).

Importantly, a range of other pathogens and physiological or mechanical disorders can also cause chlorosis, mottling, unseasonal bearing and flowering, dieback and vein corking. Some examples of these are shown in the Figures 15 and 16.

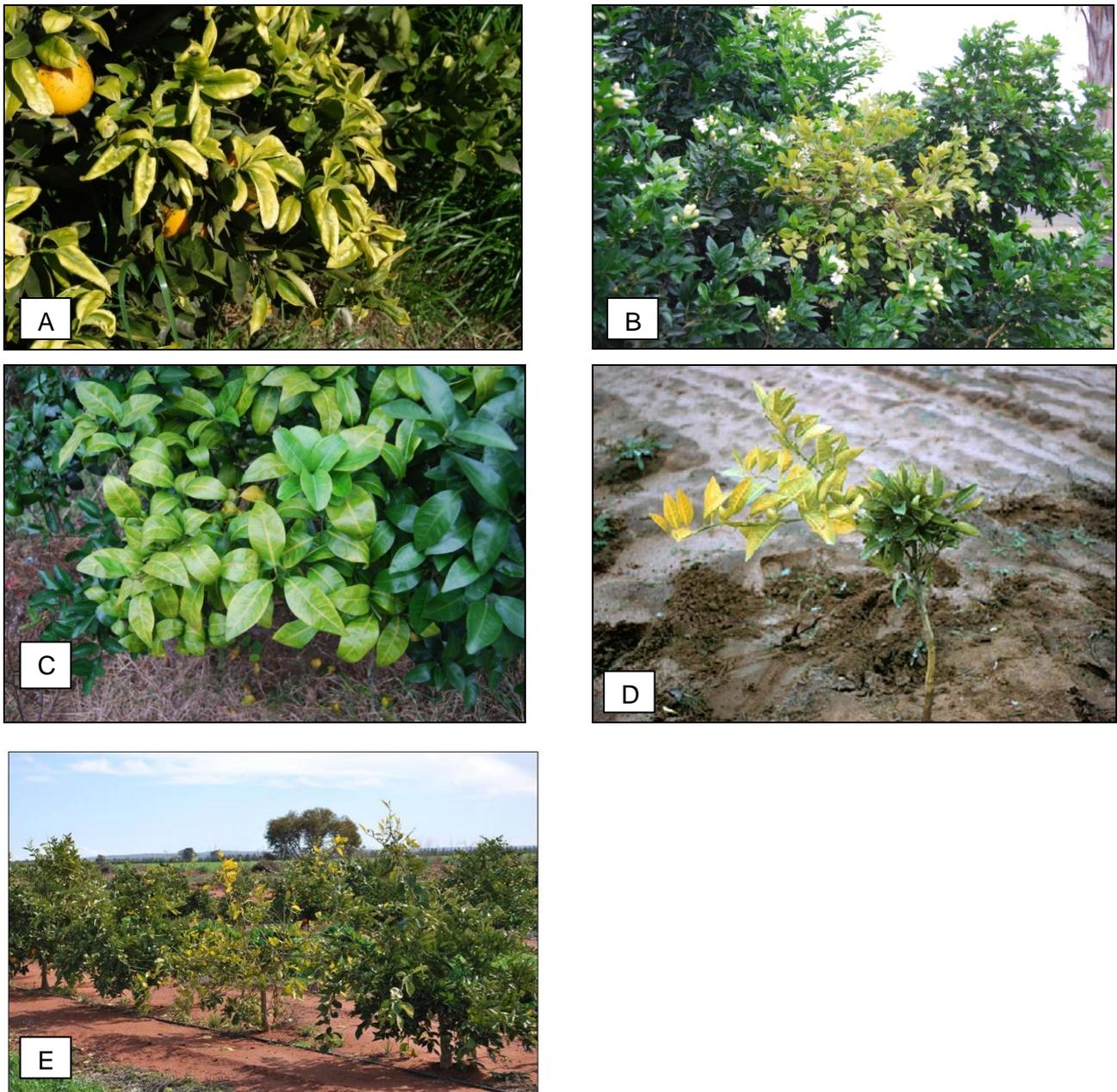


Figure 15. Symptoms similar to HLB but due to other causes. (A) cold injury, (B) borer injury, (C) girdling injury, (D) winter yellows, (E) root rot. Photos (A, B, C): GAC Beattie and (D, E): Pat Barkley.



Figure 16. Vein corking not due to HLB. (A) vein corking due to boron deficiency (GAC Beattie), (B) vein corking caused by *Citrus tristeza Virus* (Photo: DPI NSW).

3.2 Sampling

3.2.1 The parts of the plant in which HLB may be found

The liberibacters are found in immature and adult plants within the phloem of leaves, bark (Coletta-Filho *et al.* 2005; Sagaram *et al.* 2008) and roots, and in the peduncles, seed coats and columella of fruits (Albrecht & Bowman 2009, Hilf 2011). Molecular analyses detected '*Ca. Liberibacter asiaticus*' in bark tissue, leaf midrib, roots, and different floral and fruit parts (Tatineni *et al.*, 2008). Many studies show a low and uneven distribution (Su and Chang 1974; Harakava *et al.* 2000; Gottwald *et al.* 2008; Li *et al.* 2009; Tatineni *et al.* 2008). Sagaram *et al.* (2008) found that '*Ca. L. asiaticus*' was distributed in the floral and fruit tissues of citrus trees, with particularly high concentrations in the fruit peduncle, the latter also confirmed by Tatineni *et al.*, (2008). However, in the studies of Sagaram *et al.* (2008) and Tatineni *et al.*, (2008), the pathogen was not found in the endosperm and embryos. The situation regarding the presence of liberibacters in citrus seed is unclear (see Beattie and Barkley 2009) and transmission by seed appears doubtful given all the studies with negative results, so seeds are not recommended for diagnostic testing. However Hilf (2011) used real-time quantitative PCR to detect pathogen DNA in nucleic acid extracts of 36 and 100% of peduncles from two varieties, at similar percentages in extracts of the seed coats of those same varieties, and in 1.6 and 4% of extracts from the corresponding seeds. Seeds may therefore present a level of risk.

3.2.2 Interactions of HLB with climatic conditions and seasonality

The forms of the disease differ in temperature tolerance and in both field and glasshouse observations '*Ca. L. africanus*' seems better adapted to cooler temperatures than '*Ca. L. asiaticus*' (Schwarz 1968; Schwarz and Green 1972; Bové *et al.* 1974). Disease caused by the former commonly occurs in regions where the temperature remains below 30–32°C and the latter commonly in regions where temperatures are well above 30°C (Bové *et al.* 1974; Bové 2006). The temperature preferences of '*Ca. L. americanus*' appear to be similar to those of '*Ca. L. africanus*' (Lopes *et al.* 2008; Gasparoto *et al.* 2012). Folimonova *et al.* (2009) found that for infected plants incubated in a growth room with continuous light, the time before distinctive symptoms developed was reduced and that they produced much greater expressions of chlorosis than corresponding plants grown in a glasshouse.

Seasonal effects on the disease have been reported. The leaf symptoms caused by '*Ca. L. africanus*' in South Africa are more pronounced in winter (Schwarz 1968; McClean and Schwarz 1970). In Brazil, although new symptoms of HLB occurred throughout the year, they occurred with a higher prevalence during autumn and winter (Bassanezi *et al.* 2006). These differences in symptom expression are likely to be due to the effects of season on bacterial numbers. In China, nested and real-time PCR showed higher titres of '*Ca. L. asiaticus*' in the summer compared to the

winter, with bacterial numbers correlated roughly with monthly temperature (XL Deng, pers. comm.).

In Florida, where HLB is caused by the heat-tolerant form, 'Ca. *L. asiaticus*', sampling is best from early autumn to early spring. Mature trees in Florida typically produce major flushes of growth during early spring and summer and sometimes minor flushes of growth during late summer and autumn. In Australia, monthly average temperatures vary across citrus growing areas from the tropical north to the temperate conditions in southern Australia. In addition, flushing times may vary dependent on rainfall, as well as temperature (Broadbent 1992). It would therefore be expected that the optimum sampling time for detecting 'Ca. *L. asiaticus*' in Australia will vary with region.

3.3 Methods

The sampling protocols given below for symptomatic field trees are from Irey (2007) and should be followed. Note that sampling protocols for asymptomatic material, such as might be taken as part of surveillance activities, are included at Appendix 8.1.

3.3.1 Sampling symptomatic field trees

1. Samples should be collected from the symptomatic areas/branches of the trees.
2. Samples should consist of short sections (10–15 cm or greater) of symptomatic branches with the attached leaves. If fruit are present on the branches, the fruit can either be left on or they can be trimmed off. If the fruit are trimmed off, please leave the fruit stem on the sample (i.e. trim the fruit off as close to the button as possible leaving the stem on the branch).
3. If a variety of symptoms are present, the preferred samples (in order of preference) would be:
 - a. branches with mottled leaves;
 - b. branches that contain shoots that are almost entirely yellow;
 - c. branches that have leaves with yellow veins;
 - d. branches with leaves that have either green islands on a yellow background or yellow islands on a green background;
 - e. branches with nutrient deficiencies that have a "rabbit ear" appearance (small, upright leaves);
 - f. branches with leaves that show chlorosis and "vein corking";
 - g. branches with zinc and/or iron deficiencies that are not related to blight or other known causes.
4. Place the leaves/twigs into a sealable (e.g. Ziplock) plastic bag and keep the sample cool and out of the sunlight.

3.3.2 Sampling psyllids

Positive PCR results for HLB pathogens in psyllids may provide indicative information regarding the likely area exposed to infection. Due to the latency period, infected psyllids may be present one to several years before the appearance of HLB symptoms. Importantly, a negative result does not indicate the absence of HLB in the area (Manjunath *et al.* 2008). Psyllid nymphs are unlikely to move from plant to plant so can only acquire 'Ca. *L. asiaticus*' from the tree on which they live. A nymph sample with 'Ca. *L. asiaticus*' would indicate that the source plant is infected, while an adult with 'Ca. *L. asiaticus*' may indicate only the presence of infected trees in the vicinity.

In Florida, the 2007 Huanglongbing Technical Working Group agreed that the use of *D. citri* to detect incipient infections of HLB in areas where the disease is not known to occur is a potentially useful survey tool. However, the amount of bacteria in a single infected psyllid can be near or below the current detection limits of real-time PCR technology, and may have a high and possibly unacceptable false-negative rate. In Florida, the highest percentage of HLB positives from plant samples were collected in late summer and early autumn after a flush occurred. Periods when

host plants are in active flushes of growth appear to affect the ability to detect the bacterium. A drop in bacterial titre during flush is commonly observed. This corresponds to periods of the maximum psyllid population growth.

Also if the psyllids are collected from yellow sticky traps the ability to detect 'Ca. L. asiaticus' diminishes with time. Survey traps therefore need to be checked at very close intervals (every 1 to 3 days).

3.3.3 Sample desiccation

If there is a delay between sample collection and submission to a testing laboratory, then consider desiccating plant tissues using the following protocol.

1. Surface sterilise leaves with 80% ethanol or 1% available chlorine. This can be done with most household bleach (4% available chlorine) diluted 1:4 or with swimming pool chlorine (650 g/kg chlorine) at 3 g per 200 ml water as this is better than bleach, which makes a slimy and difficult to handle solution. Immerse for two minutes, then rinse the leaf material in water and blot it dry on tissue paper.
2. Using a sharp knife/blade, cut approximately 1 g fresh weight of midribs and/or petioles from leaves and chop midribs and petioles into approximately 2 mm lengths. It is important to only collect this material as any extra leaf material may reduce the sensitivity of the test
3. Wrap in paper (facial) tissue or medical gauze bandage and place over 5-7g anhydrous calcium chloride (Sigma-Aldrich product code C1016)/silica gel in 25ml plastic vials. Place immediately in the refrigerator. Calcium chloride/silica gel will dry the leaf material so it can be stored prior to testing.
4. The following day replace the tissue or gauze and moist desiccant with fresh, dry material and wrap parafilm (sealing tape), strips of cling wrap or insulation tape around the join between lid and vial. Material must be stored in the refrigerator or in a cool box with ice.

3.3.4 Sample handling following receipt into the laboratory

Following the initial receipt of the field sample to the laboratory facility:

1. Submission is received and transported (sealed) to containment area.
2. Submission number assigned, paperwork copied and filed.
3. Package opened in containment area and number of samples is ascertained.

Where possible, leaf samples should be photographed prior to dissection. Given the known concentrations of the bacterium in plant tissues, sampling tissue for nucleic acid extraction from leaf midribs and veins, or from fruit peduncles or the columella or placental tissue of fruit, could be expected to give maximum chance of detection. For each sample where both symptomatic and asymptomatic tissue is available, separately prepare at least one extract each from symptomatic and asymptomatic tissue (the latter close to but not directly next to symptomatic tissue if possible).

3.3.5 Clinical examination

For each sample:

1. Swab bench and hands, place bagged sample on bench.
2. Unpack sample.
3. Perform clinical examination and select material for photography.
4. Photograph sample.
5. Place leaf on a glass slide and with a sterile scalpel blade excise a small section of leaf tissue (maximum 5 x 5 mm). Midrib and/or other veinous tissues are likely to be optimal for detection of the bacterium. Place leaf tissue into a labelled 2 mL collection tube.
6. Prepare replicate subsamples (at least 3) for each sample in each submission.

Negative controls for use in PCR would ideally include no template controls as well as DNA extracted from known-healthy citrus.

4 IDENTIFICATION

Infection with liberibacters within tissues can be determined by biological indexing, light microscopy to determine changes in leaf tissues, electron-microscopy to detect the presence of the bacterium in phloem vessels and through the use of monoclonal antibodies. However, these methods are not used for routine diagnostics, as they are either superseded by other techniques or are compromised by the low concentration and uneven distribution of the pathogen in its hosts.

Field diagnosis based on symptoms can be supplemented by the use of the iodine starch test whilst the polymerase chain reaction and DNA sequencing are used to confirm infection.

4.1 Iodine Starch Test

The iodine starch test (IST) is a quick, presumptive field test for HLB. It is based on the accumulation of starch in infected leaves (Schneider 1968; Kawano 2006) and has given some good correlation to molecular HLB testing. The amylase in the starch stains blue with iodine while amylopectin stains purple with iodine (Shannon and Garwood 1984).

Reagents

- Iodine (1 g iodine (I₂) and 2 g potassium iodide (KI) dissolved in 100 mL distilled water)
- Course grade (120 mesh) silicon carbide abrasive paper cut into ~2 x 1 cm rectangles (do not use sand paper as there can be starch in the adhesive so test out the brand of abrasive paper before it is used)

4.1.1 Methods

(After Eng 2007 and Takushi *et al.* 2007)

1. Collect an asymptomatic leaf and five symptomatic leaves from each putatively infected tree. Do not use branches with physical damage.
2. Using the asymptomatic leaf, scratch the upper surface of the leaf 20 times with a piece of abrasive paper.
3. Place the piece of abrasive paper in a plastic bag and add 1 mL distilled water.
4. Remove the tissue scrapings from the abrasive paper by gently squeezing the plastic bag.
5. Add two drops (~30–50 µL) of iodine to the bag.
6. Repeat steps 2–5 with using the HLB-infected leaves, each being placed in a separate plastic bag.
7. Place the bags against the white card and observe the colour of the fluid in the bag, comparing the solutions obtained with the positive and negative controls. HLB-positive leaves should give a solution containing dark blue-black starch granules (Fig. 17).
8. Trees with three or more leaves giving a positive starch reaction are putatively HLB-positive and their disease status should be checked by PCR.

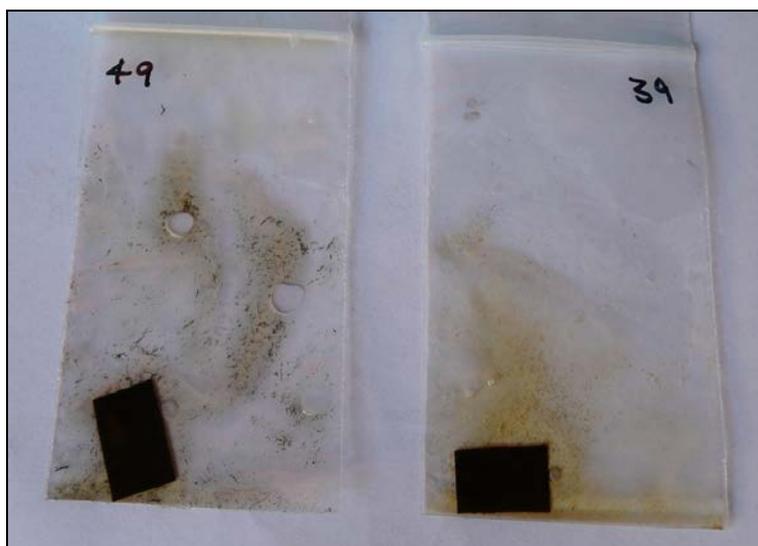


Figure 17. Positive (left) and negative (right) iodine starch reactions.

4.1.2 Accuracy

The correlations between IST and PCR test results have been variable (Su, 2008; Eng 2007; Takushi *et al.* 2007; Chamberlain and Irej 2008). Chamberlain and Irej (2008) considered the starch test a useful tool for HLB diagnosis in the field, but not as a substitute for PCR-based testing. Caution is needed to ensure that a positive IST is not caused by factors other than HLB, particularly Australian Citrus Dieback and girdling. Additionally, a recent survey of citrus orchards in Australia showed that 5 out of 25 samples showing different chlorotic symptoms gave a positive IST (Miles *et al.* 2009), but were negative for HLB. Therefore the applicability of the test in aiding the detection of HLB-infected plants in Australia needs further consideration.

4.2 DNA extraction

During the development of this protocol, many methods of DNA extraction were evaluated, including proprietary kits such as that produced by Qiagen. The Qiagen method was selected for inclusion for its reliability and ready availability, and the capacity to upgrade it to high throughput automation. In general all proprietary methods were found to be reliable and other methods could be substituted, particularly where for example, it may be preferable to test a bulked-up samples. A rapid method suitable for use in surveillance activities is described in appendix 8.2.

4.2.1 DNA extraction from leaf tissue using Qiagen DNeasy Plant Mini Kit

DNA is extracted using the Dneasy Plant Mini Kit (Qiagen, product code 69104).

Reagents

Ethanol (96-100% non-denatured).

Liquid Nitrogen

Nuclease-free sterile distilled water (NFsdH₂O)

Extraction protocol

Fresh material

1. Swab bench and hands, place bagged sample on bench.
2. Unpack sample.
3. Perform clinical examination and select material for photography.

4. Photograph sample.
5. Place leaf on a glass slide and with a sterile scalpel blade excise leaf midribs and section into ~2mm lengths. Mix segments well and weigh out 100 mg of tissue (as per DNeasy Plant Mini Kit instructions for fresh material) into a weight boat or foil. If using desiccated material, weigh out 20 mg of tissue.
6. Transfer to mortar and pestle and grind tissue under liquid nitrogen to a fine powder as per DNeasy Plant Mini Kit instructions. Do not allow tissues to thaw.
7. Follow kit instructions until elution step.
8. Follow the repeat elution steps as described in the protocol except add 50 μL NFsdH₂O each time instead of 100 μL (kit recommendation). Final volume will be 100 μL instead of 200 μL .

4.2.2 DNA extraction from a psyllid

DNA is extracted using the REExtract-N-Amp Plant PCR Kit. Preliminary experiments indicate that pooling 2–10 psyllids for extraction may hinder reliable detection of HLB positives; therefore, extraction of single psyllids is recommended.

Add 10 μL of Extraction Solution to each psyllid in 2 μL collection tube and homogenise with a sterile plastic pestle. Close the tube and vortex briefly. Make sure the psyllid is covered by the Extraction Solution.

Incubate 95°C for 10 minutes.

Add 10 μL of Dilution Solution (supplied) and vortex to mix.

Store the diluted psyllid extract at -20°C. It is not necessary to remove the psyllid tissue before storage.

4.3 Suggested “best practise” operations in diagnostic PCR

A short summary of important points, as implemented in the laboratory at EMAI and representing good practise in diagnostics based on PCR (or RT-PCR), follows:

1. Sub-aliquot all reagents (except enzymes). This reduces the potential of reagent degradation (through repeated freezing and thawing) and the potential for contamination.
2. Prepare “master mixes” (reaction cocktails) of all reagents wherever possible, and then sub-aliquot these mixes into reaction tubes. This minimises variation between the individual reaction tubes within an assay and improves accuracy of pipetting.
3. Negative controls are critical to robust results. These are of two types:
 - i. “Water controls” or no template controls (NTC) are the most important negative controls. These reaction mixes include water instead of nucleic acid to demonstrate that the components of the initial reaction mix were free of contaminating sequences (which could give rise to false positives). Include a minimum of two water controls, one at each end of a reaction set, but also intersperse others throughout the reaction set if more than 12 samples are being tested.
 - ii. “Template controls” are nucleic acids extracted from comparable tissues to the sample being tested but which are known to be negative for the agent that will be detected in the PCR. For example, this would be a healthy host plant in the case of a virus, a related but non-pathogenic strain in the case of a bacterium etc. These controls indicate if the products generated in the reaction arise from non-specific amplification of, for example, plant-derived nucleic acids, rather than the specific pathogen. Include one (or more) template controls to demonstrate that non-specific amplification has not occurred

4. One (or more) positive controls should also be included to demonstrate that the reaction has worked successfully and to indicate the size of the diagnostic band generated if the specific pathogen is present.
5. Position templates within the reaction set in order of increasing chance of being positive to minimise the chance of cross-contamination, i.e., the order should be: water control, template control, test samples, positive control and then a further last water control (Table 3).
6. Separate all work into “pre-PCR” and “plus template” phases. Use designated work spaces and equipment in these phases. Note that “pre-PCR” phase should be free of all nucleic acids except primers. Since enzymes may contain contaminating nucleic acids, they must be added only in the “plus template” phase.
7. Handle all reagents carefully, particularly the enzymes since they are highly sensitive to both temperature and mechanical damage. Remove enzymes from freezer at the last possible moment, add immediately to master mix, and return immediately to freezer. Avoid too much pipetting once enzymes are added (mix by flicking and inversion), avoid vortexing.
8. Run “intellectual controls”, e.g., cross check your own operations at all stages, ensuring correct reaction conditions (primer pair, Mg concentration, cycling program etc.), that volumes seem appropriate and that entire volumes are dispensed.
9. Ensure all volumes to be added are feasible. This may mean diluting to give more accurate pipetting volumes (e.g., add 2 μL of a 1:10 dilution, rather than 0.2 μL of an undiluted solution).
10. Use personal protective equipment and safe handling procedures at all stages and be aware of potential risks to yourself and other workers.
11. When analysing results via electrophoresis, consider the appropriate percentage of agarose/acrylamide to use in the gel and include appropriate DNA molecular weight markers.
12. An itemised protocol sheet for every reaction conducted can be used in the laboratory at reaction set-up (Table 2) and becomes the permanent record of the procedures performed. This protocol sheet may also include a few reminders about good practises, details of the samples etc.

Table 2. Example of reaction set-up template.

No	Sample no.	Notes
1	NTC	Water control
2	Negative control	Template control
3	Sample 1	
4	Sample 2	
5	Sample 3	
6	Sample 4	
7	Positive control	
8	NTC	Water control

4.4 Detection of *Candidatus Liberibacter* species by conventional PCR from plant extracts

A number of PCR assays for the detection of '*Candidatus Liberibacter asiaticus*', '*Ca. L. africanus*' and '*Ca. L. americanus*' target the 16S ribosomal operon (e.g., OI1/OI2c Jagoueix *et al.* 1994; CN265/CN266, Harakava *et al.* 2000; OI1/DC16SLibR, Subandiyah *et al.* 2000; 16SA1/16SB1, GB1/GB3, Texeira *et al.* 2005b) and PCR-based and other methods for detecting liberibacters are collated in Palacia-Bielsa *et al.* (2009). Primers described by Hocquellet *et al.* (1999) (A2/J5) generate amplification products of different sizes depending whether the fragment was amplified from '*Candidatus Liberibacter asiaticus*' or '*Ca. L. africanus*' DNA, whereas primers described by Teixeira *et al.* (2005b) specifically amplify products from '*Ca. L. americanus*'. The primer pair of Hocquellet *et al.* (1999) also successfully amplify DNA from plants infected with '*Ca. L. africanus* subsp. *capensis*' in conjunction with primers designed against the large subunit of RUBISCO as an internal control (Phahladira 2010). The A2/J5 primer pair are used in diagnostic PCR (Table 3).

Table 3. Primer sets used for the detection of '*Candidatus Liberibacter*' species using conventional PCR. (Note that in this and following tables "Las" = '*Candidatus Liberibacter asiaticus*', "Laf" = '*Ca. L. africanus*' and "Lam" = '*Ca. L. americanus*').

Target gene	Published primer	Product size	Sequence(5'-3')	Reference	Comments by authors of this diagnostic standard
rplA/rplJ	A2 J5	Las:703 bp Laf: 669 bp	TATAAAGGTTGACCTTTCGAGTTT ACAAAAGCAGAAATAGCACGAACAA	Hocquellet <i>et al.</i> (1999)	Has been used to amplify Las only, Laf has not been trialled with these primers.
16S rDNA	GB1 GB3	Lam:1027 bp	AAGTCGAGCGAGTACGCAAGTACT CCAACTTAATGATGGCAAATATAG	Teixeira <i>et al.</i> (2005b)	Has been shown to be Lam specific and does not amplify Las.
16S rDNA	fD1 rP2	bacteria approx. 1500 bp	AGAGTTTGATCCTGGCTCAG ACGGCTACCTTGTTACGACTT	Weisburg <i>et al.</i> (1991)	Used in multiplex with all the above primers as an internal control.

Plants intrinsically contain a range of substances that, if not effectively removed during extraction, will inhibit the PCR. The effect of inhibitors remaining in DNA extracts can be reduced by dilution, although the dilution required must be determined empirically.

To assist with identifying extracts that contain inhibitors at concentrations sufficient to inhibit PCR, the '*Ca. Liberibacter*' primers are used in a multiplex reaction with "universal" primers to the bacterial 16S rDNA gene (Weisburg *et al.* 1991; Table 3). These serve as an amplification control and are expected to produce a fragment in all extracts because all field samples carry some environmental bacteria. Additionally, these primers also show some cross-reaction to amplify chloroplast DNA (Weisburg *et al.* 1991).

Samples that are positive for liberibacter species will amplify both the diagnostic fragment and the fragment derived from the 16S gene. Extracts that fail to produce the 16S band may be recalcitrant to amplification and the reaction should be repeated. Dilution of the extract may be required.

4.4.1 Reagents

REExtract-N-Amp PCR ReadyMix (Sigma-Aldrich, product code R4775 if ordered separately from kit)

NFsdH₂O PCR grade

1× TBE buffer

1% (w/v) agarose gel: 1g DNA grade agarose per 100 ml in 1× TBE buffer

Loading dye

Molecular weight marker (100 bp increments; e.g. Invitrogen's "1 kb plus" ladder)

Ethidium bromide (Sigma-Aldrich) staining solution (0.8 µg/mL) or alternative stain (e.g. Invitrogen's SYBR® Safe DNA gel stain, product code S33102)

4.4.2 Method

In pre-PCR cabinet:

1. Label sterile 0.2 mL PCR tubes.
2. Prepare a "master mix" as per Table 4 in a sterile 1.5 mL centrifuge tube. Prepare sufficient master mix for at least "n+1" reactions. For example, for 9 assays (NTC, healthy citrus, 5 test extracts, 1 positive control, NTC) prepare master mix for 10 reactions, for 30 assays prepare master mix for 35 reactions.
3. Store master mix on ice.
4. Add 1 µL of NFsdH₂O to the NTC tubes.
5. Aliquot 19 µL Master Mix to the PCR tubes and close lids.

Table 4. Master mix for '*Candidatus Liberibacter*' species primers used in a multiplex reaction with 16S rDNA primers to screen DNA from plant extracts.

Reagents (initial concentration)	Volume in each PCR tube (µl)	Final concentration	Example × 10
NFsdH ₂ O	2.2		22
2× Ready mix	10.0	1×	100
MgCl ₂ (50 mM)	0.4	1 mM	4
Forward primer A2 or GB1(10 µM)	1.5	750 nM	15
Reverse primer J5 or GB3 (10 µM)	1.5	750 nM	15
Forward primer fD1(10 µM)	0.2	100 nM	2
Reverse primer rP2 (10 µM)	0.2	100 nM	2
NFsdH ₂ O/Dilution buffer (if using the Sigma kit for extractions)	3.0		30
DNA template	1.0		
Total reaction volume	20.0		

In a biosafety cabinet:

6. Open second NTC tube and leave open until template has been added to other tubes.
7. Add 1 µL DNA template to the appropriate tubes.
8. Cycle the tubes with PCR program listed in Table 5.
9. Once the PCR is complete, load 10 µL of each reaction and recommended volume of molecular weight marker into separate wells of a 1 % (w/v) agarose gel.
10. Separate the fragments in 1× TBE at 90 V for approximately 40 min.
11. Stain, visualise and photograph gel using a Gel Documentation System or similar.

Table 5. Conventional PCR program for the amplification of '*Ca. Liberibacter*' DNA using primers A2/J5 multiplexed with fD1/rP2 or GB1/GB3 multiplexed with fD1/rP2.

Step	Temperature (°C)	Time	Number of cycles
Denaturation (initial)	92	2 min	1
Denaturation	92	45 s	5
Annealing	69-65 (touchdown 1°C per cycle)	45 s	
Extension	72	1 min	
Denaturation	92	45 s	35
Annealing	65	45 s	
Extension	72	1 min	
Extension (final)	72	10 min	1

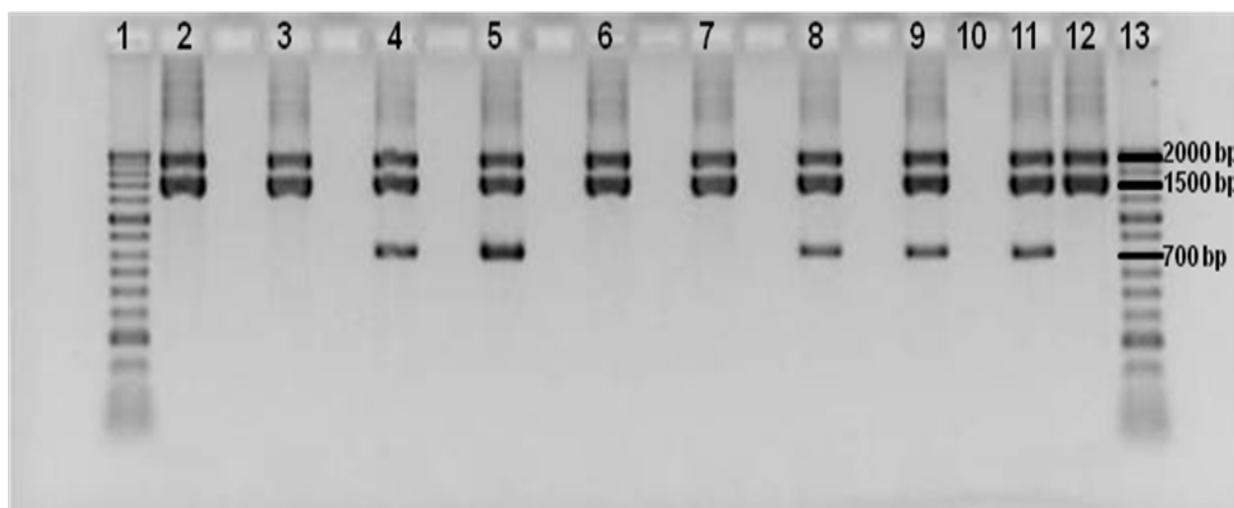


Figure 18. Agarose gel showing PCR products generated from diagnostic samples reacted with primers A2 and J5 in a multiplex reaction with fD1 and rP2. DNA Lanes 1 and 13: 1 kb+ molecular weight marker; Lane 11: positive control; Lane 12: template (negative) control Lane 10: no template control (NTC); Lanes 2-9: diagnostic samples.

A representative gel is shown in Figure 18; the samples in lanes 4, 5, 8 and 9 produce both the diagnostic band for '*Ca. L. asiaticus*' (~700 bp) and the "universal" bacterial 16S fragment (~1500 bp). These samples are assessed as positive for HLB. Samples in lanes 2, 3, 6, and 7 are amplifiable (i.e., produce the "universal" bacterial 16S fragment) but do not produce the diagnostic band; therefore, HLB is not detected in these extracts. The "universal" bacterial 16S primers also anneal to chloroplast DNA sequences, resulting in amplification of a ~ 2000 bp product from many of the plant DNA extracts. This band can be ignored when interpreting the results.

It is not unusual for the bacterial 16S fragment to be also amplified in the NTC reactions. This is most likely due to the presence of bacterial DNA contamination from the ambient environment occurring during assay set up. This is not of concern.

Any samples that failed to produce internal control fragments would need to be re-extracted and/or re-tested.

Higher throughput methods to detect and differentiate liberibacters using real-time PCR are presented at Appendix 8.2.

Amplicons generated by this and other methods can be subjected to nucleotide sequencing to additional confirmation of identity of the organism. Standard methods are applied and have not been detailed here. Nucleotide sequencing should be performed as a matter of course in the early stages of an outbreak but may be less relevant in later stages.

In seeking to verify the protocols described here, Dr Lynne Jones sought to multiplex the primers described by Teixeira *et al.* (2005b), ie those that specifically amplify products from 'Ca. L. americanus', with the internal 16S primers as described here. Whilst the product had amplified successfully in a simplex reaction, under the conditions described here the product from 'Ca. L. americanus' was not successfully amplified in this multiplex. Dr Jones therefore evaluated other internal controls and an alternative method using primers targeting the NADH dehydrogenase ND2 subunit (*ndhB* gene, Thompson *et al.* 2003) was found to work effectively, perhaps because this reduces competitive pressure between targets for the 16S region. This method has not yet been verified in a second laboratory and but is included in the Appendices to this NDP.

4.5 Criteria for the determination of a positive or negative result in PCR

The following criteria are used by the Florida Southern Gardens HLB Diagnostic Laboratory (http://www.flcitrusmutual.com/content/docs/issues/canker/sg_samplingform.pdf) and summarise the principles used universally in diagnostic laboratories:

Test results fall in one of three categories:

- HLB positive: the test results indicate that 'Ca. Liberibacter sp.' was detected in the sample, both the "universal" primers the diagnostic primers amplify a fragment of the correct size;
- no HLB found: test results did not indicate that 'Ca. Liberibacter' was present in the sample, the "universal" primers produced a fragment of the correct size but the diagnostic primers did not or;
- an inconclusive test result: neither the "universal" primers nor the diagnostic primers amplified a fragment of the correct size (re-testing should be done).

No testing procedure is 100% accurate. If a sample is designated as 'no HLB found', this does not mean that the tree/plant from which the sample was taken is disease-free—it means that no 'Ca. Liberibacter' was detected in the sample. This could be because:

- no 'Ca. Liberibacter' was present;
- 'Ca. Liberibacter' was present, but below the limit of detection;
- 'Ca. Liberibacter' was present, but the sample was inadequate for testing (sample was in poor condition, wrong tissue type was sampled, wrong sampling time); or
- the test failed.

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For contact details of additional specialists, refer to Beattie and Barkley (2009).

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8 APPENDICES

8.1 *Sampling protocols for surveillance*

8.1.1 **Sampling of asymptomatic field trees**

(No symptoms at all on the trees)

1. Samples should be collected from branches that are dark green and angular to slightly rounded in cross section. The bark should be green and not brown.
2. Leaves from the sampled branch/twig should be fully expanded and hardened.
3. Collect 6–8 leaves (including the leaf petiole) from around the tree.
4. Alternatively, cut 6–8 branches (at least 10 cm long) and trim them so that only the angular (to slightly rounded), dark green section of the branch/twig containing fully expanded hardened leaves is left. Leave the leaves on the twig.
5. Place the leaves/twigs into a sealable (e.g. Ziplock) plastic bag and keep the sample cool and out of the sunlight.

8.1.2 **Sampling of asymptomatic budwood trees**

(Scion trees, either planted in-ground or in large pots)

1. Samples should be collected from branches that are dark green and angular to slightly rounded in cross section. Essentially the samples should come from the same area of the tree where you would cut budwood that would be suitable for use in liners.
2. Leaves from the sampled location should be fully expanded and hardened.
3. Cut 4–6 branches and trim them so that only the angular (to slightly rounded), dark green section of the branch/twig is left (at least 10 cm). Leaves can be left on or removed.
4. Place the leaves/twigs into a sealable (e.g. Ziplock) plastic bag and keep the sample cool and out of the sunlight.

8.1.3 **Sampling from nursery blocks**

1. Samples from nursery blocks should consist of fully expanded and hardened leaves.
2. Leaves should be collected from branches that are dark green and angular to slightly rounded in cross section. The samples should come from the same area of the tree where you would cut budwood that would be suitable for use in liners.
3. Approximately 10% of the plants should be sampled. Samples should be more or less evenly distributed across the entire increase block. If there are symptomatic plants, it is permissible to sample this area more intensively.
4. One leaf per plant should be collected and all leaves from each individual nursery increase block can be combined into one bag and submitted as a single sample.
5. Place the leaves into a sealable (e.g. Ziplock) plastic bag and keep the sample cool and out of the sunlight.

8.2 *Higher throughput molecular detection methods*

8.2.1 **Sigma REExtract-N-Amp Plant PCR Kit**

The method selected as suitable for use in surveillance situations, because of its capacity for higher throughput extraction and reaction, is the REExtract-N-Amp Plant PCR Kit (Sigma-Aldrich, product code XNA-P) or the SYBR Green Extract-N-Amp Plant PCR Kit (Sigma-Aldrich, product

code XNAP-G). The extraction reagents supplied with these two kits are identical. Extracts resulting from these methods can however contain higher concentrations of PCR inhibitors compared to column based systems. Caution is advised and they should be considered for use in delimiting surveys as part of a possible response to a detection of HLB in Australia, rather than as methods for standard use.

Extraction protocol

1. Add 100 μ L of Extraction Solution to each leaf subsample in 2 mL collection tube. Close the tube and vortex briefly. Make sure the leaf is covered by the Extraction Solution.
2. Incubate 95°C for 10 minutes.
3. Add 100 μ L of Dilution Solution (supplied) and vortex to mix.
4. Store the diluted leaf extract at -20°C. It is not necessary to remove the leaf tissue before storage.

8.2.2 Detection of ‘*Ca. Liberibacter*’ species by quantitative real-time PCR using dual-labelled hydrolysis probes from plant extracts and psyllids.

Real-time, quantitative PCR has gained acceptance due to its improved speed, sensitivity, reproducibility, robustness and the reduced risk of carry-over contamination. Detection of ‘*Ca. Liberibacter*’ species using real-time PCR is possible using either intercalating dyes or, more specifically, using dual-labelled probes. Considerations such as the sample throughput required and cost might influence which chemistry is selected. Methods for both have been optimised and are described here, however difference in thermal platforms, optical systems and device drivers between real time PCR machines mean that probe-based assays will likely need to be evaluated on a ‘by laboratory’ basis to obtain optimal detection and sensitivity. This could include use of different fluorophores and on occasion, sequence design.

Li *et al.* (2006) developed a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan^R) assay using probe-primer sets to differentiate the ‘*Ca. Liberibacter*’ organisms. Specific forward primers to ‘*Candidatus Liberibacter asiaticus*’, ‘*Ca. L. americanus*’ and ‘*Ca. L. africanus*’ are used with a universal HLB reverse primer. These authors also describe a cytochrome oxidase (COX)-based primer-probe set as a positive control for plants. The primers described by Wang *et al.* (2006) were designed to amplify ‘*Candidatus Liberibacter asiaticus*’ only, but we found they also amplified ‘*Ca. L. americanus*’, which reduced their utility. Manjunath *et al.* (2008) targeted the nuclear gene, *wingless (wg)*, to design two primers (DCF and DCR) and a Taqman probe (DCP) that can serve as an internal positive control for psyllids. Sequences of primers and probes used in real time PCR are shown in Table 6.

This assay allows for a real-time diagnosis in around 2 hours (using LC480 Roche) and can differentiate HLB strains by using different forward primers for each HLB strain but the same reverse primer and probe.

Table 6. Primer sets used for the detection of ‘*Ca. Liberibacter*’ species using real-time PCR. (Note that in this and following tables “Las” = ‘*Candidatus Liberibacter asiaticus*’, “Laf” = ‘*Ca. L. africanus*’ and “Lam” = ‘*Ca. L. americanus*’).

Target gene	Published primer	Product size	Sequence (5'-3')	Ref.	Comments by authors of this diagnostic standard
16S rDNA	HLBas HLBaf HLBam HLBr HLB probe	approx 75 bp	TCGAGCGCGTATGCAATACG CGAGCGCGTATTTTATACGAGCG GAGCGAGTACGCAAG TACTAG GCGTTATCCCGTAGAAAAAGGTAG FAM-AGACGGGTGAGTAACGCG-BHQ-1	Li <i>et al.</i> (2006)	The Las and Lam primers are specific for that species. (The Laf-specific primer has not yet been tested)
Plant Cytochrome oxidase	COXf COXr COX probe	approx 70 bp	GTATGCCACGTCGCATTCCAGA GCCAAAAGTCTAAGGGCATTCC CAL Fluor Orange 560- ATCCAGATGCTTACGCTGG-BHQ-1	Li <i>et al.</i> (2006)	Used as a positive internal control for plant samples using dual labelled hydrolysis probes
Wingless (wg)	DCF DCR DC probe	approx 74 bp	TGGTGTAGATGGTTGTGATCTGATGTG ACCGTTCCACGACGGTGA CAL Fluor Orange 560- TGTGGGCGAGGCTACAGAAC-BHQ-1	Manjunath <i>et al.</i> (2008)	Used as a positive internal control for psyllid samples using dual labelled hydrolysis probes

8.2.2.1 Reagents

Extracts prepared as per Section 4.2.1

Primers and probes (Table 6 -labelled probes from Biosearch technologies)

NFsdH₂O

Platinum Taq and 10xBuffer

MgCl₂ PCR grade

dNTPs PCR grade

8.2.2.2 Method

In pre-PCR cabinet:

1. Prepare master mix as per Table 7 in a sterile 1.5 mL centrifuge tube. Prepare sufficient master mix for “n+1” reactions. For example, for 9 assays (NTC, healthy citrus, 5 test extracts, 1 positive control, NTC) prepare master mix for 10 reactions.
2. Store master mix on ice.
3. Add 2 µL of NFsdH₂O to the first NTC well.

Table 7. Master mix for quantitative real-time PCR.

Reagents (initial concentration)	Volume in each PCR tube (μ l)	Final concentration
NFsdH ₂ O	4.7	
10x PCR Buffer	2.5	1x
MgCl ₂ (50mM)	3.0	6.0 mM
dNTPs (10mM each)	0.6	0.24 mM
HLB primer mix *(2 μ M each)	3.0	240 nM
HLB probe (1 μ M)	3.0	120 nM
COX or DC primer mix (2 μ M each)	3.0	240 nM
COX or DC probe (1 μ M)	3.0	120 nM
Platinum Taq (5U/ μ l)	0.2	0.04 U
DNA template	2	
Total reaction volume	25	

*Note: HLB primer mix in table refers to either HLBas/HLBr or HLBaf/HLBr or HLBam/HLBr. COX primer mix refers to COXf/COXr (for plants). DC primer mix refers to DCf/DCr (for psyllids).

In a biosafety cabinet:

4. Aliquot 23 μ L master mix to the PCR plate.
5. Add 2 μ L DNA template to the appropriate well.
6. Add 2 μ L of positive control DNA to the appropriate well(s).
7. Add 2 μ L of NFsdH₂O to the second NTC well.
8. Cycle the tubes on a real-time PCR machine (see Table 8).
9. Analyse results using software appropriate to the real-time PCR machine used.

Table 8. Real-time PCR program for HLB primer-probe sets and controls using LightCycler480.

Step	Temperature ($^{\circ}$ C)	Time	Number of cycles	Ramp	Optics
Denaturation (initial)	95	10 min	1	4.4	Off
Denaturation	95	30 s	40	4.4	Off
Annealing/extension	58	40 s		2.2	On
Cooling	50	10 s	1	2	Off

8.2.2.3 Assessment of HLB qPCR using the LightCycler 480 software.

Analyse data from the run as per the thermocycler manufacturer's instructions.

The cycle threshold (Ct) or crossing point (Cp) value is defined as the cycle in which there is significant increase in reporter signal, above the threshold, i.e., the cycle in which the growth curve crosses the threshold. There are two methods to determine a Ct/Cp value, which is a cycle number in a log-linear region that is used to calculate the quantitative value of real-time PCR. One method, namely fit point, is performed by drawing a line parallel to the x-axis in the log-linear region of the real-time fluorescence intensity curve; a somewhat variable user-dependent value can be obtained by this method. The second method calculates a second derivative value of the

real-time fluorescence intensity curve and only one value is obtained. The second derivative calculation does not involve any decision by the user, because a positive peak corresponds to the beginning of the log-linear phase of the original data.

Representative results are shown in Figures 19 and 20. These results are from a multiplex, fluorescent PCR assay using probe-primer sets specific to '*Ca. L. asiaticus*' and COX-based primer-probe set as a positive control. Absolute quantification analyses using the second derivative method was used here.

Figure 19. Amplification profile for '*Candidatus Liberibacter asiaticus*' from 5 different leaf DNA extractions as well as negative and non-template control samples.

Figure 20. Amplification profile for cytochrome oxidase (COX) internal control from the same 5 samples as well as negative and non-template control samples.

Table 9. Summary of results shown in Figs. 19 and 20 from a multiplex, fluorescent PCR assay using probe-primer sets specific to '*Ca. L. asiaticus*' and COX-based primer-probe set as a positive control.

Sample	HLB Cp	COX Cp	Comments
Neg control	0.00	0.00	Water negative control
Sample 1	35.00	30.64	Suspect <i>Ca. Liberibacter asiaticus</i> sample
Sample 2	35.00	35.00	Suspect <i>Ca. Liberibacter asiaticus</i> sample
Sample 3	35.00	32.97	Suspect <i>Ca. Liberibacter asiaticus</i> sample
Sample 4	33.55	30.94	Suspect <i>Ca. Liberibacter asiaticus</i> sample
Sample 5	35.00	35.00	Suspect <i>Ca. Liberibacter asiaticus</i> sample
NTC	0.00	0.00	Non-template control

8.2.2.3.1 Evaluation of the PCR controls

Note: HLB is labelled with FAM and COX and DC are labelled with CAL.

If any of the controls do not match these requirements, then the PCR has failed (is invalid) and all samples and controls must be rerun:

- NTC Control: CAL Cp must equal 0.00 and FAM Cp must equal 0.00
- Positive Controls: CAL Cp must be greater than 0.00 but less than 37.00, and FAM Cp must be greater than 0.00 and less than 37.00.

8.2.2.3.2 Evaluation of COX or DC internal control in samples

- If a sample has a COX or DC internal control CAL Cp equal to 0.00 or greater than or equal to 37.00, the specific sample must be retested.

- If the CAL Cp remains the same after the retest, the sample is determined to have “failed quality assurance (QC)” even if the FAM Cp is in the acceptable range for HLB. This sample should be re-sampled or new DNA extracted.

8.2.2.3.3 Evaluation of sample FAM Cp

Both the PCR controls and the sample COX or DC internal controls must be valid prior to sample FAM evaluation.

- Negative samples: If a sample produces a FAM Cp equal to zero or greater than or equal to 37.00 then it is determined to be negative for HLB, if the COX or DC internal control is also acceptable ($0 < \text{CAL Cp} < 37.00$), as well as all the control reactions.
- HLB positive samples: If a sample produces a FAM Cp in the range of greater than 0.00 to less than or equal to 37.00 the sample is determined to be positive for HLB-Las, if the COX internal control is also acceptable ($0 < \text{CAL Cp} < 37.00$), as well as all the control reactions.

8.2.3 Detection of ‘Candidatus Liberibacter’ species by real-time PCR from plant extracts using SYBR Green I

A simple and economical method for a slightly higher throughput than conventional PCR would be to use real-time detection through SYBR Green Extract-N-Amp Plant PCR Readymix (Sigma-Aldrich) and a single-channel machine. Initial work used the SYBR Green Extract-N-Amp Plant PCR Readymix (Sigma-Aldrich) and a Rotor-gene 2000 from Corbett Research, but other real-time systems capable of detecting SYBR Green I would be suitable. SYBR Green I is the most commonly used dye for non-specific detection. It is a double-stranded DNA intercalating dye, that fluoresces once bound to the DNA. A pair of specific primers is required to amplify the target with this chemistry, and the amount of dye incorporated is proportional to the amount of generated target. SYBR Green I will bind to any amplified dsDNA and, consequently, primer dimers or unspecific products introduce a bias in the quantification. To check the specificity of the system, a meltcurve at the end of the PCR run is performed. The principle is that every product has a different dissociation temperature, depending on the size and base contents, so it is still possible to check the number of products amplified.

Amplification controls must be run separately in the real-time assays; in this case the 16S universal bacterial PCR, using primers fD1 and rP2, should be run as a conventional PCR assay to confirm that the sample was amplifiable. This removes some of the speed advantages of real-time detection.

8.2.3.1 Reagents

Extracts prepared as per Section 4.2.1

Primers from Li *et al.* (2006) (Table 6)

SYBR Green Extract-N-Amp PCR ReadyMix (Sigma-Aldrich, product part S4320; or other system for real-time PCR using SYBR Green I)

8.2.3.2 Method

In pre-PCR cabinet:

1. Label sterile 0.2 mL PCR tubes.
2. Prepare a “master mix” as per Table 10 in a sterile 1.5 mL centrifuge tube. Prepare sufficient master mix for at least “n+1” reactions. For example, for 9 assays (NTC, healthy citrus, 5 test extracts, 1 positive control, NTC) prepare master mix for 10 reactions, for 30 assays prepare master mix for 35 reactions.
3. Store master mix on ice.

4. Add 1 μL of NFsdH₂O to the NTC tubes.
5. Aliquot 19 μL Master Mix to the PCR tubes and close lids.

Table 10. Master mix for real-time PCR using SYBR Green.

Reagents (initial concentration)	Volume in each PCR tube (μ l)	Final concentration	Example : $\times 10$
NFsdH ₂ O	5.2		52
Forward primer HLBas/HLBaf/HLBam (10 μ M)	0.4	200 nM	4
Reverse primer HLBr (10 μ M)	0.4	200 nM	4
Dilution buffer	3.0		30
2 \times SYBR master mix	10.0	1 \times	100
DNA template	1.0		
Total reaction volume	20.0		

In a biosafety cabinet:

6. Open second NTC tube and leave open until template have been added tubes.
7. Add 1 μ L DNA template to the appropriate tubes.
8. Cycle the tubes with PCR program listed in Table 11.
9. Once the PCR is complete, load 10 μ L of each PCR reaction and recommended volume of molecular weight marker into separate wells of a 1 % (w/v) agarose gel.
10. Separate the fragments in 1 \times TBE at 90 V for approximately 40 min.
11. Visualise and photograph gel using a Gel Documentation System or similar.

Table 11. Real-time PCR program for primers from Li *et al.* 2006.

Step	Temperature ($^{\circ}$ C)	Time	Number of cycles
Denaturation (initial)	95	1 min	1
Denaturation	95	15 s	40
Annealing/extension	58	30 s	
Hold	70	1 min	1
Melt	70-95 (rising by 0.5 $^{\circ}$ C every 5 s)	-	1

Visualisation of reaction products on an agarose gel can sometimes be helpful, particularly if operators are new to real time analysis. In this case, load 8 μ L of each reaction with 2 μ L loading dye and include a suitable size marker (10 bp increments, HyperLadder V (Bioline) on 2% (w/v) agarose gel. Conduct electrophoresis and staining as per section above.

8.3 *Alternative extraction method for desiccated plant material*

This method uses sterilised Silicon Dioxide (0.1-0.5 mm granules; Sigma-Aldrich product code 31623) to macerate desiccated mid rib / petiole tissue instead of liquid nitrogen and has not yet been validated in a second lab so is not yet included formally in this NDP.

Reagents

Ethanol (96-100% non-denatured)

NFsdH₂O

Extraction protocol

Desiccated material

1. Weigh out 20 mg of tissue (as per DNeasy Plant Mini Kit instructions for dry material) into a weight boat or foil.
2. Transfer to mortar and pestle.
3. Tap or scoop a small amount of sand into the mortar and pestle (*do not* use fingers) and grind tissue to a fine powder.
4. Follow kit instructions.
5. Follow kit instructions until elution step.
6. Follow the repeat elution steps as described in the protocol except add 50 µL NFsdH₂O each time instead of 100 µL (kit recommendation). Final volume will be 100 µL instead of 200 µL.

8.4 *Alternative method for multiplexing 'Candidatus Liberibacter americanus' using conventional PCR*

Dr Lynne Jones sought to multiplex the primers described by Teixeira *et al.* (2005b) that specifically amplify products from 'Ca. L. americanus', with the internal 16S primers but was not successful. The method following has been used to successfully multiplex these primers with a different internal control that targets the NADH dehydrogenase ND2 subunit (*ndhB* gene, Thompson *et al.* 2003).

Table 12. Primer sets used for the detection of 'Candidatus Liberibacter americanus' species using conventional PCR.

Target gene	Published primer	Product size	Sequence(5'-3')	Reference	Comments
16S rDNA	GB1 GB3	Lam:1027 bp	AAGTCGAGCGAGTACGCAAGTACT CCAACCTAATGATGGCAAATATAG	Teixeira <i>et al.</i> (2005b)	Has been shown to be Lam specific and does not amplify Las.
Plant mRNA (NADH dehydrogenase ND2 subunit; <i>ndhB</i> gene)	AtropaNad2.1a AtropaNad2.2b	Plant: approx. 900 bp	GGACTCCTGACGTATACGAAGGATC AGCAATGAGATTCCCCAATATCAT	Thompson <i>et al.</i> (2003)	Possible amplicon for use as an internal control in multiplex with the above primers.

Reagents

REDExtract-N-Amp PCR ReadyMix (Sigma-Aldrich, product code R4775 if ordered separately from kit)

H₂O PCR grade

1× TBE buffer

1% (w/v) agarose gel: 1g DNA grade agarose per 100 ml in 1× TBE buffer

Loading dye

Molecular weight marker (100 bp increments; e.g. Invitrogen's "1 kb plus" ladder)

Ethidium bromide (Sigma-Aldrich) staining solution (0.8 µg/mL) or alternative stain (e.g. Invitrogen's SYBR® Safe DNA gel stain, product code S33102)

Table 13. Master mix for '*Ca. L. americanus*' primers used in a multiplex reaction with plant mRNA primers to screen DNA from plant extracts.

Reagents (initial concentration)	Volume in each PCR tube (µl)	Final concentration	Example : × 10
Nuclease-free sdH ₂ O	2.2		22
2× Ready mix	10.0	1×	100
MgCl ₂ (50 mM)	0.4	1 mM	4
Forward primer GB1(10 µM)	1.5	750 nM	15
Reverse primer GB3 (10 µM)	1.5	750 nM	15
Forward primer AtropaNad2.1a (10 µM)	0.2	100 nM	2
Reverse primer AtropaNad2.1b (10 µM)	0.2	100 nM	2
Nuclease-free sdH ₂ O /Dilution buffer (if using the Sigma kit for extractions)	3.0		30
DNA template	1.0		
Total reaction volume	20.0		

Table 14. Conventional PCR program for the amplification of '*Ca. Liberibacter*' DNA using primers GB1/GB3 multiplexed with AtropaNad2.1a/AtropaNad2.2b.

Step	Temperature (°C)	Time	Number of cycles
Denaturation (initial)	92	2 min	1
Denaturation	92	45 s	5
Annealing	59-55 (touchdown 1°C per cycle)	45 s	
Extension	72	1 min	
Denaturation	92	45 s	35
Annealing	65	45 s	
Extension	72	1 min	
Extension (final)	72	10 min	1

8.5 *Alternative extraction method for psyllids*

This method has not yet been validated in a second lab so is not yet included formally in this NDP.

The protocol for this method was supplied by Dr. Andrew Mitchell, Australian Museum.

DNA is extracted from psyllids preserved in ethanol (96-100% non-denatured) using the DNeasy Blood & Tissue Kit (Qiagen product code 69504) and additional Proteinase K (2 mL, Qiagen product code 19131).

Reagents

Ethanol (96-100% non-denatured)

NFsdH₂O

Extraction protocol

1. If psyllids have been stored in ethanol, remove and allow them to dry to before proceeding to the next step.
2. Transfer one psyllid to microcentrifuge tube.
3. Add 180 µL Buffer ATL as per the kit protocol for the purification of Total DNA from Animal tissues.
4. Add 20 µL of proteinase K. Mix thoroughly and incubate at 56°C for 2-3 hours.
5. Add an additional 20 µL of proteinase K and incubate at 56°C overnight (12 hrs).
6. Remove the psyllid, placing it back into ethanol.
7. Follow kit instructions. At the elution step, elute with 100 µL NFsdH₂O. Repeat this step but into a separate tube. Include both elutions' in the assay.

8.6 Alternative Taqman LNA based probes and primers

These methods have not yet been validated in a second lab so is not yet included formally in this NDP.

Table 15. Taqman LNA based probes and primers based on the real time assay by Li *et al.* (2006). Probes from Eurogentec (ordered through Integrated Sciences).

Target gene	Primer	Product size	Sequence (5'-3')	Ref.	Comments by authors of this diagnostic standard
16S rDNA	HLBas HLBaf HLBam HLBr HLB* probe	approx 70 bp	TCGAGCGCGTATGCAATACG CGAGCGCGTATTTTATACGAGCG GAGCGAGTACGCAAG TACTAG GCGTTATCCCGTAGAAAAAGGTAG Taqman LNA probe for Las/Laf/Lam HPLC purified labelled FAM/BHQ1 * cgtTacTcaCccGtctgcc	Li <i>et al.</i> (2006)	The Las and Lam primers are specific for that species. (The Laf-specific primer has not yet been tested)
Plant Cytochrome oxidase	COXf COXr COX* probe	approx 148 bp	TGGTCGTAACAATCACTTTAAGC TCCTTGATAGCTGGAAGTTCTC Taqman LNA probe HPLC purified labelled HEX/BHQ1 * aagAtgTgcTccAagTcct	<i>modified primers & probe sequence from Li et al. (2006)</i>	Used as a positive internal control for plant samples using dual labelled hydrolysis probes.

* Upper case letters represent LNA bases

The primer and probe sequence for the cytochrome oxidase (COX) internal control (Table 15) where re-designed by Dr Fabrice Magnino, Integrated Sciences Pty Ltd in 2009.