HUANGLONGBING AND ITS VECTORS

Information to Develop a Response Plan for the Citrus and Allied Industries

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PATHOGENS	
COMMON NAMES	huanglongbing (official common name, meaning 'yellow shoot disease'), citrus greening (informal common name), likubin or decline (Taiwan), leaf mottling (Philippines), citrus dieback (India), citrus vein-phloem degeneration (Indonesia), citrus greening, yellow branch or blotchy-mottle (South Africa)
SCIENTIFIC NAMES	'*Candidatus* Liberibacter asiaticus' (Asiatic form), '*Ca*. L. africanus' [1](African form) and '*Ca*. L. americanus' ('South American' form);[2]
SYNONYMS	'*Ca.* Liberobacter asiaticus' and '*Ca.* Liberobacter asiaticum' '*Ca*. Liberobacter africanus' and '*Ca*. Liberobacter africanum'
ASIATIC VECTOR	
COMMON NAMES	Asiatic citrus psyllid, Asian citrus psyllid, oriental citrus psyllid, citrus psylla
SCIENTIFIC NAME	*Diaphorina citri* Kuwayama [Hemiptera: Sternorrhyncha: Psylloidea:Liviidae]
SYNONYMS	*Euphalerus citri* Crawford
AFRICAN VECTOR	
COMMON NAME	African citrus psyllid, citrus psylla
SCIENTIFIC NAME	*Trioza erytreae* (del Guercio) [Hemiptera: Sternorrhyncha: Psylloidea: Triozidae]
SYNONYMS	*Aleurodes erytreae* del Guercio; *Trioza citri* Laing; *Trioza merwei* Petty; *Spanioza merwei* (Petty); *Spanioza erythreae* (del Guercio); *Spanioza erytreae* del Guercio

[1] A sub-species transmitted by *T. erytreae*, '*Ca*. L. africanus subsp. capensis', occurs in Cape chestnut (*Calodendrum capense* Thunb. [Rutaceae: Rutoideae]), an ornamental tree in southern Africa (Garnier et al. 2000, Pietersen et al. 2010). Pietersen & Viljoen (2012) and Viljoen et al (2013 a,b) found liberibacters in all genera of South African native Rutaceae analysed. Each rutaceous genus appears to harbour different specific Laf-like liberibacters. Those found in *Xanthoxylum* appear to be most closely related to Laf from *Citrus*. *Vepris* and *Clausena* harbour liberibacters more closely related to the LafC subspecies found in *Calodendrum.* This very recent suggestion of a further three subspecies of Laf (Roberts et al., 2015) can be resolved within the proposal of haplotypes rather than subspecies within Laf by giving them a biotype designation, recognising the current host plant differences. Laf subspecies vepridis is a biotype of LafA, while Laf subspecies zanthoxyli and Laf subspecies clausenae are biotypes of LafC (Nelson et al 2015). A single *Teclea gerrardii* specimen tested positive for a liberibacter and, through phylogenetic analyses of the three genes sequenced, was shown to be unique, albeit closely related to '*Ca.* L. africanus' and '*Ca.* L. africanus subsp. zanthoxyli' (Roberts & Pietersen 2016).

[2] There is potentially a 4th citrus liberibacter '*Candidatus* Liberibacter caribbeanus': see Keremane et al (2015)

Add D. communis & Cacopsylla???

Huanglongbing (HLB) and its vectors pose major disease and pest threats to the Australian citrus and nursery industries, and to rare and endangered indigenous species of *Citrus*.

"Asian citrus psyllid (ACP), Diaphorina citri, vectors the causal agent of huanglongbing (HLB), now recognized as the most debilitating and intractable disease of citrus. New emergent foliage is the site of all oviposition and development as well as transmission and acquisition of the causal bacteria, Candidatus liberibacter asiaticus (Clas). Infection of the plant and of emerging adults often occurs within a single psyllid generation, whereas symptom expression may take months or years, depending on tree age and condition as well as vector population. Therefore, rogueing symptomatic trees has proven largely ineffective where disease is established and management depends largely on vector suppression. Insecticidal control has proved effective in reducing yield loss, even from already infected trees, apparently by reducing continual re-inoculation of the causative bacteria, Candidatus liberibacter asiaticus (Clas) which reduces disease severity. Biological control by indigenous predators and the Asian parasitoid Tamarixia radiata *has demonstrated potential to greatly reduce psyllid numbers, although insufficiently to avoid disease spread. Furthermore, biological control is largely incompatible with the levels of insecticidal control that appear necessary to maintain productivity. Research into host finding behaviour, pathogen acquisition and transmission is ongoing with the ultimate objective of interfering with the vectoring process. Flight disorientation by UV reflecting ground covers has provided protection for young trees and new control methods such as RNA interference hold promise for control although practical application is still a future prospect. Ultimately, successful management of HLB will likey come with development of tolerant/resistant citrus varieties. Meanwhile, citrus growers in affected areas are largely dependent on psyllid suppression and horticultural practices to sustain sufficient tree health and productivity to remain profitable"* (Stansly & Qureshi CABI in print).

In developing the first incursion management plan for HLB and its vectors in 2009[3], we began with the premises that "*biosecurity and regulation must be built on science*" (Lyn O'Connell, Deputy Secretary DAWR, CRC Plant Biosecurity meeting, 28 March 2017) and generic plans would be of limited value. A comprehensive plan based on careful analysis of the scientific literature, of incursions elsewhere and the responses, was seen as a better approach.

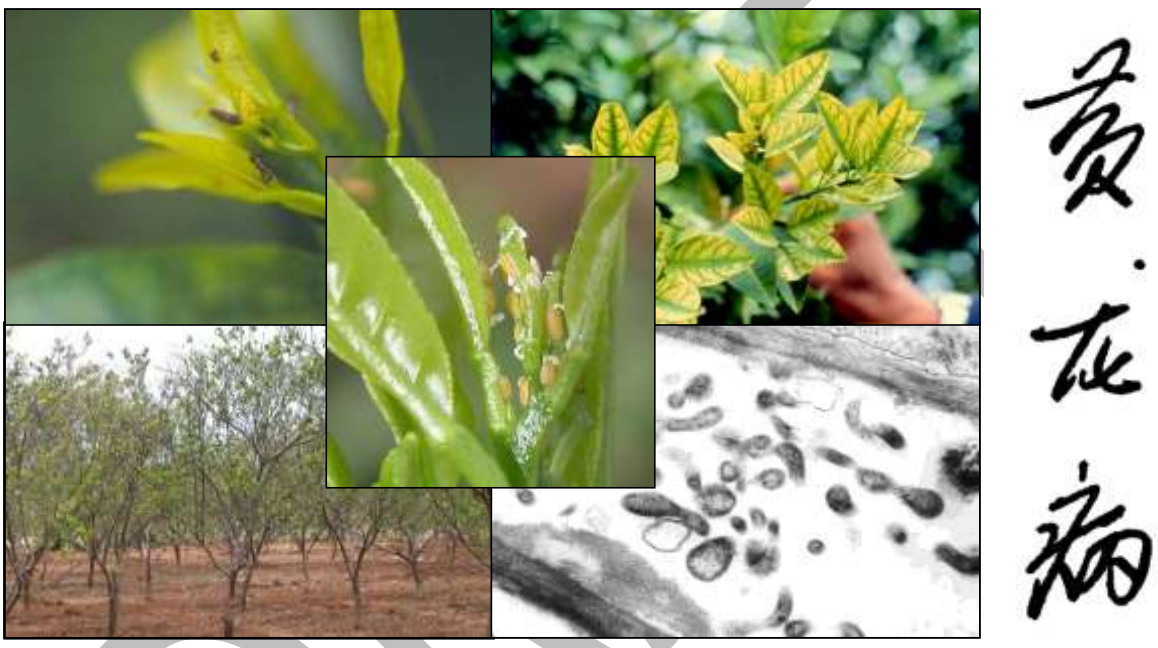
Research since 2010 has advanced our knowledge of HLB, yet the disease remains an intractable threat to citrus industries worldwide. "A single breakthrough discovery for managing HLB in the future is unlikely, since intensive research efforts over almost 20 years have not led to this result" (National Academies of Sciences, Engineering, and Medicine. 2018).

The scientific and technical content of the 2009 document has been updated to incorporate rapidly changing knowledge about host plants, advances in detection techniques for the pathogen, the distribution of the pathogens and the vectors, surveillance methodology and improvements in control methods.

The most important scientific data that should be considered in the advent of an incursion of ACP and HLB is that young flush becomes infectious within 15 d after receiving CLas inoculum. Using a microsimulation model of

[3] Beattie, G.A.C. and P. Barkley. 2009. Huanglongbing and its vectors: A pest-specific contingency plan for the citrus and nursery and garden industries (Version 2), February 2009. Horticulture Australia Ltd., Sydney. 272 pp.

asymptomatic disease spread and intensity Lee et al (2015) have shown that entire groves can become infected with up to 12,000 psyllids per tree in less than 1 y, before most of the trees show any symptoms. Transmission occurs at the feeding site among developing nymphs. The infection in the plant is transient, but the insects acquire the pathogens and can transmit immediately when they emerge as adults. Thus, the pathogen co-opts the 15 day life cycle of the bugs and their vast powers of reproduction.



[4]

Disclaimer

The information contained in this document is based on knowledge and understanding at the time of writing. However, because of advances in science, changing legislation etc, users are reminded of the need to ensure that information on which they rely is up to date and to check the currency of the information contained herein.

[4] Calligraphy: Yang Yueping. Photographs clockwise from top right: leaf symptoms, Pat Barkley, China, 1979; bacteria in phloem sieve tubes (Lafleche & Bové 1970b), dying 4-5 year-old orchard, GAC Beattie, Indonesia, 2003; adult *D. citri* and eggs, GAC Beattie, China, 1979. Centre: *D. citri* nymphs and honeydew, GAC Beattie, Việt Nam, 2006.

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The 1st contingency plan for ACP/HLB (Beattie and Barkley 2009) was prepared to provide information relevant to the implementation of *PLANTPLAN: Australian Emergency Plant Pest Response* (Plant Health Australia 2004a) in the event of incursions of huanglongbing (HLB), a serious disease of citrus, and its known vectors, the Asiatic citrus psyllid (ACP) *Diaphorina citri* Kuwayama [Hemiptera: Sternorrhyncha: Psylloidea: Psyllidae] and the African citrus psyllid *Trioza erytreae* del Guercio (Hemiptera: Sternorrhyncha: Psylloidea: Triozidae).

ACP and HLB in tandem present an entirely new threat and potential significant economic loss to the Australian citrus and nursery industries. ABARE states that with Australia's current biosecurity system, incursions of HLB are considered to occur less frequently than once in 100 years (Hafi et al. 2015). As a result, for these biosecurity threats the annual expected frequency of an incursion event is assumed to be 0.01. Without biosecurity activities, the annual expected frequencies of HLB incursions are assumed to increase to 0.2 (once in five years) (Hafi et al 2015).

'*Candidatus* L. asiaticus', which causes the Asiatic form of HLB, and its vector, *D. citri*, may represent significant threats to commercial citrus production in Australia and to the six species of *Citrus* that are native to Australia: *C. australis*, *C. australasica*, *C. garrawayi*, *C. glauca*, *C. gracilis* and *C. inodora*.[5] All citrus producing regions of Australia have climates that are favourable for '*Ca*. Liberibacter asiaticus' and *D. citri* (Aurambout et al. 2009; Narouei-Khandan et al. 2015). However Ramadugu et al. (2015, 2016[6]) claim there is HLB tolerance in the Australian native finger limes (*Microcitrus australasica*) and desert lime (*Eremocitrus glauca*)[7].

'*Ca.* L. africanus' and its natural vector, *Trioza erytreae*, also represent threats to commercial citrus production in Australia and native *Citrus* species, though, if introduced, their impact is likely to be limited, but greatest, given their susceptibility to hot, dry conditions, in the temperate southern regions of the mainland rather than in the subtropical and tropical northern regions and will be limited by the absence of their principal African alternative hosts.

'*Ca*. L. africanus' is not as aggressive as '*Ca*. L. asiaticus' and symptoms of African greening, caused by '*Ca*. L. africanus', are less severe than Asiatic HLB, caused by '*Ca*. L. asiaticus'. The two forms can be distinguished on the basis of temperature tolerance (le Roux et al. 2006a). Leaf symptoms of African HLB are more pronounced in the cool areas, than in the low-lying hot areas of southern Africa and are more pronounced in winter (Schwarz 1968a).

The vulnerability to the citrus industry Australia-wide derives from:

- Australia's proximity to the islands of the Indonesian archipelago where both HLB and *D. citri* are endemic, and to the island of New Guinea, where their eastern-most distribution is currently in the region of Vanimo in northeastern Papua New Guinea (Davis et al. 2000, 2005, Weinert et al. 2004, OEPP/EPPO 2005a, Bové 2006);
- the continuity of indigenous citrus around the Australian coastline — *Glycosmis* spp. in thickets in northern Australia (Armstrong 1975, Sykes 1997), and *C. australasica*, *C. australis*,

[5] The disease also represents a serious threat to rare and endangered native *Citrus* species in New Guinea and New Caledonia. **Good photographs of some Australasian species, excluding those from New Caledonia, can be viewed on Mike Saalfeld's website (http://www.saalfelds.freeserve.co.uk/HobbyCitrusGrowers.htm).**

[6] Ramadugu et al. **Citrograph Vol. 7, No. 2** | Spring 2016, pp. 46-51.

[7] Both are now classified as *Citrus.* Mabberley. 1998. Australian Citreae with notes on other Aurantioideae (Rutaceae). Telopea 7: 333-44.

C. *garrawayi* and *C*. *inodora* in rainforests along the east coast (Armstrong 1975, Sykes 1997) and *C. glauca* from north of Emerald, through central Queensland, and New South Wales to the Flinders Ranges in South Australia (Sykes 1999);

- the widespread occurrence of orange jasmine, *Murraya exotica/paniculata* in gardens;
- wild forms of *Murraya* including *M. heptaphylla* var. *ovatifoliolata* (Brophy et al. 1994, Mabberley 1998) in coastal and sub-coastal monsoon vine-thickets on stabilised dunes or lateritic ledges above the beach; and vine-thickets on rock outcrops in open woodland in Western Australia, Northern Territory and Queensland (Brophy et al. 1994);
- current evidence suggesting that all commercial species and varieties of *Citrus* are likely to be susceptible to HLB (Halbert & Munjanath 2004; Beattie & Barkley 2009; Appendix 1);
- suitability of plants as hosts of *D. citri* (Appendix 3) and *T. erytreae* varying with genotype, phenotype, and extent and frequency of leaf flushing; and
- all citrus producing regions of Australia having climates that are favourable for '*Ca*. L. asiaticus' and *D. citri* (Aurambout et al; Narouei-Khandan et al. 2015); the most southern regions have climates that may also be suitable in some years for '*Ca.* L. africanus' and *T. erytreae*.

The most likely pathways of entry of HLB and its vectors are:

See DAFF "Pest Risk Analysis Report for '*Candidatus* Liberibacter species' and their vectors associated with Rutaceae"[8] for additional information.

- illegal introductions of budwood from Sub-Saharan Africa, Brazil, Asia or Florida by growers seeking to gain advantage through new or improved varieties; eg In 2017, there were 16,460 citrus interceptions across Australia's international airports, mail centres and seaports. This equates to around 5.5 per cent of all biosecurity interceptions. In 2018 a passenger tried to smuggle cumquat budwood with Clas through Brisbane Airport in the inner tubing of a tyre[9]
- householders illegally importing budwood or cuttings from trees owned by friends or relatives in countries where HLB occurs[10];
- legal importation of infested or infected material that has been inadequately tested or treated and inspected, as in recent introductions of fres*h B. koenigii* leaves to California from Hawaii[11] (what is most disturbing is that further introductions o*f D. citri* came in to California from Hawaii on non-host herb shipments of malungai (*Moringa oleifera* Lam. [Brassicales: Moringaceae]) in November 2008[12], and sweet basil leaf (*Ocimum basilicum* L. [Lamiales: Lamiaceae]) January 2009); and coriander (*Coriandrum sativum* L. [Apiales: Umbelliferae] from Mexico into USA[13] (2009)).
- illegal importation of fresh leaves of kaffir lime (*Citrus hystrix*)or curry leaf (*Bergera koenigii*)[14i]

[8] http://www.daff.gov.au/__data/assets/pdf_file/0006/1771152/Draft_PRA_Candidatus_Liberibacter_species.pdf

[9] http://www.agriculture.gov.au/about/media-centre/media-releases/illegal-citrus

[10] **A lime plant detected in the luggage of a passenger arriving in Australia from Bangladesh was heavily infested with live Asian citrus psyllids DAWR Media Release 2 Dec 2016. The plant tested positive for '*Ca.* Liberibacter' (probably asiaticus) and for citrus canker (e-mail from Adrian Dinsdale , 12 Jan 2017).**

[11] See Wilkinson (2007) and Filippini (2008)

[12] California Department of Food and Agriculture, Pest Exclusion Advisory 32-2008 – Asian Citrus Psyllid Found in Hawaiian Herb Shipment.

[13] https://www.themonitor.com/article_c802f179-16e7-5cfc-abe8-b92fe24c2847.html

[14]**Fresh curry leaves surrendered by a passenger at Melbourne Airport on 21 March 2013 had eggs and nymphs of D.citri adhering to leaves and shoots. This was not the first detection of ACP on fresh curry leaves at Melbourne Airport demonstrating that these products represent a pathway for ACP to enter Australia. DAFF OSP Bulletin March 2013, p. 11.**
https://www.dallasnews.com/business/business/2017/07/28/pest-responsible-destructive-citrus-disease-found-luggage-dfw-airport

- passive transport of adult psyllids, which are strongly attracted to light (Mangan & Chapa 2013), in commercial and military aircraft (the latter being a possible explanation for the recent detection of *D. citri* in Guam, Samoa[15] and American Samoa[16]);
- air movements (e.g., cyclonic and jet streams) carrying infected psyllids from areas where HLB and *D. citri* occur e.g., the Indonesian archipelago; psyllids could also be blown on winds associated with cyclones from south pacific islands as occurred for the leucaena psyllid (Yen et al 2014)[17]
- movement of people carrying citrus fruits and other plant material across the Torres Strait from Papua New Guinea, principally by sea; and
- unregulated landings of boats carrying citrus from other areas to the north of Australia.

Plant Health Australia (PHA) and the Office of the Chief Plant Protection Officer (OCPPO) rate HLB (Plant Health Australia 2004b, Evans & Dempsey 2000) as having:

- *medium-high entry potential*, with the most likely entry pathways being via illegal importation of budwood and wind assisted movement of infective vectors into northern Australia;
- *high establishment potential*, based on suitable climate, a history of establishment overseas and the fact that symptoms are not easily recognised in the field;
- *high spread potential*, due to suitable climate, a history of spread into new areas, vector transmission with no known natural enemies of the vector in Australia and the possibility of disease transmission by other phloem feeders; and
- *high economic impact*, given that the disease is extremely difficult to control and there is no evidence elsewhere of successful eradication of the disease.

Plant Health Committee has identified 42 national priority plant pests that are exotic to Australia, are under eradication or have limited distribution including HLB[18].

Naumann (2002) rated *D. citri* and *T. erytreae* as having high potential to establish and spread, with high priority rankings as key citrus pests.

Paini et al. (2010) rated *D citri* as 6th in the top 100 Australian risk list by a quantitative modelling approach of risk utilising a Self Organising Map (SOM), which is a type of artificial neural network. By SOM, *D. citri* was given a risk category of medium.

In the PHA Emergency Plant Pest Response Deed's list of categorised Emergency Plant Pests, HLB caused by '*Ca.* Liberibacter asiaticus' is listed as a Category 2 Emergency Plant Pest (EPP)[19] (80% public funding: 20% private funding) and *D. citri* as a category 3 pest (50% public funding: 50% private funding). The American and African strains of HLB are not categorised along with the African citrus psyllid. Yet the HLB pathogen will not spread widely without the vector.

[15] R. Davis (pers. Comm.)

[16] **Peter Maddison, Pestnet, 5 Nov. 2010 stated that flights (including military operations) into Pago Pago have been responsible for entry of pests to and from Hawaii and the Pacific.**

[17] See CRC1031 Final Report 1031: Understanding the significance of natural pathways for pest entry into Australia and New Zealand. Insects from "The North" to Australia. Recognised as a very high risk pathway due to proximity and where land use changes in Indonesia and PNG are increasing propagule pressure, in particular to the expanding agriculture sector in NW Australia (e.g. Ord River Irrigation Area). The Asiatic citrus psyllid and huanglongbing is identified as a major threat along this pathway.

[18] http://www.agriculture.gov.au/pests-diseases-weeds/plant

[19] Category 2 pests are those which, if present in Australia and not eradicated would:

- cause significant public losses either directly through serious loss of amenity, and/or environmental values and/or effects on households, or indirectly through very severe economic impacts on regions and the national economy, through large trade losses with flow on effects through the economy; and
- impose major costs on the affected cropping sectors such that the cropping sectors would benefit significantly from eradication.

- Beattie, G.A.C. and P. Barkley. 2009. Huanglongbing and its vectors: A pest-specific contingency plan for the citrus and nursery and garden industries (Version 2), February 2009. Horticulture Australia Ltd., Sydney. 272 pp.
- Beattie GA, Holford P, Mabberley DJ, Haigh AM, Broadbent P. Australia and huanglongbing. Food & Fertilizer Technology Center; 2008 Sep.
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- NAPPO Discussion Document DD05. Management of Huanglongbing and Its Vector the Asian Citrus Psyllid, *Diaphorina citri.* . http://www.nappo.org/files/9314/4865/6656/DD_05_HLB__Vector_AWM_15-10-2015-e_.pdf
- CDFA. 2015. Notice of treatment for Asian Citrus Psyllid, https://www.cdfa.ca.gov/plant/pdep/treatment/notices/2016/ACPNOTSantaBarabaraAW01082016.pdf
- CDFA. Asian Citrus Psyllid Work Plan. *www.nationalcityca.gov/Modules/ShowDocument.aspx?documentid*.
- Duran-Vila N, Janse JD, Foissac X, Melgarejo P, Bové JM. Addressing the threat of Huanglongbing in the Mediterranean region: a challenge to save the citrus industry. Journal of Plant Pathology. 2014 Dec 1;96(2SUP):4-3.
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- Texas Citrus Greening and Asian Citrus Psyllid Action Plan. A Grower Sponsored Plan to Combat a Serious Disease of Citrus in Collaboration with State, Federal and International Agencies. Texas Citrus Greening Task Force 2009.
- Plan de contingencia de *Candidatus Liberibacter spp.* bacteria asociada a la enfermedad del huanglongbing o greening De Los Cítricos. 2015. Programa Nacional Para La Aplicación De La Normativa Fitosanitaria. http://www.magrama.gob.es/es/agricultura/temas/sanidad-vegetal/plancontingenciahlb_tcm7-401822.pdf
- CDFA Action Plan for Asian Citrus Psyllid and Huanglongbing (Citrus Greening) in California. August 2016. https://www.cdfa.ca.gov/citruscommittee/docs/ACP-ActionPlan-Rev-8-17-16-web.pdf; https://www.cdfa.ca.gov/citruscommittee/docs/ActionPlan.pdf

PRE-INCURSION REQUIREMENTS

Pre-incursion requirements include:

- a whole-of-supply-chain economic impact analysis to assess the costs of a possible incursion[20], and justification of eradication, and in the event that eradication is not feasible, of

[20] For a comparable economic analysis, see Richards TJ. 2009. California Citrus in 2009: Impact Analysis and Policy Simulation. California Citrus Mutual. See also Durborow (2012) and Lopez & Durborow (2014). For the potential economic cost and response to HLB in Florida see Farnsworth et al (2014).

management strategies[21]. (The benefits of the mitigation strategies should outweigh the long-term costs to the Australian citrus industry and the wider community)[22];

- at the HLB Workshop held in Melbourne on 24-25 February, 2009, it was agreed to form a national scientificTask Force to address issues related to pre-incursion preparedness for an incursion by the Asiatic citrus psyllid (ACP) and/or '*Candidatus* Liberibacter spp.' causing HLB and post-incursion action responses when national eradication is feasible. This group needs to be reformed to determine the knowledge gaps eg in occurrence of native host Rutaceae, targeted surveillance etc
- formation of a Citrus Nursery Best Management Practice Development Group to assist the HLB Task Force. The nursery industry (including general nursery/citrus nursery and both wholesale and retail sectors) should develop a voluntary 'best management practices' systems approach to citrus nursery stock propagation, increase, production and sale focused on Asiatic citrus psyllid and HLB;
- a "mock outbreak" of ACP and HLB in a major citrus growing region;
- the methods reported in the SPHDS diagnostic protocol for HLB should be used to determine if an incursion of HLB has occurred, but there is a need to evaluate the 6 published in- field detection methods (see Appendix 4 for rapid initial screening methods for suspect samples);
- The Auscitrus budwood scheme and nurseries must move to protective structures for budwood and plant production;
- prohibition of imports of fresh kaffir lime and curry leaf from any country/state with HLB and Asiatic citrus psyllid including the United States of America (USA)[23];
- updating BICON in light of recent changes to the systematics and nomenclature of the Rutaceae, and for newly determined hosts of HLB e.g. *Choisya ternata*;
- continual revision of post-entry quarantine requirements for *Citrus* and *Citrus* relatives, particularly hosts of HLB[24];
- effective quarantine, including thorough indexing of *Citrus* and related genera in post-entry quarantine—as a precaution species of *Bergera* and *Clausena* that have not been confirmed as hosts to date should be prohibited, as detection of the HLB pathogens in these genera may be problematic, as it has been in *Murraya*;
- continual updating of the HLB diagnostic protocols to reflect the latest most cost-effective and reliable detection methodologies and to utilise proven in-field detection methods;
- legislation in place nationally for mandatory removal of host plants in the absence of positive HLB determinations e.g., abandoned orchards[25];
- One critical line of defense is to monitor trees in residential areas that neighbor commercial groves. The citrus industry must work with growers, government officials and local residents to review and agree to the removal of trees that are not being properly managed and pose a threat to harbor ACP and spread HLB.
- a process implemented and supported by legislation, that ensures those involved in the nursery industry (and especially those growing *Citrus* and *Citrus* relatives, particularly *Murraya* and *Bergera*) are identified and address details are current; this must include producers and sellers at 'flea market' retail outlets;

[21] Beare S, Elliston L, Abdalla A, Davidson A. 2005. Biosecurity Systems: A Cost–Benefit Framework for Assessing Incursion. Management Decisions, ABARE eReport 05.10 Prepared for the Victorian Department of Primary Industries. *adl.brs.gov.au/data/warehouse/pe_abarebrs99001205/pc13273.pdf*

[22] Alam & Rolfe (2006) noted that there are private and social dimensions to control and eradication programs that need to be economically evaluated to justify investment in response strategies.

[23] The EU has banned entry of fresh curry leaves – see http://www.freshfruitportal.com/news/2015/08/14/u-k-turns-up-heat-on-non-eu-countries-with-fresh-curry-leaf-import-ban/?country=australia

[24] All host plants of '*Ca*. Liberibacter spp.' should be treated identically with commercial *Citrus* spp. in relation to post-entry quarantine requirements for imported nursery stock, budwood or cuttings. This is not currently the case in ICON.

[25] Without monitoring, trees that neighbor a commercial grove could easily become a host and bring the pests and HLB into an otherwise healthy area. In California the **A**bandoned **C**itrus **T**ree removal program is managed by California Citrus Mutual and allows growers to report abandoned trees that could threaten their groves. https://citrusmatters.cropscience.bayer.us/commercial-grower/act-program.

- effective avenues for ensuring industry and general public awareness of the dangers posed by illegal introductions of plant material;
- surveillance for incursions, including maintenance of current NAQS and QDPI activities in northern Australia, and establishment of surveillance programs for nurseries, orchards, urban areas and areas, where native or naturalised hosts of HLB and its vectors occur;
- training of IPM scouts and Department of Agriculture inspectors who regularly monitor pests, e.g., fruit fly traps on trees in orchards and residential properties, to recognise HLB vectors and symptoms;
- surveillance methodologies agreed upon for monitoring incursions by HLB and/or its vectors;
- orchard and nursery management, including propagation of trees using high-health status budwood and rootstocks from Auscitrus, and monitoring (self-surveillance) for psyllids and HLB symptoms by growers and nurserymen;
- State and Federal permits for the use of insecticides (e.g., the organophosphates azinphos-methyl, chlorpyrifos and methidathion, the carbamate carbaryl and the neonicotinoid imidacloprid) for control of *D. citri* and *T. erytreae* (see Bethke et al 2012, 2014, 2015 for pesticide screening against ACP; Byrne et al 2018). Note that foliar sprays and soil applications will need to be used;
- permits for importation of the primary parasitoids of *D. citri* and *T. erytreae*;
- effective avenues for ensuring general awareness of symptoms of HLB and recognising adult and immature stages of *D. citri* and *T. erytreae*;
- production and dissemination of resource material on HLB and its vectors should be co-ordinated between the states, OCPPO, PHA and industry and should be targeted at citrus nurserymen, growers, consultants and others and should be informative, without being too technical;
- awareness plan in place to be rolled out when an incursion of ACP and/or HLB occurs;
- engagement of stakeholders (industry, all levels of government, community)[26];
- maintenance of expertise in citrus pathology and entomology, particularly HLB symptomatology, epidemiology, diagnostics and the biology, ecology and control of *D. citri* and *T. erytreae*;
- training of inspectors, horticulturists, CITTgroup co-ordinators, and IPM scouts by: (a) pathologists with field experience with HLB and knowledgeable in disease symptomatology and epidemiology and awareness of diseases/disorders with which HLB can be confused and (b) entomologists with knowledge of the biology, ecology and management of the vectors and
- training of growers and nurserymen in vulnerable areas e.g., Ord River (Western Australia), Darwin (Northern Territory) and northern Queensland; and
- support for the citrus breeding programme at Bundaberg for incorporation of genes from tolerant/resistant Australian native citrus into commercial scions and rootstocks**.**

POST-INCURSION ACTION RESPONSE PLAN

When National Eradication Is Feasible

The following general recommendations should be adopted in the event of an incursion of HLB and/or its vectors when national eradication is deemed feasible:

- establishment of quarantine zones based on climate, topography, size and degree of isolation of locations (e.g., towns, cities and orchards) where citrus and/or alternative hosts (e.g., *Murraya,* desert lime, finger limes) of the disease and its vectors occur, and regions where

[26] Kruger, H, Stenekes, N, Clarke, R and Carr, A 2010, Biosecurity engagement guidelines: practical advice for involving communities, Bureau of Rural Sciences, Canberra. Also PB CRC Project 4004: **Advancing collaborative knowledge systems for plant biosecurity surveillance**

native hosts occur naturally; information on flight activity, particularly flight distance and prevailing winds, may be needed to establish the size of a quarantine area;

- responses based on where host plants are located in relation to the initial point of detection
- delimiting surveys, including trace-back and trace-forward analyses;
- controlling the movement of plants and plant parts (eg fruit) infested by ACP is the first line of defense in preventing spread of ACP and HLB to new areas;
- cleaning green waste from bins and equipment, including sprayers, tractors and those large trimmers used to trim trees. (Procedures are needed for preventing or eliminating the movement of ACP on citrus products and equipment being moved out of a quarantine area.
- Adult ACP have been observed in truck shipments of unprocessed fruit (Halbert et al. 2010) and adults may survive on harvested fruit for 10–13 days (Hall & McCollum 2011). Trapping results from residential areas, packinghouses and juice plants in California indicate transportation corridors are major pathways for ACP dispersal (Gautam et al 2018). So requirements for the movement of bulk citrus are necessary[27]. Gautam et al (2018) developed a bin-fogging treatment to disinfest fruit before it leaves the orchard to minimise vehicular transport of ACP.
- A fruit harvest protocol for organic and regular orchards in the Pest Quarantine Area (PQA) to be developed. Fruit which has undergone packing shed treatments should not pose a risk – see http://calcitrusquality.org/wp-content/uploads/2009/05/preclearence_2011_Walse.pdf;
- the movement of citrus leaves, such as those of *C. hystrix* and *B. koenigii* which are used as spices, also are of regulatory concern because of their potential as a pathway for the spread of ACP and HLB. Adult ACP have been reported to live for up to 12 days on detached leaves (Hall & McCollum 2011);
- movement of fruit with leaves attached must be regulated[28]; for orchards in an ACP quarantine area, consideration must be given to in-zone packers to clean and re-bin the fruit before trucking to the packinghouse, or manually or mechanically removing the debris in the field; a fogging system that utilizes Evergreen^{R} to kill ACP in loaded bins in the field before they move to the packinghouse. The system is envisioned to be an alternative to wet washing[29].
- identify harvesting, transport and control procedures to eliminate live ACP from citrus products and transport containers (see page 64)
- packing houses inside the quarantine area will also have to double-bag and dispose of field trash from citrus inside the eradication zone at a designated landfill;
- for situations where HLB is detected in the presence or absence[30] of one or both vectors, mandatory[31] and immediate destruction of HLB-infected plants by cutting each trunk or stem and applying glyphosate, or an alternative herbicide, to the stump to kill the roots;

[27] **http://citrusinsider.org/2017/03/new-bulk-citrus-compliance-agreements-mailed/.**
http://phpps.cdfa.ca.gov/PE/InteriorExclusion/pdf/acpgrowerinformation.pdf
https://www.cdfa.ca.gov/plant/pe/InteriorExclusion/grower-packer-hauler-information.html
https://citrusinsider.org/quarantines-restrictions/
https://citrusinsider.org/wp-content/uploads/2018/02/PEA-06-2018-Movement-of-Citrus-Fruit-From-an-HLB-Quarantine-Area.pdf

[28] See CDFA PEST EXCLUSION ADVISORY NO. 01-2014
Master Permit QC 1386 – Movement of Mandarin Fruit with Stems and/or Leaves Attached Produced Inside an ACP Quarantine Area for Shipment Into and Within a Noncontiguous ACP Quarantine Area

[29] http://citrusindustry.net/2017/07/24/field-fruit-fogging-psyllid-control/

[30] NB. Presence of HLB in the absence of its vectors will most probably lead to the death of infected host plants and the disease would, therefore, not be viewed as a severe threat to the industry. Nonetheless, tree eradication should occur as soon as possible after confirmation of infection.

[31] Legislation will be required for mandatory removal of host plants in the absence of positive HLB determinations e.g., abandoned orchards.

- for situations where eradication of *D. citri* or *T. erytreae* in the absence of HLB (determined by surveys and PCR-testing of fifth instar and adult psyllids) may be feasible the following actions are recommended:
    - immediate application of recommended insecticides[32] to all host plants;
    - skeletonisation of all *Citrus* trees in orchards, home gardens and elsewhere (excluding nurseries)[33];
    - skeletonisation of, and removal of all leaves from, grafted *Citrus* trees, and destruction of all seedling rootstocks in nurseries; and
    - elimination of all *Murraya*, *Bergera* and *Clausena* shrubs and trees from ACP infested areas;
- mandatory removal of HLB infected citrus trees, or those deemed to be infected, whether in commercial groves or in people's yards, and areas 400-800 meters around infected trees to be treated with insecticides;
- mandatory destruction of abandoned orchards within quarantine zones, without the need to determine the presence of HLB or its vectors in all trees;
- regulations prohibiting transport of hosts of HLB, *D. citri* and *T. erytreae* (e.g., nursery trees, budwood, fruit and seeds) to unaffected areas;
- are will be needed to ensure that insecticide applications for psyllid control do not result in residues that exceed the MRLs of countries importing Australian citrus fruits, and for domestic markets;
- movement of ACP and HLB on nursery stock is of concern, as discount garden centres and retail nurseries play a significant role in the widespread distribution of psyllids and plants carrying HLB pathogens (Manjunath et al. 2008; Halbert et al. 2012);
- provide nurseries with awareness material, spray schedules and with information on insect proof screenhouses;
- Sales of orange jasmine and *Choisya*[34] will be affected by an incursion of *D. citri* alone or in combination with one or more of the HLB pathogens. Depending on the distributions of the vector and disease it may be necessary to:
    - ban trade and movement of *M. paniculata* between and within states; and
    - eradicate existing *M. paniculata* plants (growing naturally, or in nurseries, home gardens, parks and elsewhere) as occurs in São Paolo, Brazil (see Lopes et al. 2008).
- The nursery industry (including general nursery/citrus nursery and both wholesale and retail sectors) should develop a voluntary 'best management practices' systems approach to citrus nursery stock propagation, increase, production and sale focused on Asiatic citrus psyllid and HLB;
- formation of a Citrus Nursery Best Management Practice Development Group to assist the National HLB Task Force. ACP-HLB Task Forces established in each citrus growing region "to coordinate an education and outreach program to alert residents to the threat posed by ACP and to mobilise broad public support for efforts to exclude, detect and eradicate the pest";

[32] Insecticides available for suppressing psyllid populations include the organophosphates azinphos-methyl, chlorpyrifos and methidathion, the carbamate carbaryl, and the neonicotinoid imidacloprid, all of which are known from overseas reports to be effective against the psyllids. Permits for the use of these chemicals need to be in place before an incursion occurs.

[33] Such action should optimise the prospect of eradication, as the psyllid cannot survive in the absence of host canopies. Skeletonising, as an alternative to tree eradication, will minimise costs and allow orchards and nurseries to return to full production sooner. It may be feasible to use defoliants in such circumstances in orchards to achieve rapid removal of foliage on the psyllid depends. However, there are currently no registered defoliants for citrus and doses (concentrations and spray volumes) required in Australia would need to be determined. Outcomes are likely to be variable depending on chemical rate, spray application method, tree age, water relations and environmental conditions at time of application. It may be possible to apply defoliants with insecticides, but the feasibility of such tank mixes would need to be carefully considered.

[34] *Choisya* spp. are not a good feeding or reproductive host for the psyllid. However the psyllid will feed on them and transmit *Ca.* Liberibacter asiaticus to it from citrus with transmission back to citrus. The species of *Choisya* were *C. ternata* Sundance and *C.* × dewitteana 'Aztec Pearl' (Brlansky, pers. comm., 20/7/2012).

- identify, contact and prohibit Internet sources from shipping host plants from/in to quarantine and buffer areas is addressed by development of software to spider, download, index, and track the sale of these plants. Internet surveillance software will allow regulators to identify offending websites, contact the owners and track compliance (Rotstein et al. 2002)[35];
- protocol for movement of fresh, mature leaves of kaffir lime, curry leaf, and bael [*Aegle marmelos* (L.) Corr. Serr.] intended for consumption[36];
- a survey of native citrus in the PQA;
- an inventory of retail, wholesale and farmers markets selling rutaceous plants in the PQA.
- a social media campaign to urge urban dwellers with citrus trees to watch out for signs of ACP[37]. In the social media campaign, participants pledge to inspect citrus trees monthly for signs of the psyllid or the disease and work with officials, including allowing traps to be hung in trees, treating trees when psyllids are detected, taking leaf samples and removing diseased trees.

In California there are FOUR KEY COMPONENTS to tackling an incursion of ACP and HLB[38]:

1. **Institutionalized Process** – a transparent and highly interactive, managed process that goes beyond simply coordinating sample collection/analysis efforts and recommending psyllid control action plans. It incorporates regular review and possibly in-field testing and deployment of early detection technologies, HLB-infected tree removal and broad industry outreach.
2. **"War Room"** – an interdisciplinary panel of researchers, industry players and regulatory representatives that meets after each data collection/analysis cycle to do a situation assessment and create and amend ACP/HLB remediation action plans within the context of the latest research and organizational capabilities.
3. **HLB Task Force** – a small accountable organization tasked with managing the process.
4. **Infrastructure** – includes CDFA, other regulatory and non-regulatory resources and capabilities.

When National Eradication Is Not Feasible

The following recommendations should be adopted to prevent occurrence and spread of HLB in post-incursion circumstances where the disease and one or both of its vectors occur in orchards in minor and major production areas, and where eradication of the disease and its vectors on a national basis is not considered feasible:

- mandatory area-wide management practices within orchards and surrounding areas;

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[35] See also CRC30062: AIMS to develop a rapid and customised (for Australia) internet web crawler which will detect organisations who would intend to market via the internet, regulated organisms and commodities (invasive species) which are prohibited entry to Australia owing to the threat they present to Australian plant health generally, and specifically, those organisms which would threaten Australia's plant-based industries.

[36] For protocol developed by USDA see http://www.aphis.usda.gov/plant_health/plant_pest_info/citrus/downloads/DA-2015-04.pdf. https://www.aphis.usda.gov/plant_health/plant_pest_info/citrus/downloads/DA-2015-04.pdf

Katayama et al. (2001) showed that methyl bromide fumigation at 48g/m^2 for 2 hrs at 15°C with 32% loading (v/v) gave 100% kill of all stages of *D. citri.* However McGuire (2000) found that leaves of the curry leaf tree subjected to fumigation with methyl bromide from 16 to 64 g·m^3 greatly increased susceptibility of leaves to postharvest decay. Cold or gamma irradiation treatments should be tolerated by this commodity. Walse (2011 http://www.calcitrusquality.org/wp-content/uploads/2009/05/preclearence_2011_Walse.pdf) states that MeBr fumigations at 55°F of 70mg h/L will control adult psylla.

Of treatments resulting in 100% D. citri nymph mortality on infested curry leaves, 40°C for 5 min with Pro-San was accompanied with the least proportion curry leaf tissue damage (Anco et al. 2015).

Eggs, nymphs, and adults of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae)—vector of citrus greening disease—were exposed to a series of gamma irradiation doses and examined for post irradiation development to the next stage and mortality. A dose of 150 Gy was found to prevent egg hatching and 185 Gy was sufficient to stop further development of 1st and 2nd instar nymphs. The 3rd and 4th instar nymphs were more tolerant, and an estimated dose of 204 Gy prevented their further development. A dose of 864 Gy was estimated to be required for complete adult mortality within 3 d after irradiation (Khan 2016).

[37] http://californiacitrusthreat.org/pledge

[38] See http://www.scribd.com/doc/294250143/CRB-Citrograph-Mag-Q1-2016-Final-Web

- quarterly, or more frequent, monitoring by trained personnel for symptoms of HLB in known host plants (*Citrus* and *Citrus* relatives: see Appendix 5 of Beattie & Barkley, 2009; Halbert & Munjanath 2004) in orchards, nurseries, home gardens and other circumstances (e.g., parks and native vegetation) where hosts of the disease and the vectors may occur within the production area;
- confirmation of HLB infection in symptomatic leaves based on use of PCR;
- mandatory[39] and immediate destruction of HLB-infected plants by cutting each trunk or stem and applying glyphosate, or an alternative herbicide, to the stump to kill the roots (this must be done without waiting to harvest any mature fruit);
- mandatory and immediate destruction of all trees in a block should the percentage of HLB-infected trees in a block reach or exceed 10% of trees within an interval of 12 months;
- mandatory destruction of abandoned orchards;
- mandatory removal of HLB-infected plants in nurseries, home gardens and other circumstances (e.g., parks and native vegetation);
- mandatory removal of alternative hosts of the vectors, particularly species of *Murraya*, *Bergera* and *Clausena*, within close proximity (2 km) of orchards;
- encouragement of urban dwellers to replace ACP host plants with non-host plants in their gardens or, failing that, to control the psyllids;
- mass release of the parasitoid *Tamarixia radiata* (especially in urban areas) for untreated citrus and hosts such as orange jasmine (Milosavljević et al 2017)
- mandatory registration of nurseries growing *Citrus* and *Citrus* relatives (particularly *Murraya* and *Bergera*);
- compulsory registration of commercial citrus producers[40];
- use of certified[41] pathogen-free buds produced under a certified budwood scheme (no use of uncertified buds or marcotts); See requirements imposed in the Florida Citrus Nursery Stock Certification Program instituted after the advent of ACP and HLB[42];
- mandated geographical isolation of budwood sources (mother trees) and their maintenance in insect-proof screenhouses;
- geographical isolation of nurseries from orchards;
- nursery production, in HLB affected regions, in secure insect-proof screenhouses;
- legislation to prohibit transport of HLB-infected or vector-infested plant parts (e.g., nursery trees, budwood, fruit and seeds) to unaffected areas;

[39] Legislation will be required for mandatory removal of host plants in the absence of positive HLB determinations e.g., abandoned orchards.

[40]The Senate Review of the citrus industry recommended:

Recommendation 2

2.89 The committee recommends that the industry work with DAFF and the LRS towards a compulsory registration system for growers to develop a central database of growers – with data including their location, contact details, area under citrus cultivation, and varieties and volumes of citrus grown – to facilitate:

- **industry planning**
- **industry policy development**
- **communication of research outcomes funded by levy payers; and**
- **communication with growers in biosecurity emergencies**

and that this database be in the custody of a body independent from the current representative bodies (such as DAFF) until such time as issues of equitable national and regional representation are resolved.

[41] A mandatory certification, or an accreditation, scheme for pathogen-free or pathogen-tested citrus budwood should be implemented as a matter of high priority in Australia.

[42] https://www.flrules.org/gateway/ChapterHome.asp?Chapter=5B-62

- management of planting densities, canopy dimensions and canopy densities to optimise yields per hectare[43] and for effective application of sprays;
- planting of windbreaks[44] to minimise movement of adult psyllids within and between orchards (Shen et al. 2013; Martini et al. 2015);
- hedging and pruning practices[45],[46] timed and undertaken to minimise the risk of enhancing vector populations through impacts on the timing and extent of flushing;
- hedging equipment cleaned thoroughly of plant material before moving between orchards
- area wide management of psyllids (Technical Working Group 2009. Area Wide Control of Asian Citrus Psyllid[47]; Rogers et al. 2014, Wright 2015) which should also target riparian habitats where native host plants are present (Setamou et al. 2016)
- orchard and nursery management of *D. citri* and/or *T. erytreae* with strategically applied insecticides and mineral oils;
    - timing of sprays should be based on host-plant phenology so as to minimise feeding and oviposition and to maximise mortality of eggs, nymphs and adults;
    - decisions of what and when to spray include prevalence of huanglongbing, pest pressure, pre-harvest intervals, overall budget, equipment availability, and conservation of beneficial arthropods (Qureshi & Stansly 2014);
    - application of sprays should be even and thorough, with sprays applied to run-off;
    - use of soil drenches and tree injections should be based on tree size and phenology, and account for potential loss or diminution of active ingredient(s) through leaching, degradation or tree growth;
    - At least one and preferably two aerial or ground applications of broadspectrum insecticides during the ‘‘dormant’’ (winter) season when most mature trees are not flushing has been shown to provide significant reduction in ACP and need for insecticides into growing season as well as conserving biological control. Additional sprays during the growing season could be based on scouting and targeted at adults prior to anticipated new growth to ensure that new growth is protected from infestation. (Qureshi & Stansly, 2010, 2014). See also UC Pest Management Guidelines. Citrus. Asian Citrus Psyllid[48];
- strategies to encourage, where feasible, biological control of the vectors by their natural enemies (e.g., planting of groundcover plants to encourage generalist predators);
- growing plants (as intercrops and/or ground-covers within orchards) that produce volatiles that repel the vectors, thereby slowing ingress of HLB into orchards;
- timing irrigation (where feasible) and fertiliser applications to regulate the timing, number and extent of flush cycles; use of supplementary overhead irrigation to reduce psyllid populations;
- education of farm and nursery personnel; pesticide manufacturers, distributors and retailers; consultants, technical advisors, and scouts; research, quarantine, regulatory and advisory staff within government departments;
- restriction of movement of unprocessed fruit from areas with *D. citri* into uninfested areas.

[43] See: Hutton RJ, Broadbent P, Bevington KB. (2000). Viroid dwarfing for high density citrus plantings. *Horticultural Reviews* 24: 277-317; Albrigo LG, Syvertsen JP, Spann TM. 2007. Canopy flush control for management of canker and greening. Citrus Industry (March) pp. 12-14.

[44] See Owen-Turner & Hardy S. 2006. Windbreaks for Citrus. CITTgroups Australia. http://www.dpi.nsw.gov.au/__data/assets/pdf_file/0005/137858/Windbreaks-for-citrus.pdf

[45] Bacon PE. 1981. The effect of hedging time on regrowth and flowering of mature Valencia orange trees. Australian Journal of Agricultural Research 32(1): 61–68.

[46] Bevington KB. 1980. Response of Valencia orange trees in Australia to hedging and topping. Proceedings of the Florida State Horticultural Society 93: 65-66.

[47] https://www.aphis.usda.gov/plant_health/plant_pest_info/citrus_greening/downloads/pdf_files/twg/Psyllid%20Area%20Wide%20Control2.09.09.pdf

[48] http://www.ipm.ucdavis.edu/PMG/r107304411.html

- small citrus growers located in areas with low HLB incidence form regional management areas of at least 500 hectares, within which ACP is controlled in a coordinated manner and diseased trees are removed (Bové, 2012). The technical reasons for implementing and maintaining area-wide control of ACP, especially in a region where HLB is present, are as follows: (1) it delays the beginning of the epidemic (by approximately 299 days); (2) it effectively reduces infection by reducing psyllid populations in neighbouring orchards; (3) it dramatically reduces the incidence (by 90%) and progression (by 75%) of HLB; (4) it reduces local psyllid populations (from 76 to 97%), even in abandoned orchards; (5) it reduces the necessity for frequent use of insecticides for local control of the psyllid; and (6) it reduces management costs for HLB, as insecticide applications are less frequent and more efficient (Bassanezi 2010, NAPPO 2015);
- A HLB working group should be created in each state or region, composed of representatives from relevant agencies, institutions, and organizations to lead the management efforts in the region (e.g. regulatory agencies, local government, the citrus chain and citrus industry (growers, packing and exporter associations, processing associations), certified nursery associations, and citrus research institutions). The working group assists in compliance with regulations related to HLB and especially in the implementation of area-wide management (Rogers et al. 2014, Rogers et al. n.d.), such as the number, size, and location of regional management areas; prioritization of areas prone to HLB endemic outbreaks; periods of total regional pesticide application; action threshold to control ACP; and rotation of pesticides. The technical group can also participate in training of stakeholders, growers, and other technical staff belonging to state, federal, or extension organizations and growers. HLB is not only a technical problem, but also must be considered from the economic, social, environmental, and commercial points of view and, as such, regional management needs the involvement of all stakeholders (NAPPO 2015).

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A fuller list of papers used in preparation of this Pest Specific Contingency Plan can be found in the reference list.

GENERAL BACKGROUND INFORMATION

HLB is the most destructive disease of citrus and is the major limiting factor for citrus production in parts of Asia and Africa (Aubert 1988b, 1990c, Otake 1990, da Graça 1991, da Graça & Korsten 2004, Bové 2006a, Gottwald et al. 2007), Brazil (Belasque et al. 2010) and Florida (Gottwald & Graham 2013). Its impact in other areas of USA is increasing as the pathogen, and *D. citri*, spread through the region.

Although huanglongbing[49] is the official name of the disease (Moreno et al. 1996, van Vuuren 1996, Bové 2006), it is also known by several other common names. These include the widely used citrus greening, likubin (decline) in Taiwan, citrus dieback in India, citrus leaf mottle yellows in the Philippines, citrus vein-phloem degeneration (CVPD) in Indonesia, greening or blotchy-mottle disease in South Africa, and greening disease in Florida and Brazil. The three Chinese characters for huanglongbing literally mean 'yellow dragon disease' (黄龙病). This has led to much confusion, as the intended meaning is 'yellow shoot disease', literally 'huangshaobing' (黄梢病).

HLB is caused by endogenous, phloem-limited α-Proteobacteria in the genus '*Candidatus* Liberibacter' that are transmitted by the citrus psyllids *D. citri* and *T. erytreae*. Three[50] liberibacter 'species' have been described from citrus namely, '*Ca.* L. asiaticus', '*Ca.* L. africanus', and '*Ca.* L. americanus' (Teixeira *et al.* 2005c), *D. citri* and *T. erytreae* are able to transmit both '*Ca.* L. asiaticus' and '*Ca.* L. africanus'; *D. citri* transmits '*Ca.* L. americanus' (Yamamoto et al. 2006), but it is not known whether *T. erytreae* can transmit it.

[49]The three Chinese characters for huanglongbing literally mean 'yellow dragon disease' (黄龙病). This has led to much confusion, as the intended meaning is 'yellow shoot disease', literally 'huangshaobing' (黄梢病). This confusion arose through the use of 'long' (dragon) by farmers in the Chaoshan (including Chaozhou and Shantou) region of Guangdong when referring to flush growth (young shoots) on citrus trees.

[50] There is potentially a 4th citrus liberibacter: see Keremane et al. (2015)

A sub-species transmitted by *T. erytreae*, '*Ca*. L. africanus subsp. capensis', occurs in Cape chestnut (*Calodendrum capense* Thunb. [Rutaceae: Rutoideae]), an ornamental tree in southern Africa (Garnier et al. 2000, Pietersen et al. 2010). Pietersen & Viljoen (2012) and Viljoen et al. (2013 a,b) found liberibacters in all genera of South African native Rutaceae analysed. Each rutaceous genus appears to harbour different specific Laf-like liberibacters. Those found in *Xanthoxylum* appear to be most closely related to 'Laf' from *Citrus*. *Vepris* and *Clausena* harbour liberibacters more closely related to the 'LafC' subspecies found in *Calodendrum.* This very recent suggestion of a further three subspecies of 'Laf' (Roberts et al. 2015) can be resolved within the proposal of haplotypes rather than subspecies within 'Laf' by giving them a biotype designation, recognising the current host plant differences. Laf subspecies vepridis is a biotype of 'LafA', while Laf subspecies zanthoxyli and Laf subspecies clausenae are biotypes of 'LafC' (Nelson et al. 2015). A single *Teclea gerrardii* specimen tested positive for a liberibacter and, through phylogenetic analyses of the three genes sequenced, was shown to be unique, albeit closely related to '*Ca.* L. africanus' and '*Ca.* L. africanus subsp. zanthoxyli' (Roberts & Pietersen 2016). *Ca*. Laf subspecies clausenae has now been found in citrus in Uganda, Kenya and Tanzania (Ronel et al. 2017)[51] and associated with HLB symptoms in this host. This subspecies was additionally detected in individual *Diaphorina citri* and *Trioza erytreae* specimens recovered from collection sites.

The presence of '*Candidatus* Liberibacter asiaticus' associated with the Asiatic form of HLB, has been confirmed in adults of the black psyllid, *Diaphorina communis* collected on citrus in Bhutan (Donovan et al. 2011). Nymphs of the pomelo psyllid *Cacophylla (Psylla) citrisuga* collected from HLB symptomatic lemon trees in Yunnan province, China were 'Las' positive (Cen et al. 2012a, 2012b). These results suggest that *Diaphorina communis* and *Cacophylla (Psylla) citrisuga* are carriers of '*Ca*. L. asiaticus'. Transmission has yet to be demonstrated for *D. communis*, but has been tentatively demonstrated for *C. citrisuga* (Cen et al. 2012b). Other psyllids have been recorded on citrus in Asia but are not known as vectors of citrus liberibacters: e.g. *Cac. citricola, Cac. murrayi, Cac*. (*P.*) *evodiae. Cac. heterogena* has been recorded in Yunnan and Bhutan (between circa 1200 and 2100m ASL) but there is little evidence that it harbours or transmits 'CLas' (Beattie pers. comm.). *T. erytreae* was reported to be the only species of *Trioza* known to feed and develop on Rutaceae (Hollis 1984), but *Trioza citroimpura* Yang & Li develops on citrus (Yang & Li 1984).

Citrus liberibacters[52] are restricted in nature to the family Rutaceae, mostly to the sub-family Aurantioideae, although dodder, tobacco, and periwinkle have been infected experimentally.

In addition to transmission by *D. citri* and *T. erytreae*, the pathogens can also be spread during propagation of host plants through infected grafting material and by marcotting (air-layering). The pathogen is not spread by contamination of people or equipment (except when infected psyllids are spread by these means), nor by movement of the pathogen in the air or in water droplets. Doubts

[51] Abstract 3.a.1. Detection of 'Candidatus Liberibacter species' from citrus in eastern Africa. Int. HLB Conf. 2017. Roberts R, Cook G, Grout TG, Khamis F, Rwomushana I, Nderitu PW, Seguni ZS, Materu CL, Steyn C, Pietersen G, Ekesi S. 2017. Resolution of the identity of 'Candidatus Liberibacter'species from Huanglongbing-affected citrus in East Africa. Plant Disease.(ja).

[52] Other liberibacters are: a liberibacter[52] occurring naturally in tomato and potato (*Solanum tuberosum* L.), and of transmission by the potato/tomato psyllid, *Bactericera* (=*Paratrioza*) *cockerelli* (Šulc) [Psyllidae], in California (Hansen et al. 2008) and New Zealand[52] (Liefting et al. 2008abcd) and in tamarillo and Cape gooseberry in New Zealand (Liefting et al. 2008b). The same pathogen, also transmitted by *B. cockerelli*, was first detected in carrots in Finland (Munyaneza 2010). The pear psyllid pest *Cacopsylla pyri* (L.) hosts '*Ca.* Liberibacter europaeus'which apparently behaves as an endophyte rather than a pathogen (Raddadi et al. 2010). One species of the genus, *Liberibacter crescens*, has recently been cultured and characterized [Fagen et al. 2014] and sequenced (Leonard et al. 2012). The initial culture of *Liberibacter crescens* BT-1 was obtained in 1995 from the peduncle of a defoliating mountain papaya in Puerto Rico. The BT-1T 16S rRNA gene sequence showed that strain BT-1T is most closely related to members of the genus 'Ca. Liberibacter' sharing 94.7% 16S rRNA gene sequence similarity with 'Ca. Liberibacter americanus' and 'Ca. Liberibacter (Fagen et al. 2014).

about seed transmission (Tirtawidjaja et al. 1981), have largely been dispelled (see Appendix 9 on seed transmission) (Graham et al. 2008, Hartung et al. 2008, Sagaram et al. 2008, Shatters 2008, Zhou et al. 2008a, Albrecht & Bowman 2009, Hartung et al. 2010).

IMPACT ON CITRUS PRODUCTION – GENERAL

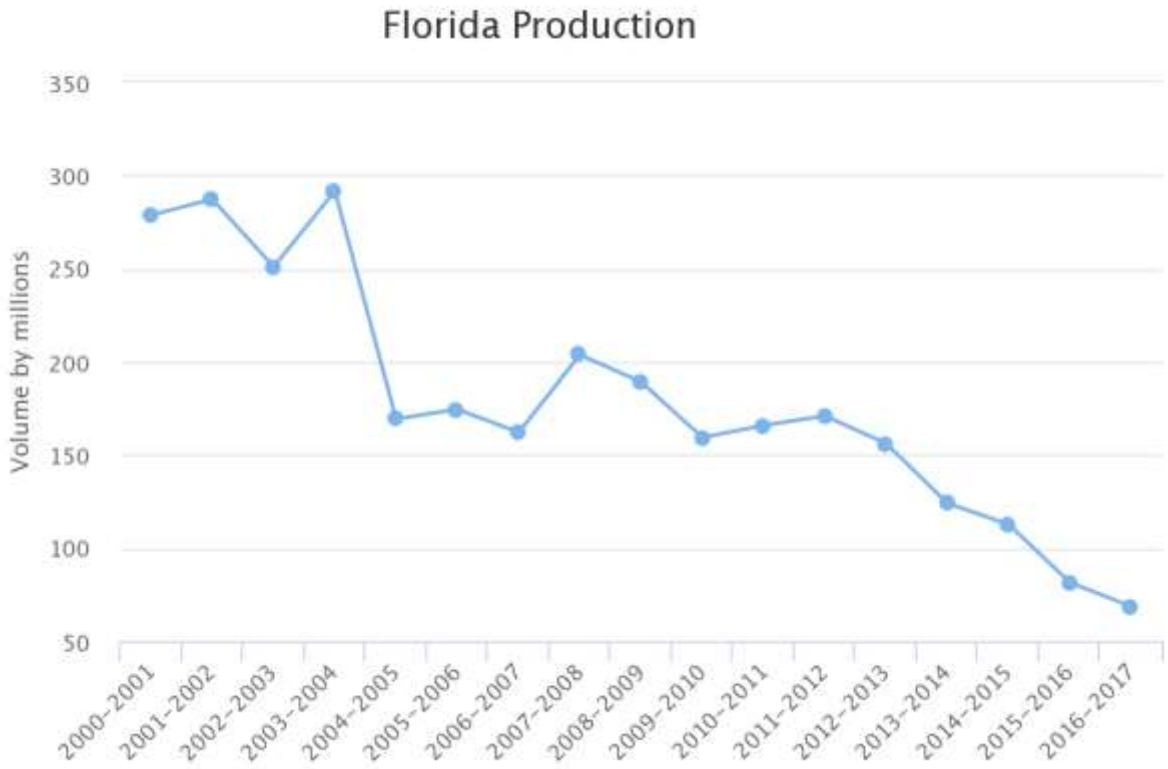
Asian form of HLB and Asiatic Citrus Psyllid

Effect on Orchard Production

Recently Setamou et al (2012) referred to HLB as an "industry killer".

Trees infected at an early age may fail to come into production while more mature trees become unproductive soon after infection. There is no successful commercial treatment of infected trees although penicillin and tetracycline have been used and in-field heat treatment is being attempted in Florida.

Since HLB was first found in 2005, orange acreage and yield in Florida have decreased by 26% and 42%, respectively. The current (2016) percentage of HLB-infected acres and HLB-infected trees in a citrus operation in Florida are 90% and 80%, respectively.



Furthermore, compared to pre-HLB levels, the average percentage of HLB-related yield loss that growers attribute to HLB is 41% (

Singerman and Useche, 2016). HLB has caused a loss of more than $2.9 billion in Florida grower revenues between 2006-07 and 2013-14 (Hodges et al 2014). This resulted in an average annual loss of more than 7,500 jobs and $975 million in industry output[53]. Neupane et al (2106) showed how production loss indicates the ineffectiveness in controlling the impact of HLB. The Florida citrus

[53] http://www.fred.ifas.ufl.edu/economic-impact-analysis/pdf/Economic_Impacts_Florida_Citrus_Industry_2012-13.pdf

industry's major economic contributions to the state's economy have declined by 31 percent in four years through the 2015-16 citrus season. That includes a 9 percent decline in 2015-16 compared to the previous season, perhaps a sign the decline is accelerating. The new study found the $8.6 billion economic impact included $6.2 billion from juice manufacturing, $2.1 billion from farm production and $308 million related to fresh citrus packing and sales. Florida citrus supported 45,422 full and part-time jobs, down 31.6 percent from the previous study. Those jobs generated almost $2.6 billion in labor income, down 31.1 percent over the same period (Court 2017).

USDA has invested more than $380 million to address HLB between fiscal years 2009 and 2015, including $43.6 million through the SCRI CDRE program since 2015. A further $22 million was granted for research into HLB/ACP in 2016[54]. See National Academies of Sciences, Engineering, and Medicine. 2018. *A Review of the Citrus Greening Research and Development Efforts Supported by the Citrus Research and Development Foundation: Fighting a Ravaging Disease*. Washington, DC: The National Academies Press. doi: https://doi.org/10.17226/25026.

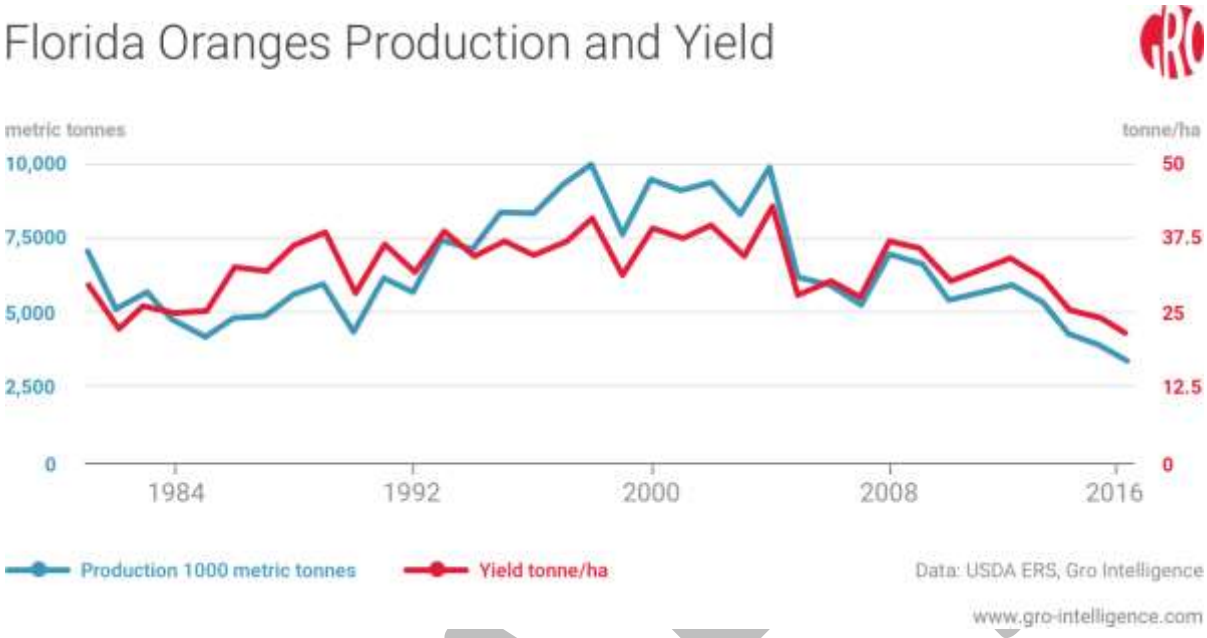
Citrus operations in central Florida experience a 12% higher yield loss to HLB relative to those in southwest Florida. This may be due to the size of the operations. Larger citrus-growing operations can attain economies of scale. These larger operations are typically owned by corporations, which can also be vertically integrated into fruit processing or packing operations. Such integration can allow a higher level of spending in the caretaking of trees in southwest Florida relative to the smaller operations found in central Florida. Another important consideration regarding the size of operations is related to Citrus Health Management Areas (CHMAs). A CHMA is a voluntary, area-wide pest management approach. Each CHMA constitutes a grouping of growers who work cooperatively to coordinate insecticide application to control the spread of ACP across neighboring commercial citrus groves. The idea behind this cooperative effort is that it provides a larger and more lasting effect relative to individual (uncoordinated) treatments because it minimizes movement of psyllids between groves (CREC 2015). However, due to the decrease in profitability, many growers have reduced inputs for caretaking of trees, including insecticide applications. Thus, they either do not participate in CHMAs or do so in a limited fashion. However, by doing so, these growers impose a cost on their neighbors. Larger operations are less dependent upon the willingness of neighboring growers to participate in CHMAs and are better able to control for ACP and manage the impact of HLB (Singerman and Useche, 2016).

the evidence indicating that higher levels of CHMA participation results in greater economic benefits has not led to proportional levels of grower participation in them (Singerman et al., 2017); rather, grower participation levels have actually fallen off over the years since CHMA establishment. In surveys of grower perspectives, the most important reason given for non-participation by CHMA members was the belief that neighboring farmers were not complying with the agreed-upon spray strategy, and the resulting doubt about whether an individual's own expense and effort would be effective in the absence of neighbor participation (Singerman et al., 2017). The secondmost important reason given was that farmers preferred to spray on their own schedule, rather than following an area-wide schedule. Thus, 'strategic uncertainty' has hindered the success of the CHMA approach.

Singerman et al. (2017) made four recommendations for improving the success of CHMAs: (1) regulatory mandates for scheduled spraying, possibly with subsidies to farmers to defray a portion of chemical costs; (2) monitoring of sprays and consequences for non-compliance; (3) a process for in-

[54] https://content.govdelivery.com/accounts/USDAOC/bulletins/144b227

kind transfers among growers and possible tax breaks, and (4) enhancement of communication and education among growers.



Florida orange production could sink by another two-thirds in the next 10 years if better solutions to HLB don't arise (Zansler 2016).

In China, citrus greening disease, in recent years, has gradually spread and [new] cases have been discovered in southern [counties]. Most of the citrus producing regions are in the area of the outbreak, which has caused a serious threat to the citrus industry - leading to a 50 per cent reduction of output and to the destruction of some areas. Planting areas [for] sugar orange, navel orange [and others] have decreased sharply - from about 300 000 acres [121 000 hectares] to 10-30 000 acres [4-12 000 hectares] of production, or even less.[55]

At present, the most serious outbreaks are in the subtropical to tropical areas. Over 10 million citrus trees were pulled out in 2014 due to HLB in Ganzhou, and more in Guangdong (Zhou pers comm 26 June 2014). The presence of HLB in many southern Jiangxi counties, may interrupt ambitious production goals, as groves are destroyed to prevent spread of the disease. (GAIN Citrus Report China 2013). China's top orange grower, Asian Citrus Holdings Ltd, is closing one of its three orange plantations, which has been devastated by HLB.The firm said HLB was first detected on its 37 sq km Xinfeng plantation in Jiangxi province in April 2015, when approximately 18% of orange trees were visibly infected. About 60% of Xinfeng's 1.6 million citrus trees were showing symptoms by November 2015[56].

In Sao Paulo State, Brazil in April 2009, 24% of all citrus blocks were affected and the number of symptomatic trees totaled ≈2 million (*ca.* 0.87%), but as surveys detect only 50% of symptomatic trees as many as 4 million trees are probably affected (Belasque et al. 2010). The number of plants

[55] http://www.freshplaza.com/article/169448/China-Citrus-greening-disease-takes-it-toll
[56] http://www.freshplaza.com/article/138404/Citrus-greening-discovered-in-China

with symptoms of the disease rose from 3.78 per cent in 2011 to almost 7 per cent in 2012[57] and 14% in 2014 (Fundecitrus[58]). The 2018 Fundecitrus data showed that 16.73% of the groves in the state of São Paulo were affected by the disease last year, with around 32 million infected trees.

Brazilian production is declining because, like Florida, it is losing hundreds of small- and medium-sized growers, those holding 500 grove acres or less. While in both countries large growers are able to relocate, the smaller growers cannot afford the additional costs of grove caretaking measures to fight greening, Spreen said. The total number of orange trees in the country declined 18 percent between 2012 and 2015, (http://www.freshplaza.com/article/172445/Brazil-losing-small-medium-sized-citrus-farms-due-to-citrus-greening).

According to the 2017 greening survey conducted by Fundecitrus, 16.73 percent of the trees in the commercial area of the state of São Paulo and the western part of Minas Gerais are affected by greening. This figure is slightly lower relative to the 2016 greening survey (16.92 percent) and shows that the spread of the disease has been stable over the last two years. Fundecitrus forecast volumes from the growing region in the southeastern state of Sao Paolo at a 28% year-on-year drop and 11% lower than the 10-year industry average. The decline is attributed to diseases, -mainly citrus canker and citrus greening (HLB). The number of properties producing oranges over the last few years has shrunk by 20%[59].

Cost of Extra Treatments - Orchards

From an economic viewpoint, the two main consequences of HLB are increased tree mortality, and increased costs of production (Morris et al. 2008). It is estimated in Florida, that post-HLB production costs have increased by approximately 40-50% compared to pre-HLB production costs (Irey et al. 2008; http://www.freshplaza.com/article/163026/Citrus-greening-has-doubled-production-costs.

Examples of direct costs are production losses due to:

- costs associated with scouting and tree removal;
- direct production losses as a result of fewer trees in production;
- fruit drop on infected trees[60], potentially exacerbated by *Lasiodiplodia theobromae* (*Diplodia natalensis*) (Zhao et al. 2015, 2016).
- costs associated with increased insecticide applications;
- costs associated with additional personnel; and
- costs associated with the growing or purchasing of nursery trees that are grown under insect-proof screenhouse and isolation requirements.

Examples of indirect costs are costs associated with:

- the impact of chemicals on non-target organisms;
- distraction of management and personnel from their core duties; and
- potential business risks due to regulatory actions, and pesticide issues.

[57] Jose Belasque Junior, Agra-net Food News [edited] <http://www.agra-net.com/portal2/home.jsp?template=newsarticle&artid=20018003864&pubid=ag005; ProMED-PLANT Digest November 14 2012 Volume 2012 : Number 057

[58] . http://www.freshfruitportal.com/2014/06/26/brazil-fundecitrus-releases-hlb-presence-forecast/?country=australia

[59] https://www.freshfruitportal.com/news/2018/05/14/orange-production-in-brazils-citrus-belt-to-fall-by-nearly-a-third/

[60] Florida citrus industry lost about 20 million boxes of fruit in the 2012-13 season through fruit drop mainly becaue of HLB. This amounts to 20-30% of the crop valued at $150 million. http://www.freshplaza.com/news_detail.asp?id=108315#SlideFrame_1

Those HLB costs often made the difference between profitability and financial loss.

"Ignoring the costs associated with the destruction of grower balance sheets, the cost to manage the spread of ACP in California – assuming 200,000 acres and at least an additional two to five dedicated pesticide sprays per year – could reach $50-120 million annually or more ($250-600 per acre) within the next five years. This equates to $.30-.70 per carton on an 850-carton per acre grove. Then, there is enhanced nutrition. From what we know to date, the best programs are costing more than $500 per acre per year. Again, assuming 200,000 acres, the additional cost to our growers is at least $100 million per annum. Therefore, the total cost to the California citrus industry could be $220 million annually. ***The cost to the state's growers could total $750-1,100 per acre per year, or about $1.30 per carton on 850 cartons per acre production.*** *Even then, such expenditures will not stop the spread of HLB in our orchards. It may slow the destruction of diseased trees, but* ***any fresh fruit infected with HLB that is sold marks the beginning of the end for us in the marketplace.*** *It should be evident that sticking with the current strategy and facing the unintended, but likely, consequences of encouraging growers to hunker down, rely primarily on bug containment, remove only regulatory-confirmed and/or symptomatic trees and wait for the commercialization of a magic bullet will only shackle growers with increasingly higher farming costs and lead to the inexorable collapse of California's productive citrus capacity".* **Citrograph *7, No. 1, pp. 9-10.***

Efforts to control of ACP/HLB will increase costs by $65 million or $248/acre if area-wide control is extended to all citrus-growing regions (Babcock report 2018 http://www.thesungazette.com/article/news/2018/08/22/regulations-cost-citrus-industry-203-million-per-year/.

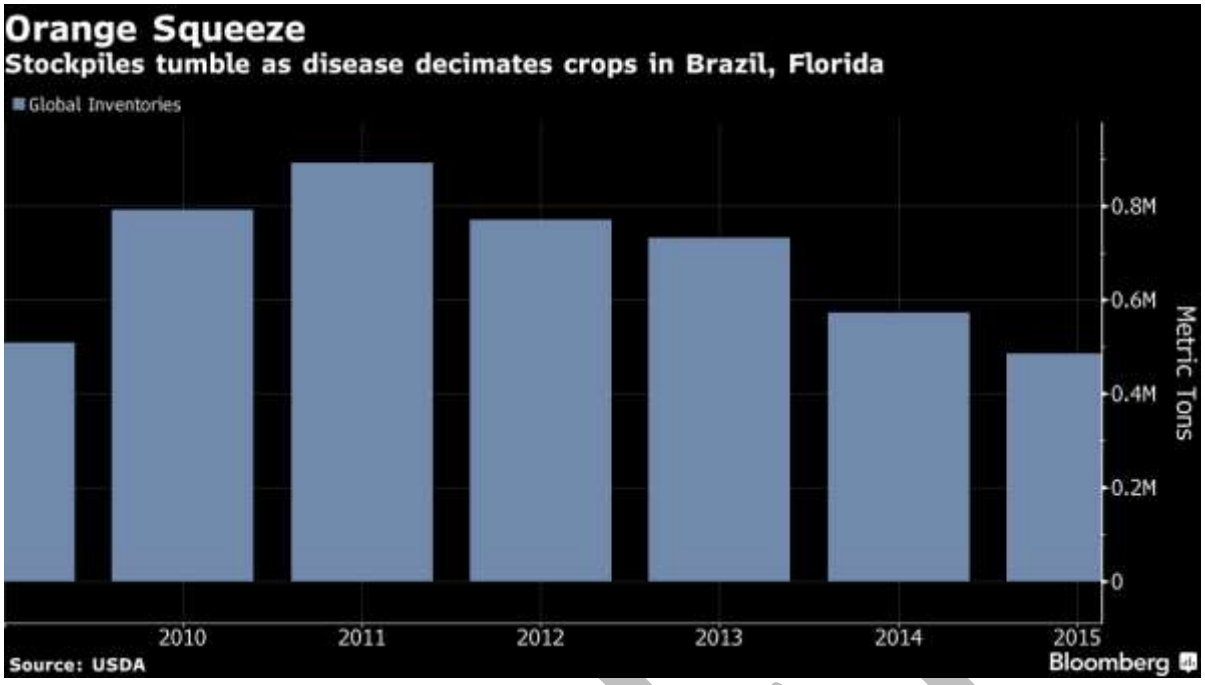
Effect on Juice Production

Fruit produced by infected trees has an unpalatable flavour (Husain & Nath 1927, McClean & Schwarz 1970; Dagulo et al. 2010) and potentially may taint extracted juice products although Baldwin et al. (2010, 2014), Melgar (2014), Raithore et al. (2014) and Plotto et al. (2010) pointed out that in a commercial situation in Florida where juices from different varieties, locations and seasons are blended, the off-flavour of HLB-symptomatic fruit would likely be diluted and also symptomatic fruit generally fall from the tree before harvest. Unfortunately, as the infection spreads, there is less healthy juice with which to blend.

In 2016, although globally we are drinking 4% to 5% less orange juice every year, orange juice production fell by a fifth in Brazil and by nearly a quarter in the world's second biggest producing region – Florida, largely due to HLB (https://www.theguardian.com/business/2016/sep/30/rude-awakening-as-price-of-coffee-and-orange-juice-shoots-up-20?CMP=share_windows_mail).

See also Kress Orange Juice: Will it be Available to Drink in the Future (Agriculturally or Commercially)? (http://nabc.cals.cornell.edu/Publications/Reports/nabc_25/25_3_1_Kress.pdf)

HLB sent fruit production in Florida down for fifth straight season in 2016, the longest slide in a century. Output in Brazil, the world's top producer and exporter, fell to the lowest in 22 years.



Florida acreage has dropped to the lowest in 50 years. In Brazil, farmers on average sold their fruit at 36 percent below market prices because they were locked in to contracts made earlier, said Gilberto Tozatti of Araras, Sao Paulo-based GCONCI-Group Citrus Consulting. https://www.bloomberg.com/news/articles/2017-01-03/thank-tiny-bugs-and-china-s-hungry-pigs-for-agriculture-winners).

In the 2000-01 citrus season, Florida had 106 citrus packinghouses ie before HLB was first confirmed in Florida in the fall of 2005. Each of the top 24 packinghouses sold more than 1 million cartons of fresh citrus that season, and all packinghouses combined shipped 55 million cartons. The 2016-17 season saw only 26 packinghouses operating in Florida, less than a quarter of the total from 16 seasons earlier. Among them, only one, Egan Fruit Packing LLC in Fort Pierce, will ship more than 1 million cartons, and the industry output in 2016-17 will total just more than 12 million cartons, a 78 percent decline during the 16 seasons. http://www.theledger.com/news/20170530/fla-packinghouses-struggle-to-maintain-supplies-amid-greening

Effect on Production Nurseries and Budwood Supply

The greatest challenge that the nursery industry in Florida had to overcome after HLB was detected in 2005 was the loss of budwood facilities that resulted in a critical shortage of budwood (Spann et al. 2008).

Both *D. citri* and '*Ca*. L. asiaticus' have been shown to move with citrus and orange jasmine nursery plants sold in Florida (Manjunath et al. 2008a, Ramadugu et al. 2008; Halbert et al. 2012). As a consequence of the rules and requirements imposed by the Florida Citrus Health Response Plan, the number of citrus nurseries declined from 70 nurseries in 2000 to 35 in 2008 (Spann et al. 2008) to just 6 or 7 in 2011 (Nelson 2011[61]). Abiding by these new rules came with a high price, and monumental risk for the nurseryman and increase the price of nursery trees (Spann et al. 2008). But in Florida as HLB continues to cause tree decline, growers are having to replant their groves in order to keep their operations economically viable. This is causing an increase in the demand for young

[61] http://www.visaliatimesdelta.com/article/20110809/NEWS01/108090302

citrus trees, especially for the new, more HLB-tolerant rootstocks and varieties[62]. These new rootstocks are mostly propagated through cuttings and tissue cultures, as opposed to the more traditional method of propagating from seed[63].

A finding of *D. citri* and/or HLB could result in the prohibition of movement of nursery trees and budwood from any nursery or budwood block in the PQA. If HLB becomes endemic in the region, tree movement to non-affected areas would be prohibited. [64,65]

The following are minimum standards of the Florida Citrus Health Response Plan[66] for citrus nurseries or budwood production in Florida (USDA, APHIS, PPQ. 2006 Citrus Health Response Plan), following the establishment of HLB:

- ***Registration of all citrus nurseries and budwood facilities*** *- includes a Citrus Nursery/Budwood Facility Compliance Agreement detailing the requirements for producing and moving citrus nursery stock, budwood and seed.*
- *Approval of citrus nursery and budwood facility sites at* ***a minimum distance from commercial citrus groves****, other citrus trees or rutaceous plants.*
- *Budwood facilities must be located* ***at least 16 km away from commercial citrus production and away from concentrations of backyard citrus.***
- ***Production of citrus nursery stock and budwood sources in approved structures.*** *(The Florida Plan specifies 'structures constructed at a minimum with poly/polycarbonate covering or screened with a maximum screen size of 266 x 818 µm, designed to exclude psyllids, melon aphid and other aphids, leafminers or other pests. The structure should be sub-divided with additional interior walls and doors to further preclude or minimize internal insect movement should insects be detected in one part of the structure. Any structure must include double entryways with positive pressure air displacement. If cooling pads and fans are used they must be enclosed with insect resistant screen as described above. Adequate construction materials should be kept available on site in the event that the structure is damaged so that timely repairs can be made. Any damages that are detected must be immediately reported to the appropriate regulatory official.').*
- *An appropriate vector control prophylaxis program must be in place to facilitate control of potential exotic vectors in the structures. Also vector spray programs must be implemented in all outdoor nurseries during the transition period.*
- *Security and sanitation measures to prevent pest or disease introductions.*
- *Training and education of employees to recognise exotic diseases. Employer to maintain a log of training.*
- *Inspections to verify pest- and disease-freedom in citrus nurseries and budwood facilities. Florida requires 'citrus plants must be inspected for pests and diseases every 30 days by state regulatory inspectors and certified disease-free prior to transport. Any citrus nursery stock or budwood source tree found infected or exposed to citrus canker, citrus greening, …psyllids, aphids or other common plant pests shall be subject to immediate quarantine action and will not be eligible for certification until treated as prescribed by the department and released from*

62 Despite the addition of two new citrus nurseries to 54 operations in 2014-15 and nine new nurseries over the past five years, the total number of new citrus propagations last year fell to 4.4 million trees, down from 4.7 million in each of the last two years, according to the report. However, propagations have risen significantly from 2009-10, when the state's 45 citrus nurseries produced just 3 million new trees. http://www.theledger.com/article/20150927/NEWS/150929643/1178?Title=New-citrus-tree-production-declined-

63 http://southeastagnet.com/2015/09/08/state-of-citrus-nurseries/

64 See Citrus Health Response Program Citrus Nursery and Budwood Protection Information at http://www.doacs.state.fl.us/pi/chrp/citrus_nursery.html

65 See Technical Working Group Commercial Production and Movement of Citrus Nursery Stock from Florida to Non-citrus Producing States: Findings and Recommendations September 18-20, 2007, Gainesville, FL. http://www.aphis.usda.gov/plant_health/plant_pest_info/citrus/downloads/cns-twg-report.pdf

66 http://www.aphis.usda.gov/plant_health/plant_pest_info/citrus/downloads/chrp.pdf

quarantine. Nursery environs shall be inspected in accordance with the Residential component of the CHRP. The presence of citrus canker or citrus greening in the environs that is determined to present a risk to the citrus nursery or budwood facility will result in an immediate quarantine of the nursery or facility until the risk can be mitigated. The approved structure containing citrus nursery stock and budwood material shall be inspected by trained nursery personnel on a weekly basis for the presence of exotic diseases and for the presence of any insects. Findings must be reported to appropriate regulatory officials. The structure's integrity will also be inspected every 30 days by the regulatory inspector to ensure that there are no holes or other damages that would compromise insect or disease exclusion. The inspector will also conduct a complete audit of the nursery or facility every quarter to verify compliance with production requirements'.

- ***Citrus budwood and seed certification**. 'Propagative material including budwood, air-layers, cuttings and all top-working material shall be from state-registered budwood source trees. Budwood shall be taken under the direct supervision of a witness authorized by the department. Budwood from each source tree shall be wrapped separately. Each bundle shall be labeled showing variety, the tree identification number, and number of buds counted or estimated. Seed from HLB-diseased trees should not be used.'*
- *All citrus budwood source trees shall be tested and indexed on a regular basis to ensure the disease-free status of citrus material.*
- *All citrus plants must remain free from arthropods and diseases of concern to maintain certification. The integrity of the structures must also be intact and the nursery or facility must be in compliance with production requirements in order to maintain certification.*
- *All citrus nursery stock and budwood moved from the facility must be accompanied by a state certificate documenting that the plant material meets state phytosanitary standards or the phytosanitary standards of the receiving state or country. This documentation must identify the citrus nursery or budwood facility site.*

The USDA-APHIS regulations on the Interstate Movement of Regulated Nursery Stock for Citrus Canker, Citrus Greening, and Asian Citrus Psyllid (Federal Register Vol. 78, No. 206, 2013[67]) have the following requirements **for movement from Asiatic Citrus Psyllid (ACP) quarantined areas:**

A. Facility: Citrus nursery stock must be grown in a facility that meets the specified requirements
B. Visual Inspection, Trapping, and Detection.
   1. Plants in the facility must be visually inspected every 30 days for the presence of ACP using approved methods and any detections of ACP or other quarantine pests must be reported immediately.
   2. Inspection methods may include, but not be limited to:
      a. Yellow sticky panels
      b. Vacuum suction of plants
      c. Tapping of plants
   3. If ACP is detected in a facility:
      a. No further shipments will be allowed from the facility.
      b. If compartmentalization of the facility exists, a risk assessment is required to determine regulatory response.
C. Treatment.
   1. All citrus nursery stock must be treated with an approved systemic insecticide (soil drench) at least 30 days but no more than 3 months (90 days) before shipment. This must be followed by an approved foliar spray no more than 10 days before shipment. Treatment must be with an

[67] https://www.aphis.usda.gov/plant_health/plant_pest_info/citrus/downloads/citrus_nurserystock_reg_final.pdf

approved product labeled for use in nurseries. Persons applying treatments must follow the product label, its applicable directions, and all restrictions and precautions.

D. Eligibility for Shipment.

Citrus nursery stock for interstate movement must be subjected to at least 3 inspection cycles (at least 2 monthly inspections and 1shipment inspection). The citrus nursery stock must be maintained inside the facility for its entire life until the nursery stock is sold.

Restricted movement from ACP quarantined areas for citrus nursery stock not grown in screenhouses is also considered.

These requirements should also apply to all ACP hosts and especially *Murraya* spp.

The APHIS rules for interstate movement were updated in March 2018: https://www.aphis.usda.gov/plant_health/plant_pest_info/citrus/downloads/citrus-nursery-stock-protocol-interstate-movement.pdf The above is now obsolete!

Should HLB become established in Australia, the following restrictions (as outlined in the Florida Citrus Health Response Plan) would have to be considered[68,69]:

- Registration of all citrus nurseries and budwood facilities - including a *Citrus Nursery/Budwood Facility Compliance Agreement* detailing the requirements for producing and moving citrus nursery stock, budwood and seed.
- Approval of citrus nursery and budwood facility sites at a minimum distance from commercial citrus groves, other citrus trees or Rutaceous plants.
- Budwood facilities located away from commercial citrus production and away from concentrations of backyard citrus.
- Production of citrus nursery stock and budwood sources in approved structures.
- An appropriate vector control prophylaxis program
- Security and sanitation measures to prevent pest or disease introductions eg clean clothing to prevent hitchhiking psyllids.
- Training and education of employees to recognise HLB and its vectors. Employer to maintain a log of training.
- Inspections to verify pest- and disease-freedom in citrus nurseries and budwood facilities[70].
- Citrus budwood and seed certification.

These minimum standards will impose a considerable impost on nurserymen and ultimately on growers from the increased cost of nursery production. Some nurserymen will go out of business, as the costs of improved infrastructure will be too great. The effect on the Australian citrus budwood scheme operated by Auscitrus, would be equally catastrophic if the outbreak occurred in Sunraysia and would involve significant costs in relocating to an area at a distance from citrus production and providing psyllid-free screenhouses for the production of all budwood and nursery stock.

[68] Minimum standards of the Florida Citrus Health Response Plan (http://www.aphis.usda.gov/plant_health/plant_pest_info/citrus/downloads/chrp.pdf) for citrus nurseries or budwood production in Florida (USDA, APHIS, PPQ.2006 Citrus Health Response Plan)

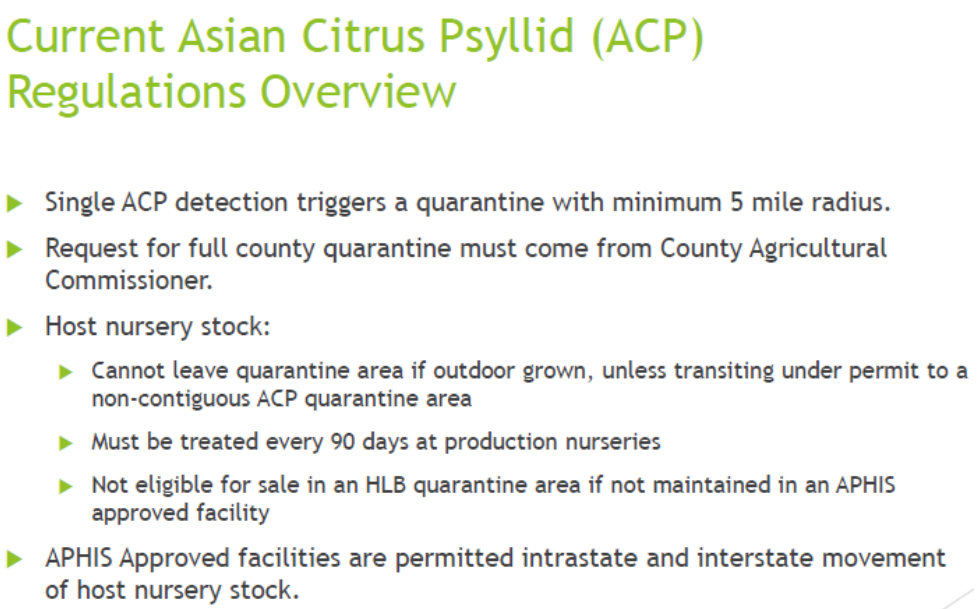
[69] Citrus Greening Control Program in Florida Nurseries. Environmental Assessment, January 2006

[70] See USDA survey protocols: http://nsu.aphis.usda.gov/plant_health/plant_pest_info/citrus_greening/downloads/pdf_files/CitrusSurveyProtocol-ExclusionaryFacilitiesInterstateMovement.pdf

Sales of orange jasmine and *Choisya*[71] will be affected by an incursion of *D. citri* alone or in combination with one or more of the HLB pathogens. Depending on the distributions of the vector and disease it may be necessary to:

- ban trade and movement of *Murraya* between and within states; and
- eradicate existing *Murraya* plants (growing naturally, or in nurseries, home gardens, parks and elsewhere in PQA) as occurs in São Paolo, Brazil (Lopes et al. 2008).

Currently in California (May 2016)[72] the regulations (https://www.cdfa.ca.gov/plant/acp/docs/mtgs/NurseryStockScopingMtg.pdf) are:



- Request for full county quarantine must come from County Agricultural Commissioner.
- Cannot leave quarantine area if outdoor grown, unless transiting under permit to a non-contiguous ACP quaratine area
- Not eligible for sale in an HLB quarantine area if not maintained in an APHIS approved facility
- APHIS Approved facilities are permitted intrastate and interstate movement of host nursery stock.

California:

When HLB was found in July 2017 in Riverside CA, site of the ARS Citrus & Date Repository and the California Citrus Clonal Protection (CCPP) indexing facilities, a 5-mile quarantine was placed around the site of the infected tree (http://agnetwest.com/2017/07/27/hlb-found-california-citrus-birthplace/). Abandoned orchards, some production nurseries and the University of California, Riverside (including the ARS Repository and CCPP) fell inside of that quarantine. Quarantine rules halt all host-plant materials from leaving the designated area.

Glasshouses were totally shut down – they could not even prune potted trees in the greenhouses. Regulators were totally unprepared! Eventually the ARS were allowed to ship seeds and fruit, but not pollen from within greenhouses and insect-proof screenhouses. All pruning material even from

[71] *Choisya* spp. are not a good feeding or reproductive host for the psyllid. However the psyllid will feed on them and transmit *Ca.* Liberibacter asiaticus to it from citrus with transmission back to citrus. The species of *Choisya* were *C. ternata* Sundance and *C. aztec* Pearl (Brlansky Pers. Comm. 20/7/2012; Setamou et al 2016). Both *Choisya* selections are hosts of '*C*.Las' (Hu 2012).

[72] https://www.cdfa.ca.gov/plant/acp/docs/mtgs/NurseryStockScopingMtg.pdf

within greenhouses and insect-proof screenhouses must be teamed/autoclaved. requiring high volume equipment. Currently (July 2017) both CCPP and the repository must follow the commercial nursery regulations stating the County Ag Commissioner must collect a sample from a percentage of stock/accessions in the protected structure. These samples are sent to CDFA for assay. This must be done twice, 6 months apart before distributions/shipments can resume (E-mail from Dr Mary-Lou Polek, Director ARS Citrus Repository to Pat Barkley, 5th August, 2017).

Retail Stores and Weekend Markets

See:

Byrne FJ, Grafton-Cardwell EE, Morse JG. Adam E. Olguin, Daugherty MP, 2018. Assessing the risk of containerized citrus contributing to Asian citrus psyllid (*Diaphorina citri*) spread in California: Residence times and insecticide residues at retail nursery outlets. *Crop Protection* 109, 33-41.

Byrne FJ, Daugherty MP, Grafton-Cardwell EE, Bethke JA, Morse JG. 2016. Evaluation of systemic neonicotinoid insecticides for the management of the Asian citrus psyllid Diaphorina citri on containerized citrus. Pest Management Science. 2016 Sep 1.

In Florida the Asiatic citrus psyllid was distributed throughout the state primarily through retail trade in orange jasmine sold as ornamental plants (Halbert et al. 2008). Psyllids are generally hard to find in large commercial nurseries in Florida, but are very common in retail nurseries (Manjunath et al. 2008a, Ramadugu et al. 2008).

D. citri was first detected in Florida in 1998 and it spread quickly to all the major citrusgrowing regions within the state in less than 3 years (Halbert et al., 2012). The unregulated movement of D. citri-infested nursery stock, both citrus and *Murraya paniculata*, is believed to have been the major contributing factor in the spread of both *D. citri* and HLB throughout Florida (Halbert et al., 2000, 2012) and in the interstate movement of *D. citri* from Florida to Texas (French et al., 2001). In 2009, over 10% of regulatory *D. citri* samples collected in Florida retail outlets tested positive for *Candidatus* Liberibacter asiaticus, and it took an average of 9 months after positive *D. citri* were detected for inspectors to find symptomatic plants that tested positive for the pathogen (Halbert et al., 2012). At many of the retail outlets where positive *D. citri*, no symptomatic plants were ever discovered, indicating that infected plants were already sold to homeowners.

Recognizing the importance of the passive dispersal of the vector on nursery stock, the detection of *D. citr*i in California triggered measures that would limit its spread. CDFA established quarantines that restricted the movement of *D. citri* host plants from areas known to be infested with *D. citri*. Production nurseries within quarantine areas are still required to treat all citrus nursery stock, and other *D. citri* host plants[73], with both an approved foliar insecticide and a systemic neonicotinoid insecticide in order to receive a 90-day certification, during which time plants may be shipped from the production facility to retail outlets. For shipments outside of the quarantine areas, including inter-state movement, nurseries must apply these treatments no more than 90 days, and no less than 30 days, prior to shipment (CDFA, 2017[74]).

In March 2018 in California (Byrne et al 2018), all existing *D. citri* quarantine control requirements apply only to production nurseries. There are no treatment requirements for retail outlets, a

[73] CDFA, 2018. Asian citrus psyllid host list. https://www.cdfa.ca.gov/plant/PDEP/target_pest_disease_profiles/hostlists/AsianCitrusPsyllid-HostList.pdf, Accessed date: 8 January 2018.

[74] CDFA, 2017. Asian citrus psyllid quarantine program. http://phpps.cdfa.ca.gov/PE/InteriorExclusion/pdf/acptreatments.pdf, Accessed date: 8 January 2018.

decision likely guided by the expectation that plants would reside at these nurseries for a short time. As a result, the residency time of citrus trees at retail nurseries may represent a critical window for *D. citri* infestation and spread, particularly if the 90-day certification period is exceeded. Furthermore, there is an increased likelihood that overwatering of trees at retail nurseries may contribute to lower neonicotinoid residues due to leaching of insecticide from pots (Cox et al., 1997; Liu et al., 2006).

Byrne et al (2018) found that that imidacloprid residues in trees grown in containers were affected by citrus species, watering level, soil mix, and time since treatment. Overall, plants had *D. citri*-effective residues for approximately 12 weeks, suggesting that imidacloprid treatments should protect the majority of containerized citrus against *D. citri* for approximately the duration of the 90 day regulatory limit.

To prevent the passive transport of *D. citri* on containerised citrus, unnecessary delays in shipping of trees after they have been treated should be avoided in order to extend the relative protective period of trees once they leave a production facility and await sale at the retail outlets. Trees treated systemically with imidacloprid could be shipped within 2 weeks of treatment, when peak residues have established within trees. To further prolong the efficacy of treatments, overwatering of trees at retail outlets should be avoided to maintain higher titers of imidacloprid, and to keep residues above critical thresholds required for *D. citri* control, particularly in citrus species that are prone to multiple flushing periods within a season. Finally, citrus stock should be protected at retail locations by placing in an enclosed shade house, and if trees have not been sold within 90 days, they should be destroyed, a measure that may require retailers to limit their inventory (Byrne et al 2018).

An unregulated and disengaged market channel in Australia is the 'flea market' retail outlet with multiple sellers at most markets across Australia. Many of these operators grow greenlife on small land parcels (urban/peri-urban) and are bypassed by mainstream horticultural information channels (industry & government) due to the inability of peak bodies and government to locate them and the resistance of these operators to engage with the peak industry bodies. These operators will be a considerable threat to any eradication efforts. Regulation and engagement of the 'flea market' retail outlets is required.

E-commerce

The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture, in conjunction with the Center for Integrated Plant Management (Northern Carolina University) developed an agricultural internet monitoring system (AIMS) for assisting APHIS to locate and regulate online sales of regulated organisms and identify potential commercial pathways for invasive species into the USA.

AIMS is an intranet-based website which integrates webcrawler software with locally developed data management software. It allows APHIS to semi-automate the process of:

- Locating internet sites selling AHPIS-regulated articles using spidering software which scans for specific key words (e.g., plant names) and applies filters to identify only those sites selling these materials. Information on these sites is then downloaded into a database to assist APHIS officers with extension and compliance activities.
- Evaluating sites to assess risk, which includes determining if the site has a valid permit – the permit database is integrated into the AIMS system. Emailing suspect sites requesting the offending articles to be removed and providing information about relevant legislation and prohibited and other invasive species. Archiving and retrieving reports and processing violations. AIMS currently tracks US based internet sites for around 600 regulated species.

In Australia, the Cooperative Research Centre for National Plant Biosecurity (CRCNPB) developed an internet surveillance system for prohibited plants and commodities. The project *'*Software development and support for invasive species internet monitoring in Australia*'* is a collaborative effort between the CRCNPB, Department of Agriculture and Food – West Australia (DAFWA) and the Centre for Integrated Pest Management (CIPM), North Carolina State University. The aim of this project was to develop a rapid and customised internet web crawler which will detect organisations who intend selling invasive species (via the internet) that are prohibited in Australia. This could be extended to include trade in any species regulated nationally or between states.

The Californian HLB Incursion Management Plan proposed implementation of regulations to control sales via E-commerce—with online monitoring of trade partially based on pop-up alerts when someone from California attempts to purchase rutaceous plants from other American states.

HUANGLONGBING

Bové JM. 2006a. Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. Journal of Plant Pathology 88: 7-37.

Gottwald TR, McCollum TG. Huanglongbing solutions and the need for anti-conventional thought. Journal of Citrus Pathology. 2017 Jan 1;4(1).

Craig A P, Cunniffe N J, Parry M, Laranjeira F F and Gilligan C A. 2018. Grower and regulator conflict in management of the citrus disease Huanglongbing in Brazil: a modelling study. J Appl Ecol. doi:10.1111/1365-2664.13122

The best way to proactively protect groves against HLB is to detect early and eradicate any incursion of the Asiatic citrus psyllid. Without the psyllid, there is no disease epidemic.

Growers, nurserymen and the general public are urged to specifically look for the psyllid and signs of HLB. The more eyes collectively monitoring, the better chance of early detection.

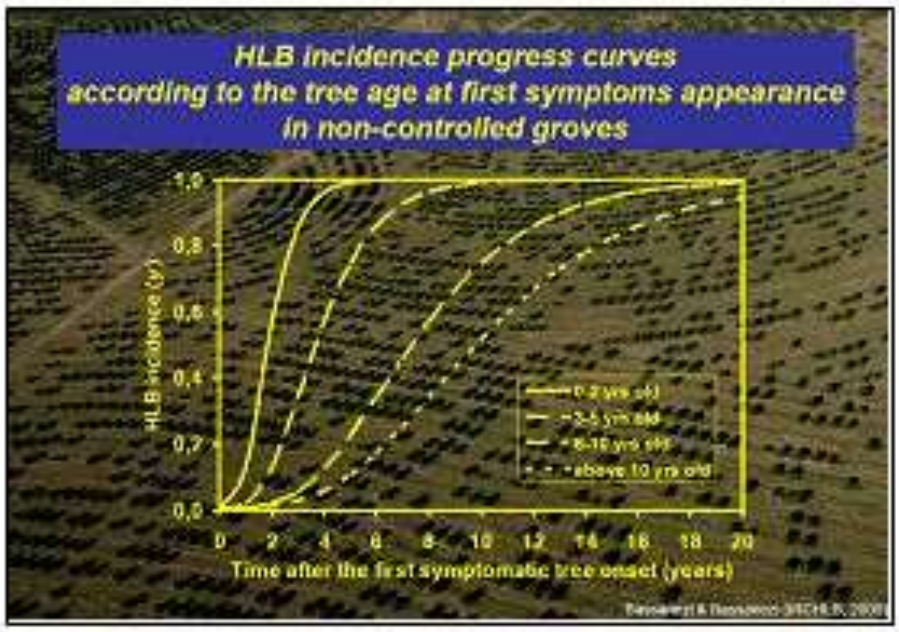
Important points about Asian form of HLB;

Diseased trees are often concentrated on the border of blocks, orchards and where there is an interface of some void of plant material immediately adjacent to areas with dense citrus planting (Bassanezi *et al*., 2005; Gottwald *et al*., 2009a). The edge effect is the result of the behavior of infective psyllids during their migration from source of inoculum trees from outside the blocks of the orchard (Boina *et al*., 2009; Setamou & Bartels, 2015). See Gasparoto et al 2018. Spatio-temporal dynamics of citrus Huanglongbing spread: A case study

- pathogen dispersal reaches long distances (up to 1500m) from the orchard border (Gottwald et al., 2009, Gasparoto et al 2018)
- natural transmission is related to high vector populations and the extensiveness of the inoculum reservoir (Aubert 1987c, Chao et al. 1979).
- CLas transmission rates are increased when citrus flush is present Young plants are therefore more likely to contract HLB if flush is present, with older flush promoting higher infection rates (Hall et al. 2016).
- acquisition and transmission efficiencies are related to the developmental stage of the insect. Nymphs are more efficient than adults (Vichin et al. 2008). Insects that complete their development on a '*Ca.* L. asiaticus' infected shoot are also more likely to acquire the bacteria, than those individuals that feed on the infected shoot as adults only (Ebert et al. 2008).

- Asiatic citrus psyllids that acquired '*Ca.* L. asiaticus' only during the adult stage were poor vectors of the pathogen, unlike adults that acquired the pathogen as nymphs (Inoue et al. 2009; Pelz-Stelinski et al. 2010).
- For adults to efficiently transmit *C*Las, the bacterium must be acquired during the nymphal stage. Two separate studies showed that no adult *D*. *citri* individuals that acquired *C*Las only during the adult stage were able to inoculate citrus seedlings (Inoue et al 2009; Ammar et al 2016))
- *C*Las replicates faster and to higher levels in nymphs than in adults (Ammar et al 2016).
- The titers of*Wolbachia* were observed to be more variable in guts of *C*Las-exposed *D*. *citri* [Kruse et al 2017] and positively correlated with *C*Las titer [Mann et al 2018, Fagen et al 2012].
- In an experiment with seedlings of a rootstock cultivar 'US-942', a 1-wk infestation of 20 Asian citrus psyllids from an infected colony resulted in 53–60% of seedlings becoming infected when flush[75] was present compared with only 7% when no flush was present. A similar experiment with 'Valencia' sweet orange resulted in 23, 80, and 3% seedlings becoming infected when young, older, or no flush was present, respectively. Young plants are therefore more likely to contract HLB if flush is present, with older flush promoting higher infection rates under the conditions of this study (Hall et al 2016).
- the time-lag between transmission of the pathogen by psyllid vectors or by propagation and the onset of visual symptoms for Asian and African HLB can be quite variable depending on the time of the year when infection took place e.g., environmental conditions, tree age, and species/cultivar (McLean & Oberholzer 1965b, Catling & Atkinson 1974, Zhao 1981, Aubert 1987b, Gottwald et al. 1991b). It appears that the same may be true for '*Ca*. L. americanus' (Bergamin-Filho et al. 2008).
- trees infected at the same time may express the onset of symptoms with great variability over one or more years. Time-lag between infection and symptoms may be 3 months to 2-3 years (Lin 1956, Zhao 1981, Xu et al. 1988a, Capoor et al. 1974, Hung et al. 2001, Gottwald et al. 2007).
- rates of spread of the disease in mature citrus trees are reported to be slower than in young trees (Lin 1956, Fraser & Singh 1969, Moll 1977, Bassanezi & Bassanezi 2008; Bassanezi 2010).

[75] Flush is any new leaf growth ranging in development from first emergence up until the leaves are fully expanded yet still tender.



- '*Ca*. Liberibacter asiaticus' is better transmitted by *D. citri* than '*Ca*. L. americanus' and the highest transmission occurred from *Murraya* (Gasparoto 2011).
- typical behaviour of *D. citri* is to jump when disturbed, followed by a short landing flight of 3-5 m, with resulting aggregative populations and clusters of HLB infected trees (Aubert 1990b).
- the establishment of secondary foci is within 24 m to 50 m (Gottwald et al. 1991a). The general pattern of aggregations can be strongly correlated with the usual traffic paths in orchards, especially in high density plantings (Aubert 1990b, Gottwald et al. 1991a).
- the results of Parnell et al (2011, 2012) showed that it is possible to generate accurate spatially explicit estimates of HLB distribution from a sample using a stochastic optimisation method.
- adult ACP are more highly attracted to '*C*las'-infected trees before feeding on them, but they are more attracted to uninfected trees after feeding on infected ones, which may promote the spread of the pathogen (Mann et al., 2012).
- uninfected adult ACP feeding on a diseased tree can acquire the pathogen within 0.5–5 h (Xu et al., 1988), but only 40% of adults feeding on diseased citrus tested positive for the pathogen after 35 days using PCR tests (Pelz-Stelinski et al., 2010). Cen et al. (2012) reported that the severity of HLB symptoms associated with a diseased tree negatively influenced phloem feeding activity by adult ACP, which could contribute to differences in acquisition rates of '*C*las'.
- a latent period of 1–25 days may be required before an adult ACP can transmit the pathogen after acquisition from diseased citrus (Xu et al. 1988), but adults developing from nymphs that acquire the pathogen can transmit as soon as they eclose (Xu et al. 1988).
- adult ACP can remain inoculative (infective) with '*Ca.* L. asiaticus' bacterium throughout their life after acquiring the pathogen as nymphs (Xu et al., 1988; Hung et al. 2004).
- Halbert et al (2015) predicted transmission which bypasses the latent period in the plant making it possible to have positive ACP throughout an orchard before ever seeing a symptomatic tree.
- plant infection levels increased rapidly over time, saturating near 200 days after inoculation – the same time at which all infected trees first showed disease symptoms. Pathogen acquisition by vectors was positively associated with plant infection level and time since inoculation, with

acquisition occurring as early as the first introduction 60 days after inoculation. These results suggest that there is ample potential for psyllids to acquire the pathogen from trees during the asymptomatic phase of infection (Coletta-Filho et al. 2013, 2014).

- Lee et al (2015) presented experimental evidence showing that young flush becomes infectious within 15 d after receiving CLas inoculum. Using a microsimulation model of asymptomatic disease spread and intensity they have shown that entire groves can become infected with up to 12,000 psyllids per tree in less than 1 y, before most of the trees show any symptoms. Transmission occurs at the feeding site among developing nymphs. The infection in the plant is transient, but the insects acquire the pathogens and can transmit immediately when they emerge as adults. Thus, the pathogen co-opts the 15 day life cycle of the bugs and their vast powers of reproduction.
- '*Ca.* L. asiaticus' was detected in bark tissue, leaf midrib, roots, and different floral and fruit parts, but not in the endosperm and embryo (Tatineni et al. 2008). Sagaram et al. (2008) found a wide variation in concentration of '*Ca*. L. asiaticus' among tissues with the highest concentration in fruit peduncles.
- To prevent the spread of HLB, especially from abandoned infected groves, the Brazilian government regulated that if 28% of a plantation unit (grove) is found to be symptomatic then the whole plantation unit must be destroyed. This decision used the best evidence available in 2008 (…..), which suggested that a 28% detectable prevalence corresponded with 100% actual prevalence, the disparity being due to asymptomatic infected trees and imperfect detection methods. Using a mathematical model with parameters estimated from field data, Craig et al (2018) evaluated the assumptions underlying the 28% threshold. They investigated the effects of spraying insecticide and removing diseased plants on the infectious pressure and potential loss of yield from an infected grove and found:

  - the relationship between detectable and actual prevalence is much wider than allowed for in the regulations. There is a high probability that groves with detectable levels of symptomatic plants substantially below 28% have a > 90% prevalence of infected plants. Paradoxically, in a well-managed orchard the threshold of 28% may not be reached at 100% prevalence.
  - Infectious pressure from an infected grove is substantially reduced when growers control disease. Individual growers failing to manage disease therefore threatens the wider grower community. Control is likely to increase yield and prolong grove productivity, but in some groves may reduce yield.
  - 5.Policy implications. Current disease thresholds aimed at restricting the spread of the citrus disease, Huanglongbing, in Brazil allow heavily infected groves to remain in the landscape, but lower thresholds would disadvantage growers who are already controlling disease. There is probably no threshold that is optimal for individual growers and regulators but roguing and spraying is beneficial to both parties. Regulations should focus less on prevalence thresholds, and instead encourage early detection and co-ordinated spraying amongst growers to control Huanglongbing on a regional level.

Important points about African form of HLB:

Since the 1960s, when approximately 38% of citrus trees in South Africa were infected with HLB, and production was virtually eliminated in three major citrus areas, the incidence has dropped to 1% in 2006 (Buitendag & von Broembsen 1993, le Roux et al. 2006a). HLB is controlled by certified greening-free nursery trees, systemic insecticides to control the psyllid vector, *T. erytreae*, and removal of diseased trees to reduce inoculum levels (Buitendag & von Broembsen 1993, le Roux et al. 2006b). There is no mandatory eradication of diseased trees, although the industry would like it.

- '*Ca*. L. africanus' is not as aggressive as '*Ca*. L. asiaticus' and symptoms of African greening, caused by '*Ca*. L. africanus', are less severe than Asiatic HLB, caused by '*Ca*. L. asiaticus', and the two forms can be distinguished on the basis of temperature tolerance (le Roux et al. 2006a).
- Leaf symptoms of African HLB are more pronounced in the cooler elevated areas, than in the low-lying hot areas and are more pronounced in winter (Schwarz 1968a). Higher temperatures for an extended period appear to inactivate the African form (Schwarz & Green 1972, Labuschagne & Kotze 1988).
- While the African form of HLB is graft transmissible, the bacterium in the host plant is irregularly distributed (Schneider 1968a) and it is possible to produce a disease-free plant from an infected parent tree (McClean & Oberholzer 1965b, Schwarz 1968a, McClean 1970, Garnier & Bové 2000a). This presents problems for confirmation of diagnosis by DNA testing.

Host range:

Known hosts of HLB are listed in Appendix 5 of Beattie & Barkley (2009) which are based on Halbert & Manjunath (2004).

See Appendix 1 of this document for the most recent information on Rutaceae and *Citrus* species/varieties with field tolerance.

Distribution of the disease

See also Beattie & Barkley (2009) Appendix IV.
Bove J. 2014. Heat-tolerant Asian HLB meets heat-sensitive African HLB in the Arabian Peninsula! Why? Journal of Citrus Pathology, 1(1) http://escholarship.org/uc/item/1665n4x9

HLB caused by '*C*Las' is known to occur:

- In South and Southeast Asia (from the Indian subcontinent, China to the Philippines, Indonesia, East Timor and Okinawa, Japan), New Guinea (Papua and Papua New Guinea) (Tirtawidjaja et al. 1965, Capoor et al. 1967, Martinez & Wallace 1967, Teaching & Research Group of Phytopathology of Guangdong Agricultural and Forest College 1977, Garnier & Bové 1996, 2000b, Weinert et al. 2004) where the heat-tolerant '*Ca*. L. asiaticus' is transmitted by *D. citri*;
- Guam (NAPIS Data Notification for 03/16/2015[76]);
- In the Arabian Peninsula, in Saudi Arabia where '*Ca*. L. asiaticus' is transmitted by *D. citri*, in Yemen where the heat-sensitive '*Ca.* L. africanus' is transmitted by *T. erytreae*, and north of the Saudi Arabia/Yemen border, where both vectors are associated with both '*Ca*. L. asiaticus' and '*Ca*. L. africanus' (Bové & Garnier 1984);
- In Sistan-Baluchistan and Hormozgan (Alizadeh 2009; Salehi et al 2012) and Kerman province (Orzooiyeh) in Iran (Mokhami et al. 2011).
- In Africa where '*Ca.* L. africanus' is transmitted by *T. erytreae* (McClean & Oberholzer 1965a, McClean 1974, Garnier & Bové 1996, Bové 2006)[77]. Samples from Uganda, Kenya and Tanzania positive in real-time PCR for '*Ca*. L. asiaticus' were shown not to contain '*Ca*. L. asiaticus' by sequencing. Sequences obtained from these samples were analogous to '*Ca*. L. africanus subsp. clausenae', identified from an indigenous Rutaceae species in South Africa, and not to '*Ca*. L. asiaticus'.[78] This is the first report of '*Ca*. L. africanus subsp. clausenae' infecting citrus and being associated with HLB symptoms in this host (Roberts et al 2017).

[76] http://www.pacificnewscenter.com/local/item/4519-new-citrus-greening-disease-threatens-lemon-calamansi-and-other-citrus-trees)
[77] '*Ca*. L. asiaticus' has recently been detected Ethiopia (Saponari et al. 2010).
[78] Results of Roberts et al (2017) indicate a non-target amplification of the real-time assay and suggest that previous reports of '*Ca*. L. asiaticus' from Uganda and Tanzania may be mis-identifications of '*Ca*. L. africanus subsp. clausenae'. This subspecies was additionally detected in individual *Diaphorina citri* and *Trioza erytreae* specimens recovered from collection sites.

- In Mauritius and Réunion where '*Ca*. L. asiaticus' and '*Ca*. L. africanus' are most probably transmitted by both vectors (Massonie et al. 1976, Lallemand et al. 1986, Aubert 1987b, Garnier et al. 1996);
- In the United States of America (Florida, Georgia[79], South Carolina, and Louisiana[80], Texas[81] (Kunta et al. 2012), Puerto Rico[82], US Virgin Is., California (Kumagai et al. 2012, Yan et al. 2015[8384]), Alabama[85]). As of December 2016, the total number of citrus trees in California that have tested positive for HLB is 22: all are in residential neighbourhoods, 20 trees in the San Gabriel region of Los Angeles County, one at the initial 2012 find site in Hacienda Heights and one in Cerritos[86], Los Angeles County. In April the number of finds increased to 53 trees with HLB confirmed in a single citrus tree in the City of La Habra in Orange County and two samples of Asian citrus psyllids in Anaheim testing positive for HLB. In October 2017 an additional 33 cases of Huanglongbing were detected in the greater Los Angeles area. Recently, a third case of HLB was detected in Riverside. The total number of disease detections in California to date is now 199[87].

  Note that the first detected California isolate of '*C*Las' was not related to the known '*Ca*. L. asiaticus' populations in Florida but was more similar to the Asian isolates (Deng et al. 2014). In August 2016, two additional trees were confirmed positive for Huanglongbing (HLB). One tree was located in San Gabriel and the other in Hacienda Heights, in very close proximity to the original HLB find from 2012[88]. By Nov. 2016 a total of 30 trees had been found[89] CDFA crews conducting intensive, risk-based surveys detected eight citrus trees confirmed to be infected with HLB in March 2017. All trees were in the core area of San Gabriel where HLB has previously been detected. This brought the total number of HLB-positive trees in California to 46[90]. In mid May 2017 the number of HLB infected trees rose to 60 with the finding of 1 infected tree in La Habra and 3 infected trees in Anaheim[91] An additional 33 cases of Huanglongbing were detected in the greater Los Angeles areain October 2017. Recently, a third case of HLB was detected in Riverside. The total number of HLB detections in California to date is now 199[92]. '*Candidatus* liberibacter americanus', was found in a citrus psyllid near Mission, Texas in April, 2013 (da Graca & Kunta 2015)[93]. NB. A total of 15 U.S. states or territories are under full or partial quarantine due to the detected presence of the Asian citrus psyllid. Those states include Alabama, American Samoa, Arizona, California,

[79] ProMED-mail post 18 June 2009 Source: Fresh Plaza, The Daily Citizen report [edited]. Also Georgia DA-2009-26 June 19, 2009.
[80] see: Animal and Plant Health Inspection Service Plant, DA-2008-24, 13 June 2008 - http://www.aphis.usda.gov/plant_health/plant_pest_info/citrus_greening/downloads/pdf_files/spro-da-2008-24.pdf
[81] http://www.pestalert.org/oprDetail.cfm?oprID=512
[82] Estevez de Jensen C, Vitoreli A, Roman F. Citrus greening in commercial orchards in Puerto Rico. Phytopathology 2010;100:S34.
[83] http://www.scribd.com/doc/294250143/CRB-Citrograph-Mag-Q1-2016-Final-Web; http://agnetwest.com/2016/03/14/additional-hlb-positive-trees-confirmed-san-gabriel/
[84] The presence of different prophages suggests that the two California CLas strains could have been introduced from different sources. Zheng Z, Wu F, Kumagai L, Polek M, Deng X, Chen J. Two "Candidatus Liberibacter asiaticus" strains recently found in California harbor different prophages. Phytopathology. 2017 Feb 20(ja).
[85] http://www.thepacker.com/news/hlb-confirmed-alabama
[86] info=citrusinsider.org@mail133.atl81.rsgsv.net
[88] http://www.citrusinsider.org/. August 11, 2016.
[89] http://ucanr.edu/blogs/blogcore/postdetail.cfm?postnum=22517
[90] http://citrusinsider.org/2017/03/hlb-detected-again-in-san-gabriel/
[91] Citrus Insider May 2017
[92] [92] The California Department of Food and Agriculture is mounting an aggressive response to all disease detections, working quickly to treat host plants in the area, remove the sources of infection, place nursery stock on hold and communicate with bulk citrus operations. Tree removal is non-negotiable. Citrus Insider Oct 2017
[93] http://www.thepacker.com/fruit-vegetable-news/Brazilian-strain-of-HLB-found-in-Texas-202405841.html

Florida, Georgia, Guam, Hawaii, Louisiana, Mississippi, Northern Mariana Islands, Puerto Rico, South Carolina, Texas, and the U.S. Virgin Islands.

- In Cuba where by '*Ca.* L. asiaticus' is transmitted by *D. citri* (Halbert 2005, Gottwald et al. 2006, Martínez et al. 2008); in Belize[94], Jamaica[95], Honduras *and* Nicaragua[96], the Dominican Republic (Matos et al. 2009), Antigua[97], Colombia[98] and Costa Rica (Pro-Med 21 Feb. 2011; Molina-Bravo et al 2015) where '*Ca*. L. asiaticus' is transmitted by *D. citri*;
- Trinidad and Tobago[ii]
- 14 states in Mexico where '*Ca*. L. asiaticus' is transmitted by *D. citri*[99,100]
- In Brazil *wh*ere '*Ca*. L. asiaticus' and the heat-sensitive '*Ca*. L. americanus'[101] are transmitted by *D. citri* (Teixeira et al. 2005a, b, Lopes 2006, Lopes et al. 2008). The rate of HLB infection in the Brazilian state of Parana almost doubled between 2015 and 2016, Regions where the infection rate amounted to 5% in 2015 have now seen that rise to 10% in 2016, while in other regions the infection rate rose from 9% last year to more than 15%."In the first five months of 2016[102],
- Isolated outbreaks in Argentina[103] (Plata et al. 2013, Outi et al. 2014, Badarraco et al 2017). HLB has been present in the country since 2012 and potentially threatens 136,000 hectares of crops in the Northwestern and Northeastern regions of Argentina, and puts the regional economies at risk[104]. Entre Rios declared a biosecurity emergency in Nov. 2018 due to finds of *D. citri* in commercial fields and in the urban area[105].
- In Paraguay[106]

[94] Caribbean Net News 15 May 2009 <http://www.caribbeannetnews.com/news-16465--27-27--.html> .
[95] http://www.freshplaza.com/article/165678/HLB-threatens-136,000-hectares-in-Argentinahttp://www.promedmail.org/pls/otn/f?p=2400:1001:53103::NO::F2400_P1001_BACK_PAGE,F2400_P1001_PUB_MAIL_ID:1000,80041
[96] ttp://www.promedmail.org/pls/otn/f?p=2400:1001:57555::NO::F2400_P1001_BACK_PAGE,F2400_P1001_PUB_MAIL_ID:1006,82336
[97] http://www.antiguaobserver.com/?p=85501
[98] http://www.freshplaza.com/article/152357/Colombia-Agriculture-severely-threatened-by-HLB
[99] NAPPO Pest Alerts 8/7/2009: http://www.pestalert.org/espanol/oprDetail.cfm?oprID=384.
[100] HLB continues its march northward along the west coast of Mexico with reports (Texas Citrus and Subtropical Fruits 24, No. 8, August 2010).
[101] Lou et al. (2008) reported detection of '*Ca*. L. americanus' in citrus, in a single leaf sample, collected in Hunan, China.
[102] https://www.agra-net.com/agra/foodnews/raw-material/fresh-fruit/oranges/citrus-greening-running-wild-in-parana-518215.htm
[103] http://www.freshplaza.com/article/152122/Argentina-Misiones-quarantined-after-HBL-discovery
[104]
[105] http://www.freshplaza.com/article/9046371/argentina-entre-rios-declares-a-phytosanitary-emergency-due-to-hlb/
[106] http://www.bernama.com.my/bernama/v7/wn/newsworld.php?id=965166

Symptomatology of HLB
Photographs of the key HLB symptoms caused by '*C*Las' in citrus and *Murraya* are given in Appendix 2.

Key HLB symptoms are also given in Lin 1956, Schneider 1968a, McClean & Schwarz 1970, Tirtawidjaja 1980, Zhao 1981, da Graça 1991, Gottwald et al. 2007 and Bové 2006a; also see http://www.imok.ufl.edu/events/field_days/0605/rouse.pdf:

- leaves with asymmetric, sometimes dull, blotchy-mottling that crosses leaf veins;
- mottled or complete yellowing of leaves and growing shoots (yellow shoots standing out from an otherwise normally green canopy);
- small upright, thickened, chlorotic leaves (sometimes resembling mineral deficiencies, particularly Zn);
- starch accumulation in the leaves (see Appendix 6);
- flushing of severely greened trees out of phase with healthy trees;
- dieback of branches;
- reduced fibrous root density[107] (Graham et al. 2013);
- symptomatic HLB-infected trees are much more affected by the extremes of temperature and moisture than trees without HLB with excessive leaf loss and premature fruit drop. This stress intolerance may be due to a loss of fibrous roots (Graham et al. 2013).
- vein-corking associated with ultrastructural changes to phloem (but note that CTV and boron deficiency can also cause vein-corking;
- unseasonal and heavy flowering on diseased branches;
- small, lopsided, bitter tasting fruit with small dark and aborted seeds;
- juice produced from HLB-affected fruit has been characterized as having negative attributes including sour, bitter, salty, metallic, astringent, tingling, with bitter, astringent and burning aftertaste (Plotto et al., 2010; 2017). Lower sugars, higher acids & flavonoids increase off taste in HLB symptomatic juice (Paula et al 2018).
- 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;
- 'silver imprint' when finger pressure is applied to the fruit.
- fruits from severely HLB-symptomatic sweet orange trees are more likely to have problems with preharvest fruit drop, and postharvest pressure damage and breakdown, but may have less puncture damage in harvesting, transportation, packing, and juice processing (Chen et al 2016). HLB

[107] Las preferentially colonizes roots before leaves, where it multiplies and quickly invades leaves when new foliar flush became a sink tissue for phloem flow. This led to the discovery that roots were damaged by root infection prior to development of visible foliar symptoms and was not associated with carbohydrate starvation caused by phloem-plugging as previously hypothesized (Johnson et al 2014).

juice has lower soluble solids but higher acidity, and limonin and nomilin levels than healthy juice. Results of Raithore et al (2015) showed that adding small amounts of HLB juice to healthy juice does not compromise taste quality. Orange juice from HLB symptomatic fruit is sour, bitter, metallic and astringent. Lower sugars, higher acids & flavonoids increase off taste in HLB symptomatic juice. Fractions of juice extracts tasted intensely bitter, astringent, harsh or herbal. Fractions of HLB juice have more bitter compounds than healthy juice fractions (Dalla Paula et al 2018).

- underlying the multitude of symptoms for HLB disease, there are dramatic physiological and anatomical changes, which may influence the susceptibility of the host eg to Diplodia fruit rot (Zhao et al 2014, 2016) and to Phytophthora root rot (Wu et al 2014) and other pathogens;

Field symptomatology

See Appendix 1 on host tolerance in-field.

Field symptomatology depends on:

- tolerance/resistance of the variety;
- whether a tree flushed during a period of psyllid activity (de Lange et al. 1985, Koizumi et al. 1994);
- attractiveness of trees to ACP, CLas establishment at ACP feeding, CLas proliferation following ACP inoculation, systemic movement of CLas with subsequent further proliferation (Stover et al. 2010a);
- resistance[108] [109]to the Asiatic citrus psyllid (Westbrook et al. 2011);
- the presence and severity of CTV strains also present (Tsai et al 2007);
- the form of '*Ca.* Liberibacter' eg '*Ca*. L. africanus' is not as aggressive as '*Ca*. L. asiaticus' and symptoms of African greening, caused by '*Ca*. L. africanus', are less severe than Asiatic HLB, caused by '*Ca*. L. asiaticus'; leaf symptoms of African HLB are more pronounced in the cool areas, than in the low-lying hot areas and are more pronounced in winter (Schwarz 1968a).
- the strain of '*Ca*. L. asiaticus' (Tsai et al. 2008).

While some symptoms vary between different citrus species and varieties (see Appendix 1), it is not significant in a diagnostic sense (Zhao 1981). In general, among the commercial varieties of citrus, HLB causes a wide range of reactions from relatively mild to extremely severe: *'The group of acid citrus (limes and lemons) is much less sensitive than the group of sweet citrus (mandarins, oranges, tangors, tangelos) and Poncirus[110] is tolerant but not immune' (*Aubert 1990b). Mandarins vary in their reaction to CLas infection (Castle pers. comm; Stover et al 2010b; Stover et al 2016). For further information see Appendix 1 on Field Symptomatology.

Because a variety is tolerant does not reduce its significance as a reservoir of infection e.g., lemons are important reservoirs of infection, in part because of their more frequent flushes, which are attractive to the vector.

[108] Ammar et al. (2013) suggested that thickness of the fibrous ring may be a barrier to stylet penetration into the vascular bundle

[109] Two types of resistance have been identified in *Citrus trifoliata*. One of these resistance types (antixenosis) greatly reduces infestation levels of the psyllid, a resistance trait that may be related to differences in volatiles used by the psyllid to find and infest plants or the presence of a volatile that repels the psyllid. The other type of resistance (antibiosis) results in reduced longevity of psyllids, possibly related to the presence of toxic secondary plant metabolites (Hall et al 2013[109], 2015; Richardson & Hall 2013).

[110] *Poncirus trifoliata* (L.) Raf. is, as originally described, *Citrus trifoliata* L.

Symptom development may be delayed for several months, or as long as 2 to 3 years after infection (Lin 1956, Zhao 1981, Capoor et al. 1974, Hung et al. 2001, Gottwald et al. 2007).

Field recognition of HLB, based on visual symptoms requires well trained personnel (Bové 2006b). Carlos et al. (2006) and Dixon (2008) reported a simple aid for the identification of suspect HLB symptoms; use a pen to draw two circles symmetric about the leaf mid rib, and compare the leaf colour within the two circle — if one is yellow, and one green, it could be HLB.

Confusion of HLB symptoms with Other Diseases and Nutrient Disorders

The distinction of HLB symptoms from nutrient deficiency symptoms can be difficult. Halbert & Manjunath (2004) stated that leaf mottling resulting from HLB infection differs from symptoms of nutrient deficiencies, as it usually crosses leaf veins, rather than occurring between or along the veins.

In Australia, similar symptoms to those of HLB are caused by Australian citrus dieback (ACD) (Broadbent et al. 1976, Broadbent 2000). ACD has been attributed to phytoplasma (Broadbent et al. 1976, Davis et al. 1997, Garnier, pers. comm., Constable, pers.comm). Recently various phytoplasmas have been associated with HLB-like symptoms overseas[111]. See Appendix 8.

HLB-like symptoms may also be caused by blight (Broadbent et al. 1996), starch accumulation associated with B deficiency (see Haas & Klotz 1931a, b)[112] or winter yellows[113],[114] (Broadbent & Fraser 1979), severe stem pitting strains of *Citrus* tristeza virus in grapefruit and Phytophthora root rot. Fruit re-greening symptoms of HLB can mimic symptoms of the rind of naturally re-greened Valencia orange fruit in late summer and autumn.

Diseases and disorders that can be confused with HLB are presented in Appendix 7.

HLB Survey Methodology

General references

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[111]Recently, phytoplasmas were reported in Brazil (pigeon pea witch's broom phytoplasma (16Sr IX) (Teixeira et al. 2008, Silva et al. 2014) and Guangdong Province, China ('Ca. phytoplasma asteri' 16Sr I) (Chen et al. 2009), India ('*Ca.* Phytoplasma trifolii' (16SrVI group) associated with blotchy-mottle leaf symptoms. These phytoplasmas can infect citrus plants alone or in combination with 'Ca. L. asiaticus' (Teixeira et al. 2008; Chen et al. 2009). Lou et al (2013) found that a few grapefruit trees with blotchy-mottle leaf symptoms in a HLB-infected orchard in China were positive for a variant (16SrII-A*) of phytoplasma subgroup 16SrII-A. Wulff et al. (2015) found that sunn hemp is a major source of inoculum of the HLB-phytoplasma in Brazil caused by 16Sr group IX phytoplasmas, and transmitted by S. marginelineatus to sweet orange.Transmission from sweet orange to sweet orange occurs only rarely, if at all. In Mexico Arratia-Castro et al reported 'Ca. Phytoplasma asteris' associated with HLB-like symptoms. A new emerging citrus decline (CDD) widely spread in Southern Kerman region of Iran, klling around 10% of cultivated citrus trees is associated with the presence of liberibacters and phytoplasmas (Alizadeh et al 2017).

[112] Boron deficiency can cause vein corking (Haas & Klotz 1931a, b) as portions of cambium of the phloem disintegrate, and the xylem tissue to lesser extent, if at all. A copious amount of gum is formed, which finds its way to the exterior through a split in the cortex (Haas & Klotz 1931b) distinguishing this form of vein corking from the effects of HLB. Boron deficiency also leads to abnormal accumulation of carbohydrates. This, coupled with destruction of phloem, interferes with translocation (Haas & Klotz 1931b).

[113] www.**citrus**australia.com.au/_literature_172463/**Winter_Yellows**

[114] Winter yellows results from starch accumulation in fully expanded late autumn flush (see Broadbent & Fraser 1979).

[115] http://www.plantmanagementnetwork.org/edcenter/seminars/outreach/Citrus/HLB/player.html and http://escholarship.org/uc/item/99c6v21q#page-1

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- USDA/APHIS **Technical Working Group Report National Surveillance Strategies for Asian Citrus Psyllid, (*Diaphorina citri*) and Huanglongbing (associated with *Candidatus* Liberibacter spp.)** https://www.aphis.usda.gov/plant_health/plant_pest_info/citrus_greening/downloads/pdf_files/twg/TWG-NationalSurvey.pdf

General:

Prior to the arrival of HLB in a region large-scale surveillance programs are usually instigated in order to detect the disease as early as possible. Early detection is necessary to minimise the impact of the disease and facilitate any containment or eradication interventions. Large-scale surveillance surveys are however expensive, covering large geographic regions and stretching fiscal and manpower resources. Available resources must thus be deployed in the most optimal way. The choice of which locations within a region to survey is a complex problem since there may be hundreds of thousands of possibilities to choose from. Predicting how the epidemic will spread through a heterogonous

landscape of citrus plantings and how this relates to where sampling resources should be deployed to find the 'needle in the haystack' is challenging and most surveys are consequently sub-optimal. Parnell et al (2014) found that the optimal pattern of sampling resources in a region is often counter-intuitive; for example simply targeting the highest risk locations is rarely the optimal course of action. We show how the optimal pattern depends subtly on epidemiological factors such as the spatial pattern of citrus plantings and vector densities in a region. They showed how geo-referenced information on likely entry points into a region, e.g.trade and travel hubs, can be incorporated to improve the probability of achieving early detection.

Since *C*Las is a pathogen vectored by *D. citri*, primary inoculum is spread rapidly by the vector and rate of spread depends on anthropogenic activity, host spatial connectivity, vector density and wind dominance. Plant flush dynamics and temperature may also affect temporal variation in disease severity and inoculum availability. Flore-Sanchez et al (2017) used a flexible diffusion model to describe HLB disease gradients in Mexico.

In Spain, extensive surveys were conducted for HLB in Canary Islands from 2009 to 2015 and in the northwest mainland Spain (Galicia) since the first detection of *T. erytreae*. During the surveys, ten leaves/tree from trees showing suspicious symptoms and from symptomless trees, as well as adult psyllids, were collected and analysed by real-time PCR using a universal '*Ca*. Liberibacter' spp. kit, according to the EPPO standard[116]. Suspected samples from other surveyed Spanish regions free of the vector were also analysed. The few samples that were positive in the screening test were tested by species-specific real-time PCR protocols, and they did not show amplification.

The CDFA routinely conducts HLB risk-based surveys throughout the state. The system considers numerous factors to determine risk of disease exposure, including proximity to other diseased trees, density of citrus in the area and proximity to transportation corridors, and it helps the CDFA focus resources on areas most at risk.(Citrograph Summer 2017).

In California Risk-Based Surveying/HLB Sample Collection is carried out as follows[117]:

Determination of HLB Survey Sites

Using risk modeling provided by Dr. Tim Gottwald, USDA, Agricultural Research Service (ARS), the following factors are considered when determining risk associated with HLB:

- Residential citrus population and distribution
- Weather effects
- Citrus transportation routes
- Potential to spread the Asian citrus psyllid (ACP) from commercial nurseries, big box stores and citrus green waste
- Areas infested with ACP
- Proximity to commercial citrus groves

Using these risk factors, total risk is determined for each square mile grid, resulting in a recommended sampling density as shown in table below. Each square mile map is identified by the

[116] EPPO, 2014. Diagnostic protocols for regulated pests. PM 7/121 (1). '*Candidatus* Liberibacter africanus', '*Candidatus* Liberibacter americanus' and '*Candidatus* Liberibacter asiaticus'. *Bulletin OEPP/EPPO Bulletin* 44 (3), 376–389.

[117] http://citrusinsider.org/wp-content/uploads/2016/08/CDFA-Handout-Final.pdf
CDFA Action Plan for Asian Citrus Psyllid and Huanglongbing (Citrus Greening) in California. August 2016. https://www.cdfa.ca.gov/citruscommittee/docs/ACP-ActionPlan-Rev-8-17-16-web.pdf

section, township, range (STR) ID (the unique index). Each STR ID is assigned a Sample Density from Table 1, which is used to determine the number of sites to survey per square mile.

Table 1. Recommended Sampling Density and Number of Survey Sites for HLB in California.

Recommended Sampling Density	Actual # of Sites to Survey	# of Square Miles with the Recommended Density	Total # of Sites to Survey
0-5	5	1,926	9,630
6-20	10	1,392	13,920
21-40	25	1,168	29,200
41-80	50	1,111	55,550
81-160	100	324	32,400
161+	200	105	21,000
Total Number of Sites			**161,700**

Key points:

- Scouting only detects 50-60% of symptomatic trees and there is one asymptomatic tree for every symptomatic tree based on PCR testing (Irey 2006a,b).
- The latency period between infection and symptom development ranges from 6 months to 2 years (Xu et al. 1988c, Wang et al. 1996, Gottwald et al. 2007) depending on tree size and age. During the latency period, psyllids can acquire the pathogen from asymptomatic trees.
- Lee et al. (2015) presented experimental evidence showing that young flush becomes infectious within 15 d after receiving psyllid transmitted '*C*Las' inoculum.
- Surveillance may also be hampered by the time-lag between infection and symptom expression.
- The period between transmission of the pathogen by psyllid vectors or plant propagation and the appearance of visual symptoms varies depends on the time of year of initial infection, environmental conditions, tree age, species/cultivar, plant health, etc. (Aubert, 1987; Catling, 1970; Gottwald et al. 1989; Gottwald et al. 2007).
- Especially during assessments of early incidence, there is a higher incidence of HLB at the edges of the orchard and associated with roads, canals, ponds. This 'edge effect' is related to psyllid movement and migration (Gottwald & Irey 2008; Luo et al. 2012, Stansly et al. 2014, Setamou & Bartels 2015).
- Scouting efforts should be initially directed towards young trees and concentrating on edges of orchards. Higher incidences of HLB infected trees were observed associated with grove and block 'edges' i.e., where there was a break in the trees that created an interface between continuous trees and an open space, there tended to be an increase in infected trees. Examples of edges include grove and block boundaries, roads, irrigation ditches, ponds, interfaces with natural areas, and interfaces between young and mature trees (Irey et al. 2008).

- Typical behaviour of *D. citri* is to jump when disturbed, followed by a short landing flight of 3-5 m, with resulting aggregative populations and clusters of HLB infected trees (Aubert 1990b).
- The range (=zone of influence) of HLB-infected trees is greater within rows (20 m) than across rows (13 m), suggesting that *D. citri* transmits HLB more readily within than across rows (Shen et al. 2013).
- 90% of asymptomatic, but RT-PCR positive, trees were within 38 m of visually symptomatic trees (Irey et al. 2006). This supports the findings of spatial autocorrelation that the establishment of secondary foci is within 24 m to 50 m (Gottwald et al. 1991a).
- The general pattern of aggregations can be strongly correlated with the usual traffic paths in orchards, especially in high density plantings (Aubert 1990b, Gottwald et al. 1991a) reflecting vector spread.
- There is mild aggregation of HLB symptomatic trees in Brazilian orchards. Secondary foci were spread all over the block indicating that there is long (4-22 tree spaces distant) and short (to nearby trees) dispersal (Bassanezi et al. 2007).
- A nymph sample with '*Ca*. L. asiaticus' would in general indicate that the source plant is infected[118], while an adult with '*Ca*. L. asiaticus' may indicate only the presence of infected trees in the vicinity (Manjunath et al. 2008a).

Data collection

The likely distribution of all known hosts (listed in Beattie & Barkley 2009; Halbert & Manjunath 2004) within a 5 km radius of the point of detections should be determined and mapped. Citrus Australia national planting statistics database for citrus, herbarium records for native *Citrus* species and *Citrus* relatives, town maps for the location of households and parks, and Google Earth should be used for this purpose.

During surveys, data, especially at the site of detection or occurrence, must be recorded according to guidelines set out in ISPM 9 (1998) Guidelines for Pest Eradication Programmes. Data collected during a preliminary investigation should be used to estimate the potential for spread and rate of spread, and to identify endangered areas. Information gathered and recorded on the Survey Form should include:

- geographical location using GPS (see QDPI&F Work Instruction ST-W-001[119]);.
- hosts infested at the site including age, variety/clone, rootstock, phenology;
- extent and impact of damage, and level of pest prevalence;
- how the pest was detected and identified;
- recent imports of plants or plant products including nursery stock movements;
- history of the pest on the property or in the area;
- movement of people, products, equipment and conveyances;
- mechanism of spread within the area including likely source of inoculum (infected trees, infected budwood, spread by storm etc.);
- climatic events and soil conditions including storms and prevailing wind directions;
- condition of infested plants including age of plant parts affected (spring flush/autumn flush etc); and
- orchard management including method of irrigation, cover crops and spray programs.

[118] NB transovarial transmission does occur for *D. citr*i at a rate of 2- 6% (Pelz-Stelinski et al. 2010; Mann et al 2011).

[119] Telford G. 2007. Global Position System (GPS) Unit Use and Calibration. QDPI&F Work Instruction ST-W-001.

Recommended Post-Incursion Pre-endemic Delimiting Survey Methodology for HLB

The following methods (or a combination) have been suggested for HLB surveys:

- Spoke survey method e.g. On confirming HLB in Texas in 2012, the first step in determining how to address this disease was for APHIS to do a delimiting survey, starting with a five mile radius around the find and then moving out an additional five miles to a ten mile radius.
- Census-based survey[120];
- Risk based[121] residential survey : see http://www.plantmanagementnetwork.org/edcenter/seminars/outreach/Citrus/HLB/ . The find of HLB and infected psyllids at Cerritos, Los Angeles County in Dec. 2016 was attributed to risk based residential survey[122]. To prevent further spread of the disease, it is necessary to design a high intensity residential survey to quickly identify additional HLB-infected trees in the area. Gottwald & Luo (2017)[123] proposed a method whereby the area surrounding HLB findings is partitioned into different sampling locations each equipped with survey strategies designed on an overall risk. This dynamic risk-based sampling plan maximizes HLB/ACP detection and adjusts to new HLB findings.
- yellow traps in cities
- graduated survey protocol[124]. The following was recommended for the CDFA Hacienda Heights survey and treatments. The area was divided into three "Zones," with specific areas and activities as follows:
  - **Zone 1** - Collect plant tissue from every host plant (100%) within a minimum of 400 m every other month (6X/year) and collect both adult psyllids and nymphs if present. Tissue should be collected from individual trees/single samples (do not pool). CDFA protocol: Adult psyllids are collected by site, nymphs are collected by tree.
  - **Zone 2** - 400-800 m survey: Survey and collect a tissue sample from 100% of the host plants by combining (pooling) 4 host plants in one PCR sample. Survey every 4 months (3X/year). If present, collect psyllids (both adults and nymphs).
  - **Zone 3** - >800 m/1 – 1.2 km: Survey 50% of the host plants twice/year. Collect plant samples from by pooling 4 host plants per sample, at a frequency of twice/year. This zone is based more on logistics/practicality. If present collect psyllids (both adults and nymphs).

  Treatments are conducted on all host plants within 800 meters of the HLB find site.

Surveys of trees are vastly better in an orchard environment with uniform cultivars and management than in a residential area, with various cultivars and varying degrees of care.

Trained surveillance teams should traverse orchards, nurseries, parks and gardens on foot looking for the following HLB symptoms:

- yellow shoots;
- mottled leaves;
- small sometimes misshapen fruits with aborted seeds and darkening of the columella; and
- evidence of citrus psyllids or their activity.

[120] For California, Gottwald recommended starting with a base of 25 hosts/sq mi but when you get into an enclave of SE Asian >20%, then you increase say 5-fold (~125 hosts/sq mi).

[121] Parnell S, Gottwald TR, Riley T, Van den Bosch F. A generic risk-based surveying method for invading plant pathogens. Ecological Applications. 2014 Jun 1;24(4):779-90.

[122] info=citrusinsider.org@mail133.atl81.rsgsv.net

[123] Abstract **8.a.5** **Risk based HLB survey for Hacienda Heights and San Gabriel in Southern CA. 2017 Int. HLB Conf.**

[124] https://www.cdfa.ca.gov/citruscommittee/docs/minutes/2014/ScienceSubco-minutes-072314.pdf

If trees are more than 2 m tall, a tractor-mounted platform (e.g., Fig. 50) should be used to supplement ground surveys (Belasque 2006, Yates et al. 2008).

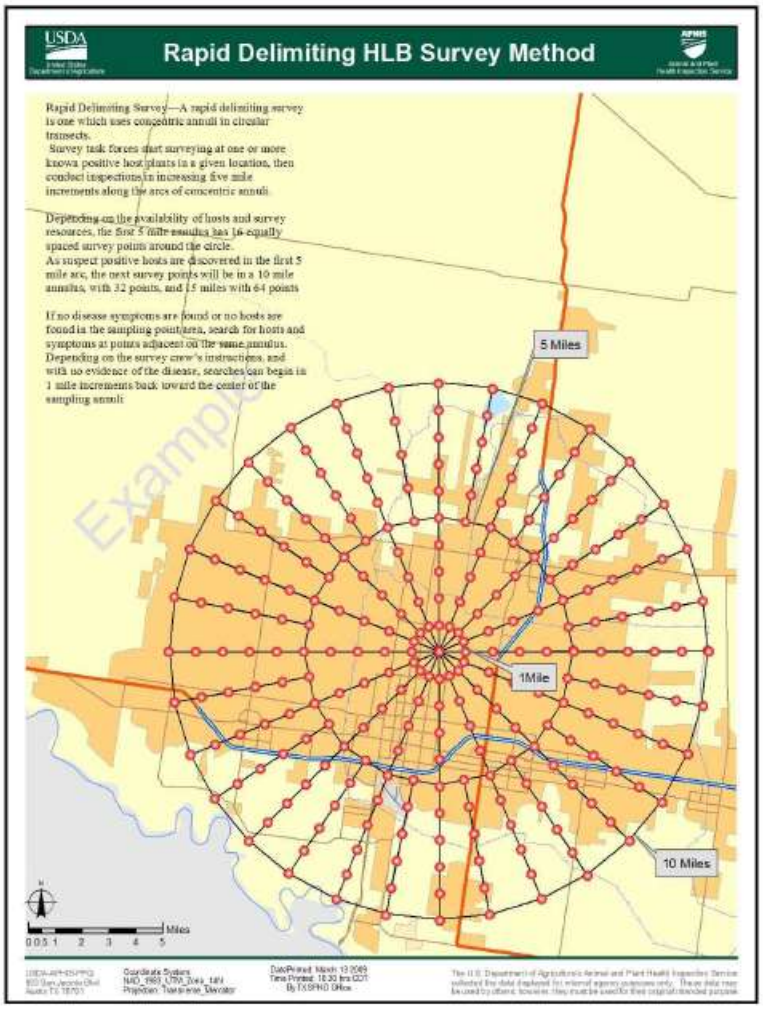
Survey platform as used in Brazil (left photo: Pat Barkley; right: Ayres[125]).

[125] http://citrusrdf.org/wp-content/uploads/2012/09/Juliano-Presentation-to-BOD-2-3-13.pdf

Survey platform as used in Brazil (Ayres[126]).

[126] http://citrusrdf.org/wp-content/uploads/2012/09/Juliano-Presentation-to-BOD-2-3-13.pdf

APHIS Procedures for Delimiting Survey



Rapid Delimiting Survey—A rapid delimiting survey is one which uses concentric annuli in circular transects. Survey task forces start surveying at one or more known positive host plants in a given location, then conduct inspections in increasing five mile increments along the arcs of concentric annuli.

Depending on the availability of hosts and survey resources, the first 5 mile annulus has 16 equally spaced survey points around the circle. As suspect positive hosts are discovered in the first 5 mile arc, the next survey points will be in a 10 mile annulus, with 32 points, and 15 miles with 64 points (**Figure 3-1 on page 3-8**).

If **no** suspect positive hosts are discovered at a five mile increment, survey crews begin to work back toward the center point to define the delimitation of the infestation. At each sampling point, surveyors will search for the nearest host tree in the immediate area for susceptible hosts (**Figure 3-1 on page 3-8**).

Use the following order of plant preference when sampling:

1. Orange, mandarin, tangelo, and tangerine
2. Pummelo, grapefruit, and sour orange
3. Lemon and lime

Examine trees for the presence of yellow shoots, foliar mottling, zinc pattern deficiency, and yellow veins. **See Symptoms On Citrus on page C-1** to view images of symptoms.

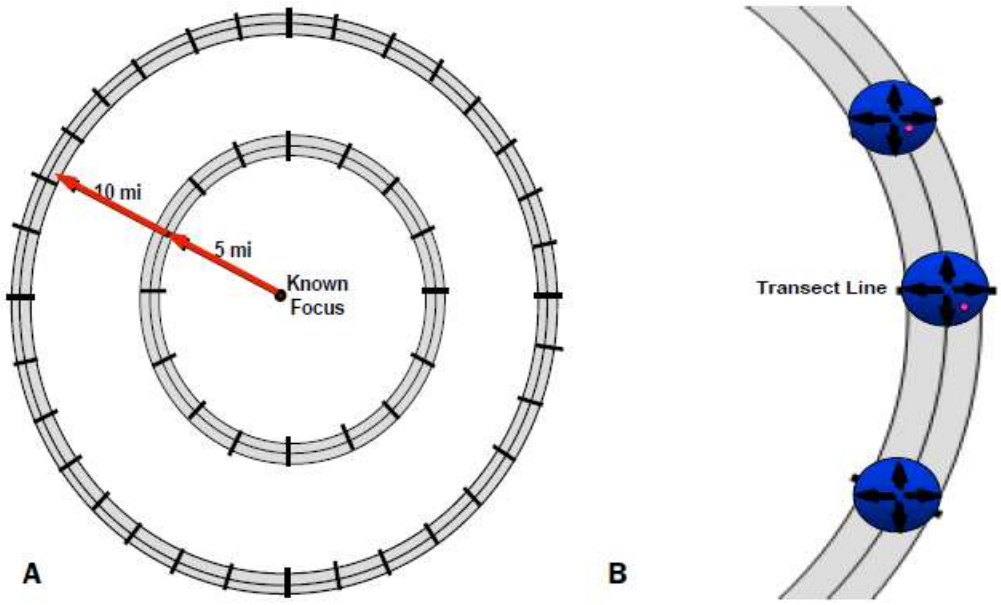


FIGURE 3-1 (A) Sampling points along concentric annuli transects at 5-mile increments away from a known positive host tree (B) Sampling points along an arc transect showing where searching begins to find the nearest host tree for survey

Survey for Satellite Infestations

After one or more infestations are delimited, regulatory and control measures may require the removal of exposed hosts around the known infested areas. Further surveys will be necessary to discover satellite infestations or other areas of potential infection. Design a sentinel or other stratified survey to accomplish this (Figure 3-3). See Sentinel Survey on page 3-5 for more information.

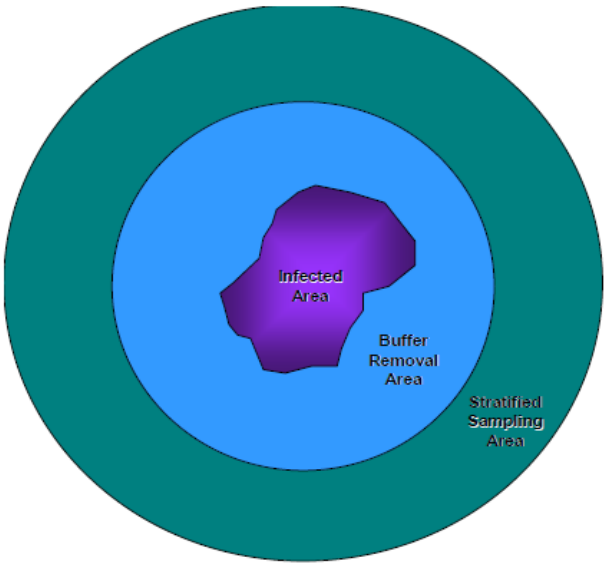


FIGURE 3-3 Stratified survey to detect satellite infestations after delimiting survey

Unmanned Aerial Vehicles

There are three main advantages in using a UAS for disease and stress detection. The first advantage is cost. Collecting images and data are less costly by UAS than by satellite or manned airplane. The second advantage is timeliness. UAS have the ability to fly and capture images on short notice or during small windows of opportunity. The third advantage is the ability to collect high-resolution aerial images by flying at a lower altitude, which results in much clearer data and images.
For some diseases, it is necessary to see an individual leaf on a tree. For example, in huanglongbing only one branch may show evidence of the disease, while the rest of the tree canopy visually appears healthy (Ehsani 2015).

In spite of the potential advantages of UAS in agriculture, there are several challenges that must be addressed before these machines truly can be used for disease and pest scouting. The first challenge is the lack of suitable, lightweight and cos teffective sensors. The common multi-band cameras that are commercially available have limitations, either in optical or spectral resolution. Most of them are limited to three to six bands that are good for detecting general plant stress, but cannot distinguish specific symptoms that would indicate infection by a particular pathogen. Algorithms need to be developed to identify unique signatures for each and every insect pest and disease. Another challenge is that certain sensors work better at night, and current regulations do not allow flying these types of equipment during that timeframe. Furthermore, to detect certain diseases, UAS must fly very close to the top or side of the tree canopy while avoiding hitting obstacles, such as wind machines. The ability to fly between the tree rows or very close to the canopy requires certain navigation capabilities that currently do not exist (Ehsani 2015).

Scaling up sensor packages to a UAV platform presents novel considerations and challenges eg the problem of fluctuations in distance between the sensor and leaves, lower intensity counts overall at the relatively larger distances to trees used during UAV operation, the degree of spectral selectivity that is optimal for discrimination between healthy and infected specimens (Sarkar et al 2016).

Diagnosis

As the National Academies of Sciences, Engineering, and Medicine (2018) wrote in *A Review of the Citrus Greening Research and Development Efforts:*
Molecular and serological diagnostic technologies for CLas are ultrasensitive but, on their own, are not ideal for epidemiological and regulatory purposes because of uneven pathogen distribution
in the tree.
No single diagnostic method will be sufficient to identify recently infected trees.
Detection of infection prior to symptom development is possible through detection of changes in host metabolites and volatiles.

"A quantitative PCR-(qPCR)-based assay (Li et al., 2006) for amplifying CLas 16S RNA has become the standard assay accepted by many laboratories and, more importantly, by regulatory agencies to provide an initial determination of CLas infection. This is followed by conventional PCR assays and DNA sequencing for final verification. Although many reports have been published in the past decade on other methods to detect CLas in plant and insect tissues (Valdes et al., 2016; Ghosh et al., 2017), none of the mechanistically similar technologies (e.g., digital PCR, immunoblots, LAMP, CANARY) have proven to be more sensitive than qPCR. Furthermore, since qPCR can detect as little as one copy of bacterial DNA the issue for CLas detection is not the sensitivity of bacterial detection but rather the uneven spatial and temporal distribution of the pathogen in trees and insects (Tatineni et al., 2008; Li et al., 2009; Kunta et al., 2014; Louzada et al., 2016)". "The qPCR test is unsatisfactory since trees are a source of inoculum long before the regulatory-approved diagnostics can provide information on the infection status".

The primary continuing need for CLas diagnostics are methods that detect infection shortly after inoculation, well before any symptoms of disease can be observed. One promising detection method is based on the chemical analysis of volatile organic compounds (VOCs) that are released by HLB-infected trees. Biomarkers specific to CLas have been found and could be analyzed using gas chromatography/mass spectrometry and gas chromatography/differential mobility spectrometry (Aksenov et al., 2014). Greenhouse tests showed that a mobile differential mobility spectrometry system was able to distinguish volatile differences between closely-related citrus cultivars and show volatile-profile differences between healthy and infected citrus (McCartney et al., 2016).

Identifying ACP carrying CLas is more a problem of sampling than of detection since qPCR and other conventional assays can easily detect small copy numbers of bacterial DNA if it can be recovered from the insects. Because only a relatively small proportion of the ACP individuals are actually competent vectors (Coy and Stelinski, 2015), the insect sample size must be large enough to determine infection pressure accurately. Furthermore, the trapping method used to collect ACP must allow quality DNA to be recovered from the insects. Sticky cards are effective for trapping insects but not for the recovery of quality DNA.

Refer to outdated SPHDS Diagnostic Protocols for HLB by Hailstones & Holford for PCR methodology to be used during a suspected incursion.

Also see:

- Appendix 4 at end of this document: SUMMARY OF PAPERS ON PCR DETECTION METHODS FOR CITRUS LIBERIBACTERS

- NAPPO diagnostic protocol[127]
- EPPO (2015) PM 7/121 (1) 'Candidatus Liberibacter africanus', 'Candidatus Liberibacter americanus' and 'Candidatus Liberibacter asiaticus'. Diagnostics. Bulletin OEPP/EPPO Bulletin (2014) 44 (3), 376–389
- US National Plant Diagnostic Network Standard Operating Procedure for Plant Diagnostic Laboratories Citrus Greening and the Citrus Psyllid version 2[128]
- The sampling protocol for the Florida Southern Gardens HLB Diagnostic Laboratory (http://www.flcitrusmutual.com/content/docs/issues/canker/sg_samplingform.pdf
- Arredondo Valdés R, Delgado Ortiz JC, Beltrán Beache M, Anguiano Cabello J, Cerna Chavez E, Rodriguez Pagaza Y, Ochoa Y. 2016. A review of techniques for detecting Huanglongbing (greening) in citrus. *Canadian Journal of Microbiology*, http://www.nrcresearchpress.com/doi/abs/10.1139/cjm-2016-0022#.V2uJ8DIkrcs
- LeVesque C, McRoberts N. 2017. Comparative Study of Early Detection Techniques: Texas 2 Study. Citrograph Spring edition, pp. 44-47.
- Ding F, Paul C, Brlansky R, Hartung JS. Immune Tissue Print and Immune Capture-PCR for Diagnosis and Detection of Candidatus Liberibacter Asiaticus. 2017. Scientific Reports 18;7:46467.
- Ghosh DK, Bhose S, Warghane A, Motghare M, Sharma AK, Dhar AK, Gowda S. Loop-mediated isothermal amplification (LAMP) based method for rapid and sensitive detection of 'Candidatus Liberibacter asiaticus' in citrus and the psyllid vector, Diaphorina citri Kuwayama. Journal of plant biochemistry and biotechnology. 2016 Apr 1;25(2):219-23.
- Siverio F, Marco-Noales E, Bertolini E, Teresani GR, Peñalver J, Mansilla P, Aguín O, Pérez-Otero R, Abelleira A, Guerra-García JA, HernÁndez E. Survey of huanglongbing associated with'Candidatus Liberibacter'species in Spain: analyses of citrus plants and Trioza erytreae. Phytopathologia Mediterranea. 2017 Apr 1;56(1):98.
- Pagliaccia D, Shi J, Pang Z, Hawara E, Clark K, De Francesco A, Liu J, Tran TT, Bodaghi S, Thapa SP, Folimonova SY. 2017. A Pathogen Secreted Protein as a Detection Marker for Citrus Huanglongbing. Frontiers in Microbiology 8:2041.
- Lou B, Song Y, RoyChowdhury M, Deng C, Niu Y, Fan Q, Tang Y, Zhou C. 2017. Development of a Tandem Repeat-based Polymerase Chain Displacement Reaction Method for Highly Sensitive Detection of 'Candidatus Liberibacter asiaticus'. Phytopathology Oct 11(ja).
- Gottwald TR, McCollum TG. Huanglongbing solutions and the need for anti-conventional thought. Journal of Citrus Pathology. 2017 Jan 1;4(1).
-

Detection relies on recognition of symptoms in the field by trained staff and then molecular testing of samples. Methods used to detect the pathogens have included light microscopy (Tirtawidjaja et al. 1965, Schneider 1968, Heredia et al. 2006), the iodine starch test (Le & Nguyen 2002, Onuki et al. 2002; see Appendix 6), electron microscopy and ELISA (monoclonal antibodies, Garnier et al. 1991, Gao et al. 1993), but detection methods based on polymerase chain reaction (PCR) and DNA hybridisation are at present the most successful methods available.

Other methods under trial include:

- chemical analysis of released volatile organic compounds (VOCs) that emanate from infected trees (Askenov et al. 2014),

[127] http://www.aphis.usda.gov/import_export/plants/plant_exports/downloads/NAPPO_HLB_DP_2_2012-05-30-e.pdf

[128] https://crdn.ifas.ufl.edu/workshop/pdf/Carrie%20Harmon%20docs%20for%20CPDN%20traiing%20CD/SOP_HLB.pdf

- spectral characteristics (Ehsani et al. 2007; Poole et al. 2008; Hawkins et al. 2010a,b; Sankaran et al. 2010; 2013; Mishra et al. 2011; Sankaran & Ehsani, 2012; Cardinali et al. 2012; Garcia-Ruiz et al. 2013; Pourrera et al. 2015;Pourreza et al 2016; Sarkar et al 2016, Wetterich et al 2017).
- other potential methods[129] include metabolic changes, small RNA markers and protein secretions (Pagliaccia et al 2017).

Biological indexing, electron microscopy, as well as PCR techniques are sometimes needed to provide a more robust general initial detection of HLB (Okuda et al. 2005). The possibility of a phytoplasma causing HLB-like symptoms should not be discounted (see Appendix 8) especially as Australian Citrus Dieback (prevalent in grapefruit) is associated with a phytoplasma. A purely molecular approach to diagnosis is not wise (especially if negatives are obtained), since primers for '*Ca.* L. africanus and '*Ca.* L. asiaticus' did not detect '*Ca.* L. americanus' (Teixeira et al. 2005a, c). More recently false positives were picked up in Florida nursery samples with the internationally accepted Li primers (Li et al., 2006). Other variants may exist. Pietersen & Viljoen (2012), Viljoen et al (2013) and Roberts et al (2015) found liberibacters in all genera of South African native Rutaceae analysed. Subsequently a novel subspecies of '*Candidatus* Liberibacter africanus' ('*Candidatus* Liberibacter africanus subsp. tecleae') was found on native *Teclea gerrardii* (Family: Rutaceae) from South Africa (Roberts and Pietersen 2016). Nelson et al (2014) claimed that the suggestion of a further three subspecies of '*C*Laf' could be resolved within a proposal of haplotypes rather than subspecies within '*C*Laf' by giving them a biotype designation, recognising the current host plant differences. Thus '*C*Laf' subspecies vepridis is a biotype of LafA, while '*C*Laf' subspecies zanthoxyli and '*C*Laf' subspecies clausenae are biotypes of LafC (Nelson et al. 2015). However, the five Laf subspecies described (LafC, LafCl, LafV, LafZ and now LafT) were generally identified from multiple specimens, LafT being the exception, of specific host species only, suggesting that gene-flow between these various liberibacters is limited, supporting the higher taxonomic status afforded by subspecies classification. LafCl, LafV and LafZ were identified from the native hosts of the triozid, *Trioza erytreae* del Guercio (order Hemiptera, family Triozidae) (Moran 1968; Burckhard and Ouvrard 2012), the vector of Laf (McClean and Oberholzer 1965). As commercial citrus species are not indigenous to Africa, it is hypothesised that Laf either made a direct host jump from an indigenous rutaceous species to citrus (da Graca 2008) or evolved from a liberibacter species present on the African continent prior to the introduction of commercial citrus species (Phahladira et al. 2012; Roberts et al. 2015). The evolutionary theory of Laf is supported by the current lack of evidence of Laf occurring in indigenous rutaceous species tested thus far, along with the occurrence of four subspecies to Laf from South Africa identified from indigenous rutaceous species. However

Keremane et al. (2015) reported a new liberibacter species ('*Ca*. L. caribbeanus') from Colombia, South America. In addition Fleites et al. (2014) have reported a non-pathogenic strain of '*Ca.* L. asiaticus' not associated with prophages.

For an extensive itemisation of methods used to detect citrus liberibacter species, **including in-field detection methods,** see Appendix 4.

Currently (Feb 2016), PCR is the primary tool for detecting the CLas in psyllids and citrus plants. There are two types of PCR being utilized, conventional PCR and quantitative PCR (qPCR). Both Texas and California are using qPCR for processing samples, because this method can rapidly process very large numbers of samples and potentially detect lower amounts of bacterial DNA in samples. However, conventional PCR provides the regulatory confirmation of HLB infections since the resulting product can be sequenced to provide a DNA match.

[129] http://ucanr.edu/News/Asian_citrus_psyllid_and_huanglongbing_disease/Detecting_HLB-infected_trees/

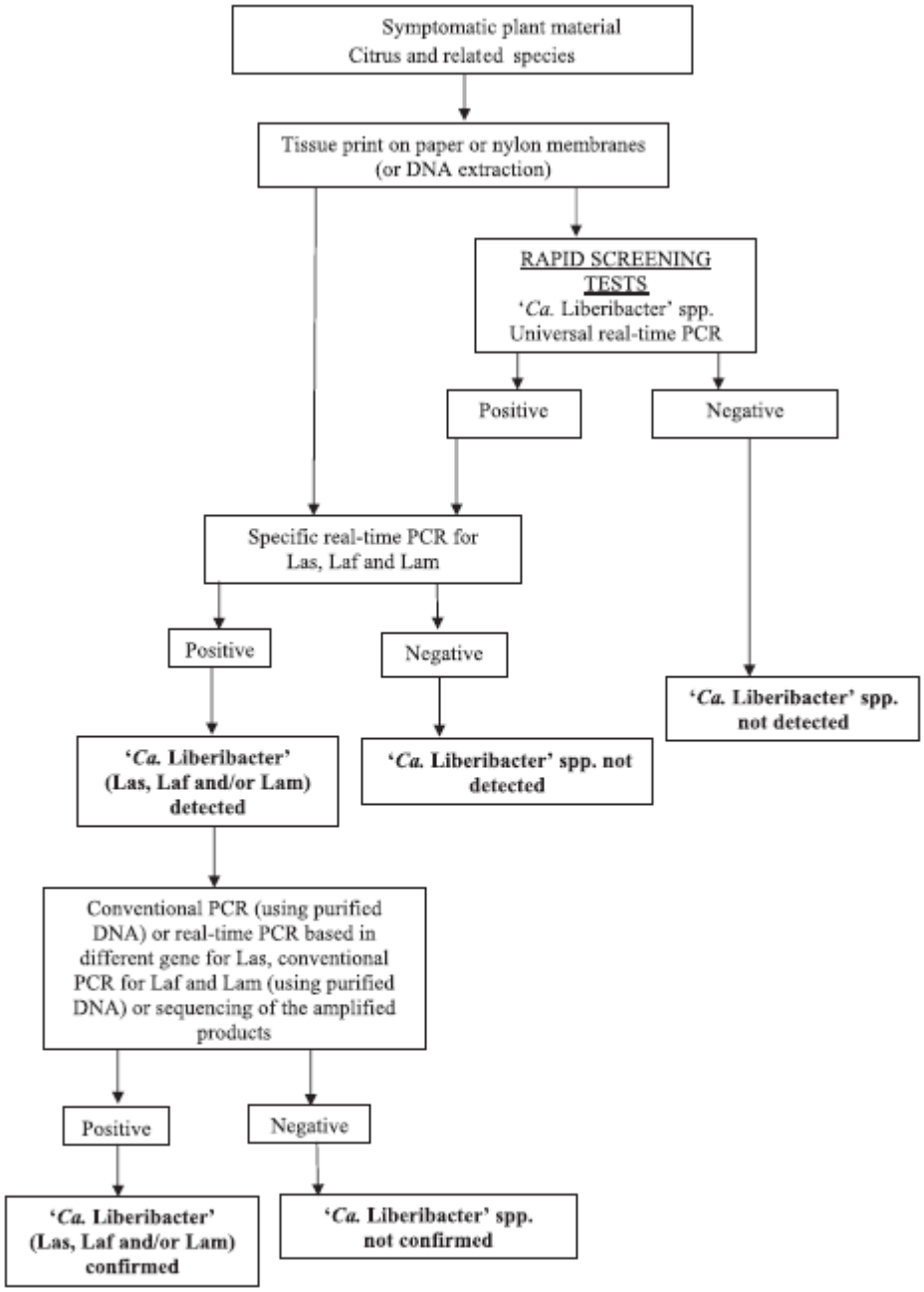


Fig. 1 Flow diagram for the detection of '*Ca.* Liberibacter' spp. in plant material. Laf = '*Ca.* Liberibacter africanus', Lam = '*Ca.* Liberibacter americanus' and Las = '*Ca.* Liberibacter asiaticus'.

© 2014 OEPP/EPPO, *Bulletin OEPP/EPPO Bulletin* **44**, 376–389

From Gottwald and McCollum (2017):

***CLas* titer, detection and population dynamics:** Currently, *CLas* infections in citrus can only be confirmed by detection of *CLas*-specific DNA sequences via PCR technology. Most frequently, detection of *CLas* DNA is based on quantitative polymerase chain reaction (qPCR). Detection of a *CLas* 16S rDNA fragment (Li et al. 2006) is perhaps the most widely reported qPCR method in the published literature and is part of the USDA, APHIS protocol for *CLas* diagnostics, although multiple additional PCR primers have subsequently been developed.

Considerable evidence has been reported to support that: 1) the sensitivity of qPCR for detection of *CLas* 16S rDNA is in the range of 1-9 target copies (McCollum et al. 2017; McCollum et al. 2014a), and that 2) detection of *CLas* 16S rDNA is highly specific. In addition to sensitivity and specificity of qPCR, there is a linear relationship between Log target copy number and Ct value between 10^0 and 10^7 copies per assay making qPCR ideal for estimation of *CLas* titer.

CLas infections are never uniformly distributed within the citrus canopy, coupled with the "loading capacity" (amount of tissue equivalents that can be tested in a single assay) of qPCR (approximately 1 mg tissue equivalent per assay), sampling becomes the overriding limitation of qPCR for detection of *CLas* infections. Citrus petioles are the tissue of choice for *CLas* diagnostics because they have a high proportion of phloem (*CLas* is phloem-limited) compared to other tissues. However, a moderate size citrus tree will have thousands of leaves. In the absence of suspect HLB symptoms, CLas infections within the canopy are most likely rare and of low titer. Therefore, determining where to sample is problematic and there is a low probability of randomly or even systematically selecting rare infected tissue and thus detecting *CLas*.

Detection of *CLas* infection via qPCR in samples with suspect HLB symptoms cannot be considered "early" detection, especially if the objective is to curtail the development of an HLB epidemic. Following infection of a citrus tree with *CLas*, there is a latency period of at least several months, and perhaps longer, prior to the appearance of HLB symptoms depending on the age of the tree (Gottwald 2010). Symptom expression in potted greenhouse trees can be more rapid (Fig. 2). During the latency period, *CLas* populations increase within infected shoots, and can serve as inoculum for subsequent infections. For each tree that is infected with *CLas* and eventually develops HLB symptoms, there are likely multiple neighboring trees infected *CLas*, but are in the cryptic phase, i.e. not yet HLB symptomatic (Gottwald 2010). Therefore, to be considered "early" detection, *CLas* infection must be confirmed prior to the appearance of HLB symptoms.

Movement of *CLas*-infected ACP into new areas is far from uniform at the regional or orchard level (Parry et al. 2014; Gottwald et al. 2010), this contributes to the heterogeneous distribution of infections both within regions and among and within trees. At the time when *CLas*-infected ACP first invade an area, the distribution of nascent infections in citrus is highly erratic both among and within individual trees (Gottwald et al. 2010, Bassanezi et al. 2005).

Fast and simple procedures for accurate detection of *Candidatus* (*Ca.*) Liberibacter (L.) spp. ('*Ca.* L. africanus', '*Ca.* L. asiaticus' and '*Ca.* L. americanus') by Rt-PCR in plant tissues and in individual psyllids (Bertolini et al. 2010, 2014; Keremane et al. 2015, AGDIA[130]) that do not require plant extract preparation nor purification of nucleic acids could greatly help to increase the number of analyses required to ensure the efficiency and success of large surveys for eradication and/or certification

130 https://www.agdia.com/testing-services/Citrus-Greening.cfm

programs. The Bertolini system has been used in Spain for '*Ca*. Liberibacter' spp. field surveys[131]. There are a few growers using the Keremane system in Tx and CA, but there are a few issues[132]:

1. researchers are still providing the kits for detection; the company has not yet come up with a commercial version.
2. many growers find the technology a bit too much for their comfort. Things may change when they find HLB closer to them. The goal is to come up with a much simpler technology (eg. Dipstick assay).

Recently an anti-outer membrane protein A (OmpA) polyclonal antibody (Ding et al 2015) was highly effective for the detection of Ca Las from citrus tissues in a simple tissue printing format. The antibody was also used to capture bacteria from periwinkle extracts. About 80% of all field samples analyzed tested positive with both immune tissue printing and qPCR; whereas 95% were positive with at least one of these two methods. When asymptomatic citrus tissues were tested, the tissue printing method gave a higher rate of detection (83%) than the qPCR method (64%). This is consistent with a lower concentration of Ca Las DNA, but a higher proportion of viable cells, in the asymptomatic tissues. The immune tissue printing method also highlights the detail of the spatial distribution of '*Ca*. Liberibacter asiaticus' in diseased citrus tissues. Both the immune capture PCR and immune tissue printing methods offer the advantages of low cost, high throughput, ease of scaling for multiple samples and simplicity over current PCR-based methods for the detection of '*Ca*. Liberibacter asiaticus' (Ding et al. 2017).

Pagliazzia et al (2017) reported that a CLas secreted protein can be used as a biomarker for detecting HLB infected citrus. Proteins secreted from CLas cells can presumably move along the phloem, beyond the site of ACP inoculation and CLas colonized plant cells, thereby increasing the chance of detecting infected trees. They generated a polyclonal antibody that effectively binds to the secreted protein and developed serological assays that can successfully detect CLas infection. This work demonstrates that antibody-based diagnosis using a CLas secreted protein as the detection marker for HLB infected trees offers a high-throughput and economic approach that complements the approved quantitative polymerase chain reaction-based methods to enhance HLB management programs.

Texas qPCR psyllid testing showed a shift in psyllid sample results from suspect (Ct-values 33-39) to clearly positive (Ct-values < 32) over a two-year period; then one to two years later, many trees with HLB disease were detected. "*To act conservatively and get ahead of the disease spread, needs follow up on the areas that have had ACP with Ct values in the suspect 33-39 range and test more psyllids and trees in those areas. Testing ACP samples is extremely useful for locating regions with HLB infection, since psyllids are accumulating bacteria as they feed on infected trees. A CLas-positive adult psyllid doesn't tell us exactly which tree is positive, because the adults move around, but it tells us that the bacterium is in the area. Because adult psyllids tend to be on the borders, citrus growers should initially focus their HLB detection efforts on the borders of their orchards*" (Bartels 2016)[133][134].

In both Texas and California spatial clustering of psyllid samples with inconclusive Ct-values have been shown to cluster around known positive HLB infected trees. The San Gabriel area quarantine in

[131] See Siverio et al. **Threat Of huanglongbing in the Mediterranean Region: surveys and analyses of 'Candidatus Liberibacter' species in plants and in Trioza erytreae.** http://www.neppo.org/wp-content/uploads/2014/05/m.m._lopez_hlb_agadir_1-11-13_2013110610_12_5.96-MB1.pdf

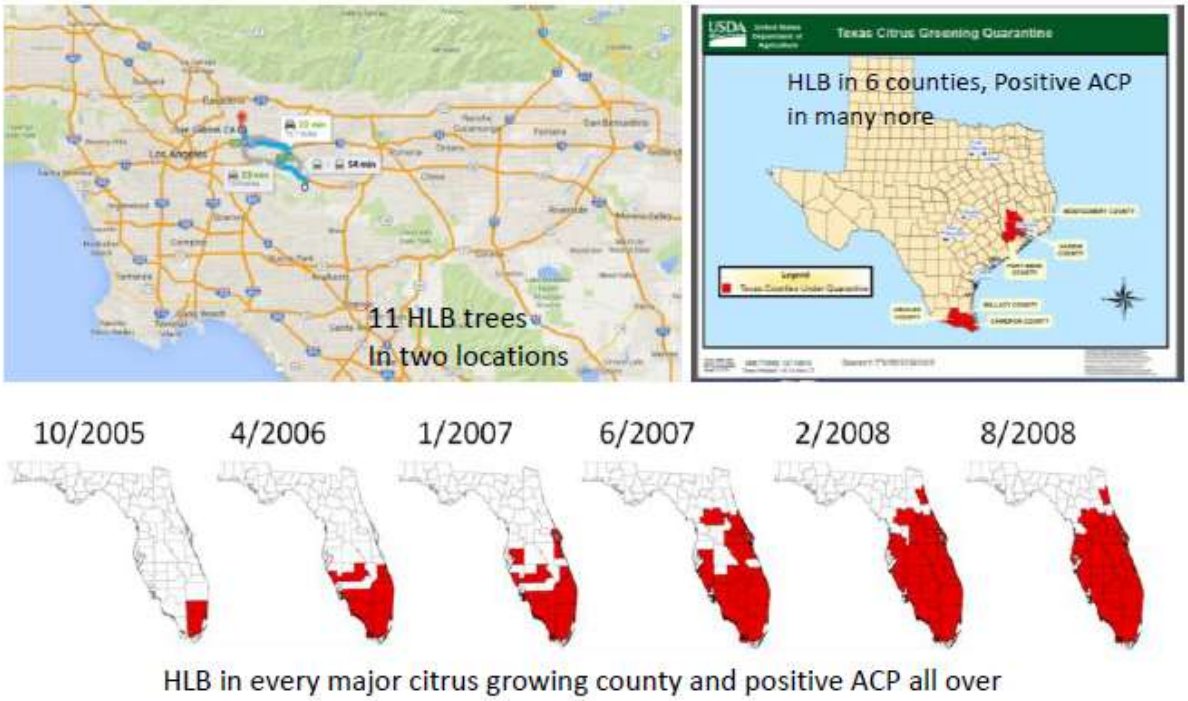
[132] Personal communication of Manjunath Keremane to Pat Barkley on 30 May 2016

[133] https://www.cdfa.ca.gov/citruscommittee/docs/minutes/2015/04-27-15StatewideQWorkingGroupMinutes.pdf

[134] Immediate Action is Necessary. Summary of the HLB Morning Session. **Citrograph Vol. 7, No. 2** | Spring 2016, pp. 24-25

California was found after more intensive survey around an inconclusive psyllid sample (Bartels & Cook 2017[135]).

The CDFA is rigorously testing psyllids and by regulation only those psyllid samples with a Ct of below 32 are followed for regulatory action/s. However, CDFA follows up on all samples with any amplification even at 38 with resampling and repeat analyses. If you compare what happened three years after the first find in FL, TX and CA, Manjunath (Pers. Comm. To Pat Barkley 30 May 2016) believes they have postponed the CA epidemic by a few years:



Legislation

Critical powers in response to incursions of HLB and/or its vectors should:

- **Legislation forcing growers and homeowners to apply sprays for the Asian citrus psyllid when a find is made in the vicinity.**
- '**An emergency rule that requires all bulk citrus loads to be fully tarped during transport regardless of where the load originates from or its destination to prevent the spread of the Asian citrus psyllid[136].** In California psyllid finds are along transportation corridors. Tarps or mesh coverings must not have holes larger than 0.3 square millimeters (0.547mm x 0.547 mm or 0.6mm x 0.5mm[137].
- **'Require occupiers of any place within a declared area to take specified measures, including the treatment or destruction of plants and plant products, necessary for the control or eradication of an exotic pest.** As part of a containment or eradication programme for an exotic pest, occupiers of affected properties will need to implement control measures that may include the application of pesticides and destruction of host plants. All State plant protection agencies have the legislative authority to require land owners or occupiers to take

[135] Abstract **6.a.4** **Update on the Hot Spot Cluster Analysis of Ct-values from Asian Citrus Psyllid Samples. Int. HLB Conf. 2017**

[136] For Californian equirements see http://files.constantcontact.com/921f7f1a301/9e7f2ea2-f481-4e0b-bca7-48262ebd6323.pdf

[137] http://citrusinsider.org/2017/01/new-bulk-citrus-compliance-agreements-mailed/
http://phpps.cdfa.ca.gov/PE/InteriorExclusion/pdf/acpgrowerinformation.pdf

specified measures to control or eradicate an exotic pest and penalties may be imposed on a person for non-compliance.'

- **'Prohibit the planting or propagation of plants within a declared area.** In order to contain or eradicate a newly introduced exotic pest, it is necessary to implement a range of measures to prevent the establishment and spread of the organism. This may include preventing the planting of host crops within the affected area for a period of one or more growing seasons. South Australia, Victoria, Tasmania and the Northern Territory have specific powers to prohibit the planting or propagation of plants, or reduce the number of plants, within a declared area over a specified time; for other States, legislative authority may be provided under general powers associated with control or eradication programmes for exotic pests.' This legislation may be needed to eradicate existing *Murraya* plants (growing naturally, or in nurseries, home gardens, parks and elsewhere in PQA) as occurs in São Paolo, Brazil (Lopes et al. 2008).
- **'Able to destroy healthy or apparently uninfested plants to prevent the spread of an exotic pest.** For exotic pests that pose a high risk of spread, it may be necessary to destroy healthy host and volunteer plants surrounding the outbreak to create a buffer zone for containment or eradication. In some cases an eradication programme may not be able to proceed unless such a buffer can be established. The provisions of the State plant health Acts vary in relation to the removal of healthy plants and none has specific provisions for the creation of buffer zones.
- Unfortunately, not every sample collected from a citrus tree with HLB will contain the bacteria that causes the disease. This can delay the detection of an infected tree and provide more opportunity for ACP to spread the disease. Hence the need for Agriculture officials to be able to remove all citrus trees where a % of trees within the block have been shown to be infected without the need for laboratory tests to confirm that each tree has HLB.
- **Legislation should include destruction of abandoned/neglected orchards and home garden trees without the need to prove plants are infected or infested.** Abandoned citrus groves are a significant source of '*C*Las' and dispersing *D. citri* move this pathogen into nearby managed groves (Tiwari *et al*. 2010). **A uniform definition for an abandoned orchard is required in Australia.** ', an abandoned orchard[138] is defined as an orchard where there has been:
  - no commercial fruit harvest during the last two seasons; and
  - no production care during the past two years, including weed control and mowing; and
  - where orchard use has been transferred to other uses (e.g., livestock).

  In Florida a proposal for a cost-sharing program for the removal or destruction of abandoned citrus groves to eliminate material that harbors citrus greening and the vector that spread the bacterial disease is currently working through the legislative process as HB 7007 and SB 1010.

The ability to destroy asymptomatic plants without testing to verify infection may arise because

1. young flush becomes infectious within 15 d after receiving 'CLas' inoculum (Lee et al. 2015)
2. incidence of infection in asymptomatic samples based on PCR testing may be up twice the estimated incidence of infection based on visual symptoms alone (Irey et al. 2006a,b) and a decision may have to be made at what percent infection the whole orchard is deemed to be infected or at risk of infection.

Eradication/Control

[138] Citrus Health Response Program Abandoned Grove Initiative. 2009.

Craig AP, Cunniffe NJ, Parry M, Laranjeira FF, Gilligan CA. Grower and regulator conflict in management of the citrus disease Huanglongbing in Brazil: a modelling study. Journal of Applied Ecology. 2018.

ACP was found in Brazil in the 1940's but HLB was not detected until 2004 (Texeira et al. 2005a). As Gottwald & Graham (2014) pointed out, "*it is highly likely that HLB has been introduced into many areas within the Western Hemisphere over time but due to the absence of vector has tended to die out as infected trees declined and eventually died*". ACP was first detected in Florida in 1998 but no attempts were made to eradicate and within 2 years spread across the entire state. It is believed that the vector encountered the prior introductions of HLB eventually resulting in the current epidemic.

In California a group of three ACP adults tested positive for '*Ca.* L. asiaticus' with the Li et al. (2006) real-time PCR assay. ACP adults were collected from a residential citrus tree located in the Hacienda Heights area of Los Angeles County, California. The tree had 23 graft unions, primarily of Meyer lemon and pomelo varieties and showed HLB symptoms and tested positive for '*Ca.* L. asiaticus' (Kumagai et al. 2013). In June 2015, CDFA again detected several HLB '*C*Las' positive citrus plants (kumquat, Mexican lime, and mandarin respectively)[139] in neighboring Los Angeles residential blocks. The two detections[140] had distinct '*C*Las' evolutionarily divergent characters, implying their different origins of introduction into California (Yan et al. 2015). The implication is that HLB has been present in California well before the advent of ACP into the state. Attempts are being made to control ACP in the citrus growing region of Central California but the psyllid is out of control in the Los Angeles Basin and southern California. In early 2016 there are 53,000 sq miles (137,269 sq km) under quarantine for ACP in California. The two findings of HLB to date have been eradicated. Through the Citrus Matters Abandoned Citrus Tree Removals program, California Citrus Mutual works with growers, government officials and local residents to seek the voluntary removal of trees at no cost to the homeowner[141].

While HLB has not been eradicated in any country, it is because ACP has not been eradicated when introduced. The only country to carry out a successful eradication of ACP has been Australia. *D. citri* was recorded in Australia, in the Northern Territory in 1915, but it was eradicated by chance during the 1916-1922 eradication campaign for citrus canker (*Xanthomonas citri* subsp. *citri* (Bellis et al. 2005) presumably introduced on infected trees from China or Japan.

To eradicate HLB, it is necessary to prevent the introduction and establishment of ACP.

To attempt HLB control after the establishment of ACP:

- Regional management of ACP.
- Speedy elimination of symptomatic trees with frequent re-surveys. This means that once HLB has been detected in a region, or on a farm, growers must be constantly alert. They must be aware of the problem, symptoms of the disease and characteristics of the vector, and they must co-operate in the surveillance and eradication (Bergamaschi 2006, Lopes et al. 2008).

[139] http://www.citrusinsider.org/2016/02/hlb-confirmed-in-san-gabriel/#more-2009

[140] These incursions, presumably of Asian origins, led to the development of risk-based residential survey methodologies: Gottwald et al 2013. Risk-Based Residential HLB/ACP Survey for California, Texas, and Arizona: http://www.plantmanagementnetwork.org/edcenter/seminars/outreach/Citrus/HLB/player.html; Gottwald T, Luo W, McRoberts N. Risk-based residential HLB/ACP survey for California, Texas and Arizona. Journal of Citrus Pathology, 1(1): 121-125. http://escholarship.org/uc/item/99c6v21q

[141] http://www.thepacker.com/news/residential-citrus-program-targets-psyllid

- Pathogen acquisition by vectors was positively associated with pathogen titer and time since inoculation, with acquisition occurring as early as the first measurement, at 60 days after inoculation. This result is problematic from a disease management perspective because it means that infected citrus trees may be sources of infectious vectors well before the disease symptoms become evident. Therefore, there is ample potential for psyllids to acquire the pathogen from trees during the asymptomatic (or latent) phase of infection, reducing the efficacy of diseased tree removal (rouging).
- In Brazil there is mild aggregation of HLB symptomatic trees with secondary foci spread all over the block indicating that there is long (4-22 tree spaces distant) and short (to nearby trees) dispersal. This impedes adoption of an eradication radius of exposed trees and the entire block would need to be eliminated (Bassanezi et al. 2007).
- HLB spread occurs as an incessant mixture of these two processes with a continuous introduction of inoculum by ACP from outside the plot and local spread from within the plot occurring simultaneously. The overarching influence in HLB epidemics is the migration and transmission of '*Ca.* L. asiaticus' via psyllids from outside the block, i.e., the influence of primary spread (Gottwald et al. 2008a).
-

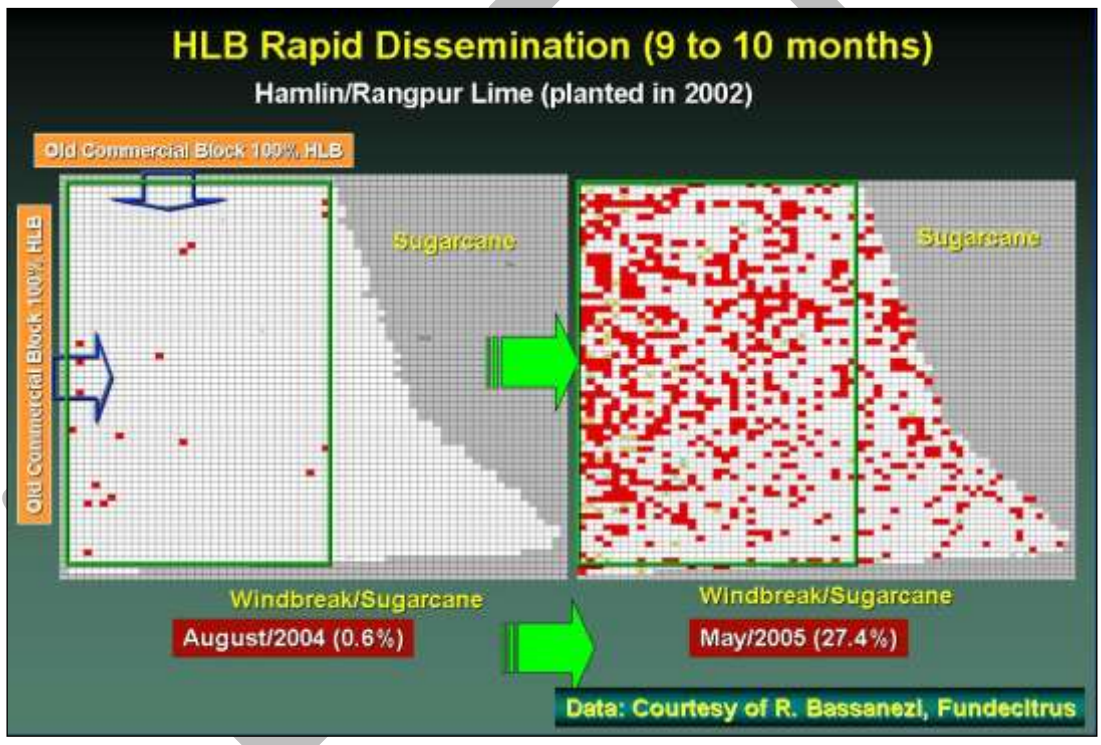


Figure 45. Rapid dissemination of HLB over 9-10 months from a 100% infected orchard to an adjacent orchard in Brazil (T Gottwald: Citrus HLB Research Overview for NAS/ARS Combined Workshop, 2007).

- Significant control will likely only be achieved from regional disease and psyllid management strategies (Bassanezi et al 2013 a,b).
- Eradication mode should be continued as long as possible because it is very difficult to control HLB once it established (Irey 2016)[142]. Early HLB detection is the key to getting ahead of the disease spread. Having a large psyllid and plant sampling volume (large number of samples, wide area tested, etc.) is the most important factor to maximize detection of HLB. Irey recommended the use of both validated tests and new technologies – not relying on just one or the other.

[142] Immediate Action is Necessary. Summary of the HLB Morning Session. **Citrograph Vol. 7, No. 2** | Spring 2016, pp. 24-25

- Irey et al. (2006a,b) reported detection from asymptomatic samples with initial results indicating that actual incidence of infection based on PCR testing may be up twice the estimated incidence of infection based on the presence of visual symptoms alone.
- Simulations of Laranjeira et al. (2010)[143] showed that the 28% criterion for orchard eradication used in Brazil corresponds to 100% of infected plants only in a very limited range of infection rate x incubation period. For incubation periods ranging from 6 to 12 months, maximum infection is reached only when ~45% are symptomatic. Moreover, when the 28% criterion is applied ~40% of the plants are still productive (most of them asymptomatic) no matter the infection rate or the incubation period.
- To prevent the spread of HLB in Brazil, especially from abandoned infected groves, the government regulated that if 28% of a plantation unit (grove) is found to be symptomatic, then the whole plantation unit must be destroyed. This decision used the best evidence available in 2008, which suggested that a 28% detectable prevalence corresponded with 100% actual prevalence, the disparity being due to asymptomatic infected trees and imperfect detection methods. Using a mathematical model with parameters estimated from field data, Craig et al (2018) evaluated the assumptions underlying the 28% threshold. The relationship between detectable and actual prevalence is much wider than allowed for in the Brazilian regulations. There is a high probability that groves with detectable levels of symptomatic plants substantially below 28% have a >90% prevalence of infected plants. Paradoxically, in a well-managed orchard, the threshold of 28% may not be reached at 100% prevalence. There is probably no threshold that is optimal for individual growers and regulators but roguing and spraying is beneficial to both parties. Regulations should focus less on prevalence thresholds, and instead encourage early detection and co-ordinated spraying among growers to control Huanglongbing on a regional level (Craig et al 2018)

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Factors influencing decisions on eradication or containment will be:

- a pre-incursion cost/benefit analysis establishing significant economic loss to the citrus, nursery and allied industries or the community if the organisms establish;
- physical barriers and/or discontinuity of hosts that occur between production;
- the degree of isolation of the area affected by an incursion, size of orchards, and a relatively low number of growers and households;
- difficult to achieve cost-effective control of the disease and its vectors;
- no primary parasitoids in Australia that will parasitise the vectors;
- outbreak(s) few and confined;
- trace back information indicating few opportunities for secondary spread;
- weather records show unfavourable conditions for pest and disease development and wider spread; and
- ease of access to outbreak site and location of alternative hosts.

Prior response and eradication agreements must be established between industry and all levels of government and the immediate response must assume that eradication is possible until delimiting surveys have been completed.

HLB Response in Urban Area in California:

[143] Laranjeira FF, DeSimone ER, Gilligan CA. 2010. Modelling Huanglongbing (HLB) Spread and Eradication Procedures in Brazil. Paper to be presented to 18th IOCV Conference Sao Paulo, Brazil.

• Any citrus tree that tests positive for HLB using the federally recognized PCR diagnostic system is removed and destroyed.
– Tree removal is mandatory – homeowners cannot opt out.
• All host plants within 800 meters around an HLB detection site are treated and receive intensive visual survey.
– Treatments and visual survey are mandatory – homeowners cannot opt out.
– Plant samples and ACP samples are collected.
• CDFA continues to treat properties in the area on a routine basis.

HLB Response in Commercial Grove in California:

• Any citrus tree that tests positive for HLB using the federally recognized PCR diagnostic system is removed and destroyed.
– Tree removal is mandatory – growers cannot opt out.
• In a commercial grove, CDFA will follow a hierarchical sampling protocol to test trees surrounding the HLB find site.
– All trees with symptomatic leaves will be sampled, and perimeters and edges of open spaces will receive added focus, as HLB is often found in higher concentrations in these areas.

Pre-Incursion Awareness/Engagement

The following are required:

- training of inspectors, horticulturists, CITTgroup co-ordinators, and IPM scouts by (a) pathologists with field experience with HLB and knowledgeable in disease symptomatology and epidemiology and awareness of diseases/disorders with which HLB can be confused, and (b) entomologists with knowledge of the biology, ecology and management of the vectors;
- training of growers and nurserymen in vulnerable areas, e.g., Ord River, Darwin, northern Queensland;
- collation of internationally available DVDs, pamphlets and other extension material on HLB and the vectors of the pathogens for immediate distribution in the event of an incursion; and\collaboration of PHA, OCPPO, AQIS and state departments and industry to produce one set of informative awareness material aimed specifically at growers, nurserymen and the general public;
- an awareness plan it place to be rolled out when there is an incursion of ACP and/or HLB;
- a ‘spotting guide’ for symptoms of HLB, and with photos of the Asiatic citrus psyllid, was produced by Citrus Australia as a poster and distributed to citrus growers through the Australian Citrus News 85 June/July Issue 2009, to nurserymen through the Auscitrus Newsletter and the NSW Newsletter of NGIA and to state departments of agriculture. These need to be improved and updated.

Examples of some awareness websites:

- Citrus Greening/Huanglonbing (Factsheet). View
- **Texas citrus greening official site. View**
- Huanglongbing/Citrus Greening Pathology Training (CHRP) – Spanish. View
- Citrus Greening Sampling. View
- Citrus Greening Sampling – Spanish. View
- Citrus Greening Submission form – Spanish. View
- Huanglongbing/Citrus Greening Pathology Training (CHRP). View
- Citrus Health Response Program (Huanglongbing/Greening-Florida). View
- Asian Citrus Psyllid and Citrus Greening Disease (IPM-Florida). View
- Interactive Greening Training (Florida). View
- Be on the Lookout for Citrus Greening Disease (Texas). View
- Signs of Citrus Greening (L-5505, Texas). View
- Citrus Greening Photos by Host (Florida). View
- Scouting for Citrus Greening (Florida). View
- Citrus Greening: Questions and Answers (USDA). View
- Citrus Greening: A Serious Threat to the Florida Citrus Industry. View
- Citrus Greening (Enverdecimiento de los citrícos – Florida). View

- http://californiacitrusthreat.org/materials.php
- www.californiacitrusthreat.org
- www.cdfa.ca.gov/plant/pdep/target_pest_disease_profiles/ACP_PestProfile.html
- Save our Citrus: www.saveourcitrus.org
- USDA: www.aphis.usda.gov/plant_health/plant_pest_info/citrus_greening/index.shtml

- San Diego County: www.sdcounty.ca.gov/awm/acp.html
- Florida Department of Plant Industry Citrus Health Response program: www.doacs.state.fl.us/pi/chrp/greening/citrusgreening.html
- http://citrusinsider.org/wp-content/uploads/2014/07/Asian-Citrus-Psyllid-and-Huanglongbing-Disease-English.pdf
- http://www.californiacitrusthreat.com/
- http://www.citrusgreeningtraining.org/
- http://www.valleyag.org/texascitrusgreening/signs.php
- http://amarillo.tamu.edu/amarillo-center-programs/extension-plant-pathology/citrus-publications/citrus-greeninghuanglongbing/
- **http://www.ipm.ucdavis.edu/QT/asiancitruscard.html**
- Information video for homeowners: https://www.youtube.com/watch?v=2VuuHzKHIiE
-

http://californiacitrusthreat.org/pdf/CRB-FlipBooksm.pdf gives ready access to pictures and the identifying features of 25 plants that are hosts of Asian citrus psyllid.

Post-Incursion Awareness/Engagement

https://citrusmatters.cropscience.bayer.us/homeowners
http://www.ucanr.edu
http://californiacitrusthreat.org/asian-citrus-psyllid.php
http://www.californiacitrusthreat.org/asian-citrus-psyllid.php
http://californiacitrusthreat.org/huanglongbing-citrus-greening.php
https://www.cdfa.ca.gov/plant/PE/InteriorExclusion/acp_quarantine.html
http://californiacitrusthreat.org/asian-citrus-psyllid.php
http://www.growingproduce.com/citrus/saving-california-citrus-means-thinking-outside-the-carton-opinion/
https://www.acgov.org/cda/awm/documents/Save_Your_Citrus_Ala_Co1.pdf

PB CRC Project 4004: Advancing collaborative knowledge systems for plant biosecurity surveillance (http://www.pbcrc.com.au/research/project/4004):

Engagement for Collaboration tool – Overview

Stage 1

Who to engage? – Identify key stakeholders

Key stakeholders include those who exert influence over collaboration and decision-making processes (e.g. because of their knowledge, role, skills, or relationships with other key players), and whose support (or lack thereof) is critical to the success or failure of any proposed biosecurity effort.

Why engage? – Develop desired objectives for stakeholder engagement

What are the major concerns of the key stakeholders? It is important to develop objectives that reflect these concerns. All objectives should be considered, not just those thought to be appropriate by a small group or those that are based on science. Eliciting and clearly defining a set of fundamental objectives is vital. If the objectives are vague or incomplete, then we will be working to resolve the wrong problem.

How to engage? – Create a suite of appropriate stakeholder engagement strategies

Create a range of possible engagement strategies that represent potential ways to meet the fundamental objectives. Engagement strategies need to suit the (human and biophysical) biosecurity context. They need to support processes to enable the use of both scientific and other kinds of knowledge to inform decisions and build capacities and resources. Importantly, different engagement strategies may be required for different stakeholders, rather than a 'one-size-fits-all' approach.

Stage 2

Success?– Evaluate the performance of each stakeholder engagement strategy

Present a selection of key stakeholders with the engagement strategies developed in Stage 1 to evaluate how successful these options might be.

Return on investment (Bang-for-buck) – Build consensus on which engagement strategies will give the best return on investment

Build consensus among stakeholders on which engagement strategies will be most cost-effective. All stakeholder views are considered, compared and combined.

In practice, these stages are overlapping and iterative

An effective public outreach program will be required to create awareness and cooperation with a focus on commercial growers, picking teams, packing shed and juice plant operators, homeowners and public officials in infested areas, retail nurseries and outlets such as Bunnings, gardening clubs, garden care operators and traditional and social media.
Realizing the pivotal role of homeowners, a public outreach plan should be designed to create an environment of cooperation whereby homeowners would be willing to inspect their citrus trees and allow agriculture officials to inspect and, if needed, treat their trees. The challenge is finding a positioning strategy and message platform that resonates and fuels support.

On finding ACP and/or HLB, the following should occur (Krist 2011):

- ACP-HLB Task Force established in the citrus growing region "to coordinate an education and outreach program to alert residents to the threat posed by ACP and to mobilise broad public support for efforts to exclude, detect and eradicate the pest";
- presentations to elected officials (local, federal and state governments) and civic groups;
- distribution of a pamphlet for home gardeners, landscapers, park managers, retail nurseries, chain stores (e.g., Bunnings and Woolworths), to address quarantine risks posed by orange jasmine as well as citrus;
- distribution of educational materials to the general public e.g. bookmarks, door hangers, mouse pads, fliers, rates notices inserts;
- give-away ACP identification card with a small hand lens;
- operation of a hotline;
- public outreach activities could also include agricultural shows, field days, community fairs, school events, career days, participation in botanical garden activities;
- involvement of TV and radio gardening show hosts e.g. Gardening Australia;
- preparation of material for print, radio and on-line distribution and
- Facebook page

Awareness strategies should be undertaken by Citrus Australia and the nursery industry to provide advice to growers, packers, retail and wholesale nurserymen on what to look for and what to do. Advice should be targeted to the audience:

- distribution, to citrus growers and nurserymen and allied groups, of folders/pamphlets containing information about the importance of the disease, symptom and pest recognition;
- hosting of educational workshops for growers, packers, IPM scouts etc.;
- orchard and nursery visits to increase the knowledge and consciousness of citrus growers and nurserymen about the disease, its potential economic impact and the vector;
- free diagnostic checks on suspect material;
- radio and TV, e.g., Country Hour, Landline, and ABC Gardening Show to reach home gardeners, landscapers as well as growers and nurserymen;
- print media, e.g., Australian Citrus News, rural newsletters, and the Land, Good Fruit & Vegetable, Australian Horticulture;
- social media: encourage people to get connected on Facebook, Twitter and Flickr in order to support the cause, spread the word and stay updated on the latest citrus alerts. Adding information on these social media sites will grant followers access to the latest citrus news, helpful links, tips, and citrus disease;
- on-line information e.g., Citrus Australia's weekly emails to growers.

What growers can do[144]:

- **Make coordinated, area wide treatments a priority**by getting them done within the designated time frames.
- **Regularly monitor trees for signs of the Asian citrus psyllid and treat again** if you see it. Use a systemic in the warmer months, if possible. In areas where the psyllid is established, two to three treatments per year is the bare minimum recommended. Four or five treatments per year is more realistic for effective suppression to the levels needed to protect against HLB infection.
- **Remove trees you are not willing or unable to protect.**
- **Talk to your neighbors**about what they are doing to protect or remove their citrus. Help inform others and look for opportunities to work together. Be sure to follow best management practices when out in the field.

From growers and shippers to field workers and truck drivers, every level of the industry must work together to stop the ACP and HLB:

- Field equipment such as forklifts, ladder trailers, portable restrooms, picking bags, and field bins are continuously transported between orchards. Packinghouses, growers, and their contractors must take action to ensure this equipment is free of leaves and stems, and consequently ACP, before it leaves the field.
- Packinghouses, growers, and their contractors must take action to ensure this equipment is free of leaves and stems, and consequently ACP, before it leaves the field.
- Pick and pack locally
- Tarping loads
- washing or fumigation that cleans the fruit before it leaves an area."
- brushing equipment off at the end of the day or shaking out picking bags

[144] https://www.cacitrusmutual.com/santa-barbara-county-acp-area-wide-management-update/

For town residents, activities should include:

- placing billboard advertisements,
- placing signage at bus shelters and rail stations,
- distributing a door hanger at residences in the core HLB area,
- exhibiting at community events and
- running the public service announcement in movie theaters, on TV and in newspapers.

See https://citrusmatters.cropscience.bayer.us/homeowners

All communications should ask residents to cooperate with agriculture officials working to protect the community's citrus.

Management if eradication fails:
Craig A P, Cunniffe N J, Parry M, Laranjeira F F and Gilligan C A. 2018. Grower and regulator conflict in management of the citrus disease Huanglongbing in Brazil: a modelling study. J Appl Ecol. doi:10.1111/1365-2664.13122

The following has been implemented in Texas (J. da Graca pers. comm. Nov. 2015):

1. **A mandated budwood certification program.** All budwood source trees under screen and tested twice annually. All nurseries in the commercial production areas under screen, and movement of trees into the area from other areas forbidden. Commercial nurseries inspected monthly and trees drenched with systemic insecticide before shipping.
2. **A voluntary coordinated spray program.** In autumn/winter, all growers are contacted and strongly encouraged to spray against ACP in April and July (Aust equivalent to Texas) (2-3 week periods). NB these dates may be varied depending on host phenology. Add a coordinated spray after rains. Integrate psyllid treatment with measures against other pests.
3. **Tree removals** especially removal of infected residential trees and abandoned orchards.
4. **Early detection**. Psyllids are a good indicator of potential future symptom appearance. Test psyllids for '*C*Las' by PCR.
5. Transgenic citrus carrying spinach defensin gene? – this is backed by Southern gardens in FL who are sponsoring the de-regulation of transgenic citrus. The trees still need to be tested extensively in the field. There is anti-GMO pushback, so consumer acceptance will be a problem.
6. **Establish a Pest & Disease Management Corporation.**

The Strategic Plan for Combatting HLB in California[145]:

1. Quickly detect and eradicate diseased trees by improving the urban survey and sampling processes, continuing quick mandatory tree removal of infected trees, and collaborating with the scientific community on early detection efforts.
2. Control movement of psyllids around the state and enforce regulations by increasing enforcement staff with emphasis in HLB quarantine areas and implementing a regional ACP quarantine with performance standards.
3. Suppress psyllid populations by promoting grower participation in area-wide treatment programs, removing uncared for host plants, continuing to using biocontrol and continually assessing urban treatment protocol.
4. Improve data technology, analysis and sharing and explore new solutions for digitization of data, including Pesticide Usage Reports.

[145] https://citrusinsider.org/2018/04/citrus-pest-disease-prevention-committee-creates-strategic-plan-for-combatting-huanglongbing/

5. Use outreach and collaboration to encourage homeowner and industry participation in program efforts, and foster local governments' support for program activities.

For management of HLB/ACP in **Brazil,** see:

- http://citrusrdf.org/wp-content/uploads/2012/09/Juliano-Presentation-to-BOD-2-3-13.pdf
- Futch & Singerman 2018. An inside look at Brazil's citrus production practices. Citrus Industry News. http://citrusindustry.net/2018/01/30/inside-look-brazils-citrus-production-practices/

Futch & Singerman state:
"A recent survey conducted by Fundecitrus found that the average incidence of HLB in 2017 was 16.73 percent, slightly down from 16.92 percent in 2016. Fundecitrus also found that the smaller the farm, the larger the impact of HLB. In farms with up to 10,000 trees, the incidence of HLB is 36 percent. In farms with up to 500,000 trees, the incidence is 2.37 percent. Researchers argue that this is the result of the "border" effect; the largest number of infected trees is usually within 656 feet of the grove border.

Many of the larger growers in the Southwest citrus production region of São Paulo have found it economically beneficial to work with nearby property owners in their management of psyllids and HLB. Their strategy addresses both psyllids and HLB in a regional approach and can extend as far as 1.25 to 3 miles beyond their property. In one case, a grower hopes to extend this management plan as far as 12 miles over time.

This regional approach includes offering adjacent property owners (usually residential properties) a fruit tree (other than citrus) free of charge in exchange for removing their citrus tree. In cases where neighbors do not have the ability to physically remove the tree themselves, the grove company will remove the tree and then plant the alternative fruit tree in its place. If the property owner is not willing to remove the citrus but allows for spraying, the company will spray the citrus tree monthly at no cost to the property owner.

In cases in which the neighbor does not accept the tree replacement or does not allow for spraying, growers release a parasitic wasp (*Tamarixia radiata*) of citrus psyllids. Releasing the wasps in their own groves is unlikely to be beneficial due to the frequent psyllid sprays, but releasing them in areas with infrequent sprays seems to provide some benefit in reducing psyllid numbers.

By reducing psyllids and sources of HLB around their groves, growers in Brazil can better manage production impacts within their properties. While this process can be expensive, one grower indicated that for every $1 spent on external actions, $8 or more in internal actions can be saved, thereby providing a greater return on investment.

In addition to using the sticky traps, growers will spray the borders weekly using a smaller sprayer that sprays rows up to 328 feet from the grove border to keep psyllids from further spreading into the grove.

Most growers also place yellow sticky trap cards along their property borders to detect where the psyllids are coming from into their groves, and also to concentrate their external actions as well as border sprays. When growers detect psyllids, they apply pesticide. In most cases, these border

sprays are triggered by having on average just one psyllid per trap. In addition to using the sticky traps, growers will spray the borders weekly using a smaller sprayer that sprays rows up to 328 feet from the grove border to keep psyllids from further spreading into the grove.

Border rows within a grove are planted at higher tree density to allow trees to act as a trap crop. When any tree in the border is found to be HLB positive, it is quickly removed and then replanted. This removal and replanting keeps the border fully functional in acting as a trap for entering psyllids.

Most of the growers we visited time their insecticidal sprays to coincide with new vegetative growth flushes. Female psyllids lay their eggs onto new flush, and if young flush is not present, then they forego reproduction. By treating new flushes, growers are better able to control psyllid numbers over time. Brazilian growers are thus monitoring psyllid nymphs on flushes on a frequent basis. Some growers monitor for both adults and nymphs. Other growers will monitor only for nymphs, as the adults are more difficult to detect consistently".

To prevent the spread of HLB, especially from abandoned infected groves, the Brazilian government regulated that if 28% of a plantation unit (grove) is found to be symptomatic then the whole plantation unit must be destroyed. This decision used the best evidence available in 2008 (.....), which suggested that a 28% detectable prevalence corresponded with 100% actual prevalence, the disparity being due to asymptomatic infected trees and imperfect detection methods. Using a mathematical model with parameters estimated from field data, Craig et al (2018) evaluated the assumptions underlying the 28% threshold. They investigated the effects of spraying insecticide and removing diseased plants on the infectious pressure and potential loss of yield from an infected grove and found:

- the relationship between detectable and actual prevalence is much wider than allowed for in the regulations. There is a high probability that groves with detectable levels of symptomatic plants substantially below 28% have a > 90% prevalence of infected plants. Paradoxically, in a well-managed orchard the threshold of 28% may not be reached at 100% prevalence.
- Infectious pressure from an infected grove is substantially reduced when growers control disease. Individual growers failing to manage disease therefore threatens the wider grower community. Control is likely to increase yield and prolong grove productivity, but in some groves may reduce yield.
- 5.Policy implications. Current disease thresholds aimed at restricting the spread of the citrus disease, Huanglongbing, in Brazil allow heavily infected groves to remain in the landscape, but lower thresholds would disadvantage growers who are already controlling disease. There is probably no threshold that is optimal for individual growers and regulators but roguing and spraying is beneficial to both parties. Regulations should focus less on prevalence thresholds, and instead encourage early detection and co-ordinated spraying amongst growers to control Huanglongbing on a regional level.

For 2015 **Florida** Citrus Pest Management Guide: Huanglongbing see: http://www.crec.ifas.ufl.edu/extension/pest/PDF/2015/Huanglongbing.pdf and for the Asian citrus psyllid see https://edis.ifas.ufl.edu/in686

Management options are influenced by:

- potential for ingress of infected psyllids from surrounding areas (abandoned orchards, area-wide management);
- climate (rainfall, and seasonal temperatures and humidities);
- irrigation and nutrient management practices;
- flushing phenology and extent, including the number, size and shape of leaves, in relation to host plant species or hybrid, climate and management practices;
- impacts of tree size, canopy shape and planting densities on spread of the pathogen in trees;
- impacts of tree size, canopy shape and planting densities on application of chemicals to trees;
- hedging and pruning practices;
- the presence and effectiveness of natural enemies of the vectors;
- the extent of feeding and oviposition suppression by deposits of chemicals applied as sprays (e.g., mineral oils);
- doses of chemicals applied for psyllid control (e.g., for foliar applications, deposition of active ingredients on foliage in relation to concentrations of active ingredients in sprays and spray volumes per hectare);
- timing (e.g., as a new growth flush cycle commences) and methods of application (e.g., foliar, trunk or soil) of chemicals applied for psyllid control;
- number, frequency and thoroughness of insecticide applications
- sprayer type, efficacy, calibration etc.

Effective management of HLB requires:

- use of pathogen-free rootstocks and scions in lieu of potentially infected budwood, scions, and marcotts;
- frequent (e.g., 6 times annually) monitoring for HLB symptoms;
- prompt destruction (cutting down and poisoning of roots) of diseased trees within orchards;
- destruction of abandoned orchards;
- use of windbreaks to restrict and slow psyllid dispersal[146];
- propagation of plants, as interplants or ground-covers within orchards, that produce volatiles that repel adult psyllids;
- ensure that fruit bins are free of leaves and other debris;
- effective psyllid control[147], (Bassanezi et al. 2013);
- use of sprayers (e.g., oscillating booms rather than airblast sprayers) that do not assist, or at least minimise, dispersal of adult psyllids in air currents; regional area-wide management (Rogers et al 2014, Wright 2015; CREC 2015); in California CDFA treats residential properties 400 meters around commercial properties. Residential properties are only treated if 75 percent of commercial citrus is treated as part of an area-wide management program. There are two applications per year.
- good nutrition and management (Xu et al. 2014);
- UV light reflection from metalized film (Imaflex)[148] slows spread of ACP by deterring ACP from landing on trees and therefore reduces HLB pressure on young citrus trees compared to whiteface mulch or bare ground. Metalized mulch can thereby augment current control measures for young trees based primarily on systemic insecticides. Additional costs could be compensated for by increased tree growth rate which would shorten time to crop

[146] The effectiveness of windbreaks will depend on several conditions including canopy density, height, and spacing within and between breaks.
[147] See http://www.imok.ufl.edu/docs/pdf/entomology/ppt_0001.pdf
[148] http://www.prnewswire.com/news-releases/imaflex-developing-novel-agricultural-film-to-prevent-citrus-greening-207513531.html

profitability (Croxton & Stansly 2013). In addition, metalized mulch, together with the associated drip irrigation and fertigation system, increases soil moisture, reduces weed pressure and increases tree growth rate;

- the key recommendation for **organic citrus growers** is area-wide ACP treatment programs. "Area-wide" would mean that pest control activities would be coordinated throughout the area to target the 2-3 week periods around growth flushes when all trees can be treated at a similar time. Because this is control and not eradication, there are some organic materials that are approved for this program in the USA. They are Pyganic plus oil, Trilogy (neem oil), PFR - 97 (*Isaria fumosoroseus* fungus), and oils from petroleum and other sources[149] (Barthe et al. 2014; Qureshi & Stansly 2014). The efficacy of petroleum spray oil against citrus psylla was tested by Rae et al. (1997). As the oil concentration increased from 0×25% to 1×0% there was a linear decrease in the number of psylla of each stage present on foliar shoots after 8 days. Psylla survival was stage-dependent, with 1st± 2nd instars being the most susceptible and eggs being the most tolerant to oil. In Florida, Tansey et al. (2015) concluded that ACP suppression, higher yields and eventual production gains indicated that frequent, low-volume application of HMO may be a viable alternative for suppressing ACP populations. See also http://www.citrusinsider.org/2016/07/ucanr-addresses-organic-control-of-the-asian-citrus-psyllid/#more-2268
- Some growers in Florida are turning to thermotherapy of trees (Al-Jamaili & Ehsani 2015) in an attempt to maintain production[150]. For a summary of the effects of heat treatment temperatures on nursery plants and budwood see Beattie and Barkley. 2009. Huanglongbing and its vectors: A pest-specific contingency plan for the citrus and nursery and garden industries (Version 2), February 2009.

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[151] http://www.cabi.org/isc/?compid=5&dsid=18615&loadmodule=datasheet&page=481&site=144.
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[153] http://www.ipm.ucdavis.edu/PMG/r107304411.html

Integrated Pest Management for Home Gardeners and Landscape Professionals
http://ipm.ucanr.edu/PDF/PESTNOTES/pnasiancitruspsyllid.pdf

General Background Information

Diaphorina citri was recorded in Australia, in the Northern Territory in 1915, but it was eradicated by chance during the 1918-1922 eradication campaign for citrus canker (*Xanthomonas citri* subsp. *citri* (Bellis et al. 2005). In 1922 a decision was made to eradicate all citrus trees in NT north of latitude 19°S, which lies about 700 km south of Darwin, and to prohibit the replanting of citrus trees until 1925. The source of the ACP is believed to have been plants imported into the Darwin Botanic Gardens, probably from China or Japan.

If introduced again *D. citri* would pose a major threat to the viability of the Australian citrus industry, and native species of *Citrus* due to its potential to vector '*Ca.* Liberibacter' spp. particularly '*Ca.* Liberibacter asiaticus'. Analyses of mitogenome sequences revealed that ACP from California were related to those from Florida (Wu et al. 2017).

Distribution of *Diaphorina citri*

Diaphorina citri occurs in the following regions and countries: in Asia, the Arabian Peninsula (Saudi Arabia and Yemen), and from Afghanistan through the Indian Subcontinent, Southeast Asia and East Asia (the Ryukyu Archipelago and Kyushu in Japan, in China, particularly Taiwan and the coastal provinces of Guangxi, Guangdong, Fujian and Zhejiang), the Philippines and **through the Indonesian archipelago to north eastern Papua New Guinea; in Guam; Northern Mariana Islands[154], Samoa (Upolu and Savaii [2 big islands of Samoa)[155], American Samoa[156],** in the US[157] states of Alabama, Arizona, California[158], Florida, Georgia, Hawaii[159], Louisiana, Mississippi, South Carolina, and Texas, as well as the Caribbean, South America; in the Indian Ocean, the Mascarenes (Mauritius and Réunion)[160]. Recently *D. citri* was reported from Tanzania (Shimwela et al 2016) and Kenya (Rwomushana et al 2017[161]).

There is little genetic diversity among the populations worldwide (Boykin et al. 2012). Global phylogenetic analyses showed that *D. citri* populations in Iran, India, Saudi Arabia, Brazil, Mexico, Florida and Texas (USA) are similar (Lashkari et al 2013). Analyses of mitogenome sequences revealed that Asian Citrus psyllids from California were related to those from Florida (Wu et al 2017).

Description of *Diaphorina citri*

A key to the genera of Psyllidae is given by Yang (1984). Keys for the species of *Diaphorina* include Mathur (1975) for Indian species, Hollis (1987) for the '*amoena*' group and Burckhardt (1984) for Mediterranean species. The draft diagnostic protocols for the detection of the Asian citrus psyllid (*D.*

[154] Federal Domestic Quarantine Order Huang Long Bing (Citrus Greening) http://www.tnla.info/pdf_files/citrusgreening/citrusgreen2.pdf
[155] R. Davis (pers.comm.)
[156] Pestnet 2 November 2010
[157] Phylogeographic studies suggest that *D. citri* populations did not invade North America from South America (de Leon *et al.* 2011). The *Wolbachia* strain in the Florida *D. citri* isolate falls into a sub-clade of supergroup B, distinct from *Wolbachia* present in *D.citri* isolates supporting the hypothesis that the *D.citri* introduced into Florida did not originate from China (Saha et al 2012).
[158] It has taken 7 years for ACP to build up and spread to the Central Valley of California. In early 2016 there are 53000sq miles under quarantine in California.
[159] http://hawaii.gov/hdoa/pi/ppc/2006-annual-report/asian-citrus-psyllid
[160]Aubert 1987c, Halbert & Manjunath 2004, Halbert & Núñez 2004, Weinert et al. 2004, Villalobos et al. 2005, OEPP/EPPO 2005a, Conant et al. 2007, Poe 2007, Poe & Shea 2007, US Federal Domestic Quarantine Order: Citrus Greening Disease (CG) and Asian Citrus Psyllid (ACP), 16 December 2009[160], NAPPO Pest Alert, May 2010.

[161] Rwomushana I, Khamis FM, Grout TG, Mohamed SA, Sétamou M, Borgemeister C, Heya HM, Tanga CM, Nderitu PW, Seguni ZS, Materu CL. 2017. Detection of Diaphorina citri Kuwayama (Hemiptera: Liviidae) in Kenya and potential implication for the spread of Huanglongbing disease in East Africa. Biological Invasions.:1-1.

citri) and the African citrus psyllid (*T. erytreae*) (Malipatil & Semeraro 2007) must be peer reviewed and revised to provide a diagnostic key for Australian use.

The European and Mediterranean Plant Protection Organization diagnostic standard for *D. citri* (OEPP/EPPO 2005a) contains a description of the adults (including their genitalia), the egg and the nymphs of the psyllid based on Yang (1984). Detailed illustrations of all stages are contained within Husain & Nath (1927) and Yang (1984). Less detailed illustrations of all stages are based on Catling (1970). Wings and genitalia based on Yang (1984), are illustrated in OEPP/EPPO (2005a).

A positive identification is possible on adult females and advanced larval instars. Microscope-slide preparation of stages of *D. citri* is strongly advised to allow proper identification (OEPP/EPPO 2005).

D. citri can be readily separated from most other species reported on citrus and its relatives by a distinct pattern on the forewings (Halbert & Manjunath 2004): distinct bars on the top and bottom resulting in the insects having the appearance of a flattened X-pattern when viewed from the side. *D. citri* can be separated from many of the Australian Psylloidea, specifically the Psyllidae subfamilies Spondyliaspidinae and Triozidae, by its mottled wings, general 'squat' habitus and distinct wing venation. A few spondyliaspidines and some triozids have patterned or mottled wings, but most species are distinctly elongate and have characteristic venation, i.e., trifurcating venation in the Triozidae (Hollis 2004).

Some key points about *D. citri* life cycle:

- Husain & Nath (1927) reported total life-cycle lengths of 15-47 days, depending on season;
- annual generations depend on regional climates: 6-16 (Husain & Hath 1927; Atwal et al. 1970; Yang et al. 2006);
- *D. citri* reproduces sexually (Husain & Nath 1927, Mann et al 2011);
- females may produce up to 1900 eggs: unfertilised eggs do not hatch;
- females lay their eggs in the growing tips of young host plants, preferring flush growth < 6 mm in length to longer flush lengths: numbers of eggs laid on a flush decline rapidly as the length of flush increases, and when individual leaves attain lengths > 10 mm, and flushes 50 mm (Lin et al. 1973, Chen & Liao 1982, Leong 2006);
- numbers of eggs laid per leaf are greatest on the first leaf, and then progressively lower on the second and subsequent leaves (Xu et al. 1994);
- the psyllid has five nymphal instars with no diapause;

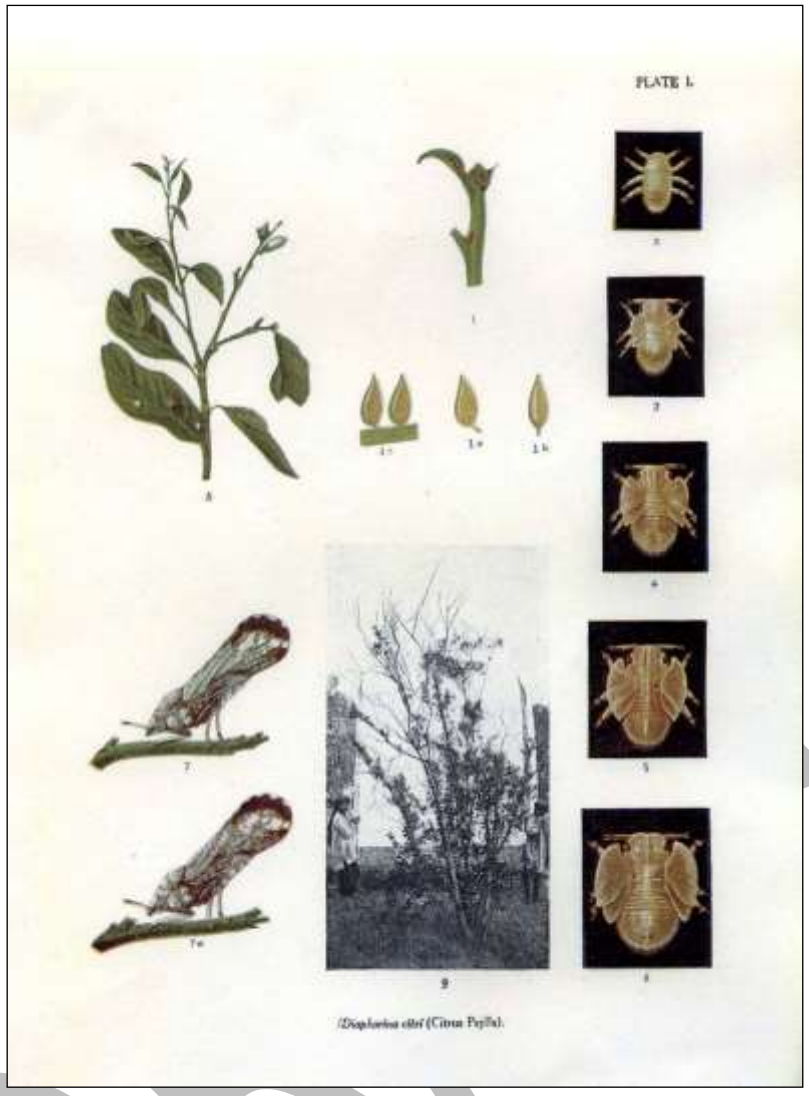
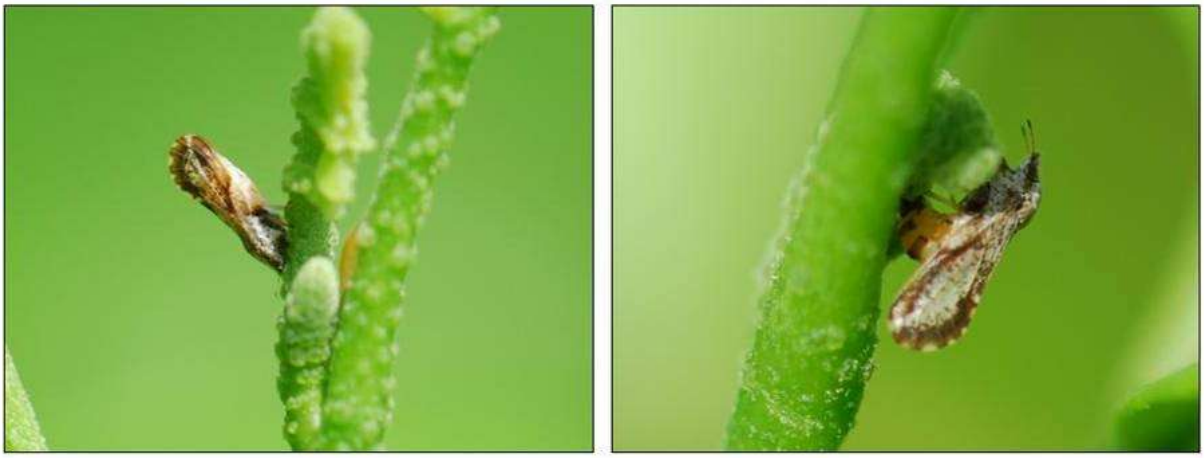


Plate I from Husain & Nath (1927), with illustrations of *D. citri* and its impact on citrus in the Punjab.



Adult *D. citri* (left) and ovipositing female: both on *M. paniculata* var. *exotica*, South China Agricultural University, Guangzhou (GAC Beattie).

Mating adult *D. citri* (left) and honeydew being produced by adult female: both on *M. paniculata* var. *exotica*, Guangdong Entomological Institute (Yang Yueping).

Teneral adult *D. citri* (light green), nymphs and copious honeydew on *M. exotica* sheltered from rain (left) and eggs on young citrus flush (right) (GAC Beattie).

D. citri nymphs and honeydew on *M. exotica*: Guangzhou, September 2009 (left), Kolkata, October 2009 (right) (GAC Beattie).

Hosts of *Diaphorina citri*

Known hosts of *D. citri* are listed in Appendix 3**.**

Recently the term host has also been applied by some authors to any plant on which immature or adults feed. Burckhardt et al (2014) has proposed a terminology to clarify associated plant definitions, and suggest restricting the use of the term *host-plant* to plants on which a psyllid species completes its immature to adult life cycle. For the other plant associations we suggest the terms *overwintering* or *shelter plant* (plants on which adult psyllids overwinter and on which they may feed), *food plant* (plants on which adult psyllids feed, but do not breed and do not spend an extended period of time) and *casual plant* (plants on which adult psyllids land but do not feed).

Germplasm in the following major groups of Rutaceae does not have any significant resistance to infestations of the Asian citrus psyllid: sweet oranges, citrons, pomelos, limes, lemons, sour oranges, papedas, mandarins and hybrids among these groups including grapefruit. However, within the trifoliate orange group, most (but not all) accessions of *Citrus* (*Poncirus) trifoliata* as well as a number of its hybrids were shown to have natural resistance[162] to ACP (Hall et al 2013[163], 2015; Richardson & Hall 2013, Hall et al 2017).

Besides *Murraya*, another common rutaceous hedge plant in Australia is *Choisya ternata* which along with *C. arizonica* were found to be feeding hosts for the Asian citrus psyllid. Egg laying and nymphal development were found on *C. ternata*[164] (Hu 2012, Sandoval 2009).

Seedlings of the native Australian *Citrus* (Eremocitrus*) glauca, Citrus (*Microcitrus*) inodora* and *C. australasica* and a Microcitrus hybrid were avoided by adults as food and resting hosts, but the *Microcitrus* hybrid was in the most susceptible egg group. *Citrus glauca* and *C. inodora* had a moderately high rank for nymphs and *C. australasica* had a moderately high rank for eggs (Westbrook et al. 2011).

The suitability of hosts is influenced by cultivar, ambient temperature, nutrition, soil moisture, light (within shaded forests, on the edge of forests, under cloud or in sunny conditions), plant density (in native vegetation or in relatively dense citrus monoculture), the nutritional value and the frequency of flush growth and local biotypes of the psyllid.

Young citrus trees that flush prolifically under ideal conditions are more suitable resources for the psyllid than older trees growing under identical conditions, and young and old trees grown under poor conditions. *Murraya* growing in a shaded forest will be a less suitable host than the same plants growing as an ornamental in a well-maintained garden (Aubert 1988a).

All known field hosts of *D. citri* belong to the family Rutaceae, most within the Aurantioideae. *Diaphorina citri* occasionally might colonise extraneous hosts. In thousands of samples of *D. citri* over the past 14 years in Florida Halbert (pers.comm.), has found only one or two credible samples (with nymphs) from outside the Rutaceae, so regulating Rutaceae is sufficient. Hitchhikers are another matter entirely. Clearly, *D. citri* adults can be found in many places, both on plants, and on inanimate objects.

[162]Two types of resistance have been identified. One of these resistance types (antixenosis) greatly reduces infestation levels of the psyllid, a resistance trait that may be related to differences in volatiles used by the psyllid to find and infest plants or the presence of a volatile that repels the psyllid. The other type of resistance (antibiosis) results in reduced longevity of psyllids, possibly related to the presence of toxic secondary plant metabolites.

[163]http://research.citrusrdf.org/reports/2013/07/10/Hall-315-final-report-June_2013.pdf

[164]http://research.citrusrdf.org/reports/2013/01/18/progress-report-final_Alternative_host_16.pdf

Important points about *Diaphorina citri* behaviour*:*

- ACP phenology is directly linked to citrus phenology, as the insect requires new growth tissue to complete its life cycle. In Florida two annual peaks of ACP adults were observed, one following the first major flushing event of the season and then a secondary peak of activity in late summer and sometimes prolonged through early fall (Hall *et al.* (2008), Stansly *et al.* (2014). The seasonal decline of flush in a typical flushing cycle was a major factor limiting ACP populations (Udell et al 2017).
- Flush shoots at emerging and developmental phases should be the focus of any chemical or biological control strategy to reduce the biotic potential of *D. citri*, to protect citrus tree from Liberibacter infection and to minimize HLB dissemination (Cifuentes-Arenas et al 2017).
- CLas transmission rates are increased when citrus flush[165] is present. In an experiment with seedlings of a rootstock cultivar 'US-942', a 1-wk infestation of 20 Asian citrus psyllids from an infected colony resulted in 53–60% of seedlings becoming infected when flush was present compared with only 7% when no flush was present. A similar experiment with 'Valencia' sweet orange resulted in 23, 80, and 3% seedlings becoming infected when young, older, or no flush was present, respectively. Young plants are therefore more likely to contract HLB if flush is present, with older flush promoting higher infection rates under the conditions of this study (Hall et al 2016).
- psyllid populations can increase rapidly, particularly in spring when the N content of usually abundant flush growth is high and when competition between females for flush growth is low (Atwal et al. 1970, Pande 1972, Koli et al. 1981, Khan et al. 1984, Huang et al. 1990, Hung 2008);
- a slight presence of flush (from no flush to 10% of the branches) during winter was sufficient to significantly increase the density of *D. citri* populations within citrus trees (Martini et al 2016).
- *D. citri* females only lay eggs on young shoots because nymphs cannot develop on mature citrus leaves (Hall and Albrigo 2007)
- Flush has been found to be a strong attractant for *D. citri* in the field (Lewis-Rosenblum et al. 2015).
- Aubert (1987a) suggested that *D. citri* exhibits a *k*-type of self dispersal and that its common behaviour is to jump when disturbed, and then fly towards neighbouring targets, particularly HLB-affected trees or yellow traps;
- Aubert (1987a) reported observations that suggested possible medium to long distance transport by strong winds, since upsurges of *D. citri* had been recorded in open orchards without windbreak protection after typhoons. Aubert (1987a) considered passive transport through 'advective winds' to be much more occasional;
- psyllid migrations appear to be highest when plants are flushing; psyllid populations are sedentary and feeding when foliage is mature (Aubert 1987c);
- *D. citri* dispersed more as barometric pressure increased, and less when barometric pressure decreased. Psyllids dispersal increased linearly with temperature. Changes in humidity did not affect dispersal of *D. citri*. Less than 1% of psyllids dispersed at 15 °C, compared with 7.7% at 21 °C and 27% at 25 °C (Martini & Stelinski 2017).
- host-specific psyllid densities under field conditions may be attributed to the frequency and availability of flush shoots rather than host preference (Setamou et al 2016).
- physical characteristics and nutritional composition of flush shoots and their phloem sap are important factors regulating host colonization and behavior of *D. citri*, and this interaction can impact the dynamics and spread of HLB (Setamou et al 2016)

[165] Flush is any new leaf growth ranging in development from first emergence up until the leaves are fully expanded yet still tender.

- *D. citri* exhibit strong phototaxis and tend to aggregate where light is most intense (Se´tamou et al. 2011)
- Diaphorina citri females only lay eggs on young shoots because nymphs cannot develop on mature citrus leaves (Hall and Albrigo 2007), which likely explains the greater movement by females (Martini & Stelinski 2017).
- the developmental host plant species influences adult host plant preference, with female psyllids preferring the species on which they were reared (Stockton et al. 2016). However, such preferences are subject to change with the introduction of an alternative host plant within 24–48 hrs. *D. citri* demonstrate host plant preference based on developmental and adult experience and can learn to recognize olfactory and visual host plant stimuli in ways that may be sex specific. These experience-based associations are likely used by adults to locate and select suitable host plants for feeding and reproduction and may suggest the need for more tailored lures and traps, which reflect region-specific cultivars or predominate Rutaceae in the area being monitored.
- tolerance of temperatures as high as 50°C in the field (Husain & Nath 1927); under laboratory conditions (that are not representative of field conditions) the optimum temperature for adult ACP survival is 25 °C and 20 °C while ACP adults seem to be able to tolerate low temperatures of 0 °C and 5 °C for several days and at warmer temperatures of 40 °C and 45 °C, one day of exposure was sufficient to reach 95% and 100% mortality respectively. (El-Shesheny et al. 2015). 25 °C was the most suitable for ACP population growth under laboratory conditions (Liu & Tsai 2000);
- *"Opportunist,* D.citri *is an extremely stedious insect surviving a wide range of temperatures extremes:from 45°C in Saudi Arabia to -7 or -8°C in subtropical China ,the latter minimum being actually lethal for several citrus. But more than temperatures, high relative humidity and rainfall are certainly serious limiting factors for the Asian citrus psyllid: rain by washing off eggs and young instar nymphs, humidity by favouring fungal epizotics. But endowed of a high rate of fertility and short life cycle at warm temperatures, the insect vector will .invariably, take advantage of any hot/dry season .to build up very rapidly.This fertility rate can be eventually counterbalanced by natural parasitism of insect enemies.However.appropriate manipulation of selected exotic enemies is more adapted to recent invaded oceanic islands,while continental situations with rich".Aubert 1988.*
- mild to moderate freeze events usually are non-lethal to adult ACP even if they are not cold acclimated (Hall et al. 2011), but a freeze that kills flush would be expected to result in mass mortality of young ACP (Hall et al. 2012; Hall et al. 2014). Tolerance of low ambient temperatures (e.g., mid winter average daily temperatures between 5.4°C to 6.9°C, with minimums from -5.3°C to -7.5°C (Xie et al. 1988); all three life stages can survive short periods of cold: relatively many adults and nymphs survive after being exposed for several hours to temperatures as low as −6 °C and relatively many eggs hatch after being exposed for several hours to temperatures as low as −8 °C (Hall et al. 2011);
- impacts of climate on adult longevity vary and adults can live for up 6 to 9 months (Husain & Nath 1927, Xie et al. 1989) in regions with cool to cold winters;
- the host range is mostly within the Aurantioideae with a preference for *M. paniculata*/*exotica* and *Citrus* (see Beattie & Barkley 2009, Halbert & Manjunath 2004); When stimulated with odor sources of 22 genotypes in a Y-tube olfactometer D. citri preferentially entered the arm containing the volatile oils of Murraya paniculata, confirming orange jasmine as its best host (Andrade et al 2016).
- host preference is influenced by season, variety, flush morphology, abundance, frequency and duration of flushing;

- oviposition preferences reflect the need for the five nymphal instars of the psyllid to complete their development on immature growth; most eggs are laid within 14 days of new growth commencing (Lin et al. 1973);
- breeding activity is largely suspended when citrus trees are dormant (Waterhouse 1998);
- flight is limited generally to a few metres where hosts are abundant, and possibly limited to 0.5-2 km when searching for a host; dispersal over 90 to 470 km is, or may be, possible in strong winds, such as those associated with cyclones (Aubert & Xia 1990, Sakamaki 2006, Gottwald et al. 2007, Halbert et al. 2008, Boina et al. 2009);
- adult ACP is active during daylight, but flight activity is pronounced during sunny afternoon hours (Sétamou et al., 2011). Flight activity regularly peaks in spring (Hall & Hentz, 2011); Asian citrus psyllid also regularly flies 60–100 m between pairs of managed and unmanaged groves, with net movement toward managed groves (Boina et al., 2009);
- Kobori et al (2011) suggest that once D. citri, carried by the wind, had arrived on the host plants,the psyllids hardly moved again;
- vibratory, substrate-borne signals are used by adult male and female ACP to communicate over 10-50-cm distances within their citrus tree hosts (Mankin et al. 2014);
- *D. citri* movement was greatest during the spring and summer months and decreased significantly during the colder months. *D. citri* were able to traverse potential geographic barriers such as roads and fallow fields. In the absence of severe weather events, *D. citri* were able to disperse at least 2 km within 12 d (Lewis-Rosenblum et al 2015);
- movement between unmanaged and managed orchards of marked *D. citri* adults has been reported for distances of 60 to 100 m over a 3 day period (Boina et al. 2009) and 400 m and 2000 m over 4 and 12 day periods, respectively (Lewis-Rosenblum 2011);
- *D. citri* is positively phototropic, and higher populations have been found along edges of orchards exposed to the sun (Anco & Gottwald 2015). Air movement, microclimates within the orchard, and nutrient content and relative health of citrus trees (Anco & Gottwald 2015) may also contribute to localized abundance of *D. citri*.
- From Setamou & Bartels (2015):

tribution. In both cultivars, significantly more psyllids were found on perimeter trees throughout the study period suggesting a strong edge effect in *D. citri* distribution in the groves. *D. citri* densities and infestation levels gradually declined from the edge to the center of grove. Higher numbers of *D. citri* were recorded on trees located on the east and south sides of the groves than those on the west and north sides. Citrus groves located at the outer edge of the study with at least one side non-surrounded to other citrus groves harbored significantly more *D. citri* than groves located within the block cluster and entirely surrounded by other groves. In detailed field studies during 2012, infestation of *D. citri* started from border trees in the grove where possibly one generation is completed before inner trees become infested. In addition, psyllid densities decreased significantly with increasing distance from the grove edge. Using the selection index, *D citri* exhibited a strong niche occupation preference for border trees.

- Probing and feeding behavior were monitored using the electrical penetration graph (EPG) technique, and pathogen acquisition efficiencies were tested by qPCR. The results showed that some EPG variables were significantly influenced by host-plant leaf maturity. The duration of waveform C (pathway phase) on new shoots was significantly longer than that on young leaves and mature leaves. In contrast, the duration of waveform E2 (phloem ingestion) was significantly shorter on new shoots and young leaves than on mature leaves. However, the duration taken for stylets of adult *D. citri* to reach the phloem and commence ingestion was not related to leaf

maturity status. The qPCR results indicated that 23 of the 24 adults for which E2 waveforms were recorded harbored '*C*Las'. In addition, the minimum period of E2 waveform of these individuals was only 2 min. Proportions of '*C*Las'-positive adults feeding on mature leaves, young leaves and new shoots, were 55%, 40% and 35%, respectively. The main EPG variables were not significantly different between the males and females. These results of Luo et al (2015) suggest that the acquisition of '*C*Las' by adult *D. citri* is highly efficient, even when feeding on mature leaves. Therefore to effectively manage both vector and pathogen, *D. citri* populations should be monitored carefully, even when the trees stop producing new growth.

- The finite rate of population increase and net reproductive rate were both greater among C. Las-infected *D. citri* as compared with uninfected counterparts, indicating that overall population fitness of infected psyllids was improved given the greater number of offspring produced. The survival of Las-infected adult *D. citri* was lower compared with uninfected *D. citri,* which suggests that there may be a fitness trade-off in response to Las infection. A beneficial effect of a plant pathogen on vector fitness may indicate that the pathogen developed a relationship with the insect before secondarily moving to plants (Pelz-Stelinski & Killiney 2016).
- Latitude and row orientation both had a significant effect on psyllid density during winter. *Diaphorina citri* abundance was higher when more than 20% of the surrounding landscape was urbanized (Pelz-Stelinski et al. 2016).
- The median latent period (LP50, i.e acquisition time after which 50% of the individuals can inoculate) of 16.8 and 17.8 days for psyllids that acquired Las as nymphs and adults, respectively, was determined by transferring single individuals in 48-h IAPs. Inoculation events were intermittent and randomly distributed over the IAPs, but were more frequent after acquisition by nymphs. A minimum latent period of 7-10 days was observed by transferring groups of 10 psyllids in 48-h IAPs, after a 96-h AAP by nymphs. Psyllids transmitted for up to 5 weeks, when submitted to sequential 1-week IAPs after a 14-day AAP as nymphs. The long latent period and persistence of transmission are indirect evidences of circulative propagation of Las in *D. citri* (Canale et al 2016).
- Acquisition efficiency of CLas by *D. citri* was highest in nymphs reared at 25 ◦C on a host plant with high CLas titers but was independent of the host genotypes assessed and of vector sex.We further observed that *D. citri* nymphs acquired CLas more rapidly than adults based on acquisition access periods (AAPs). CLas did not multiply in the alimentary canal, hemolymph, and salivary glands of adults for 18 days after a 3-day AAP as adult. However, CLas multiplication was detected in hemolymph and salivary gland of adults after the bacterium was acquired by nymphs. Eighty percent of salivary glands of adults contained CLas 18 days after a 3-day AAP as nymph compared to 10% 18 days after a 3-day AAP as adults (Wu et al 2018).
- Results of Ammar et al (2016) strongly suggest that Las multiplies in both nymphs and adults of *D. citri* but attains much higher levels in a shorter period of time post-acquisition when acquired by nymphs than when acquired by adults, and that adults may require longer access to infected plants compared to nymphs for Las to reach higher levels in the vector. However, under the conditions of their experiments, only *D. citri* that had access to infected plants as nymphs were able to inoculate Las into healthy citrus seedlings or excised leaves.
- Build-up of ACP in high density spatial aggregations (and therefore in HLB transmission risk) before dispersal has implications for increased local and regional spread of the disease (Udell et al 2017). Insecticide applications during the tree dormancy have been shown to effectively reduce ACP densities before the growing season begins (Qureshi & Stansly, 2010). Coordinated insecticide vector management (CHMA www.flchma.org) can help to decrease ACP metapoplations and eliminate hotspots with high infective potential (Bassanezi *et al.*, 2013). Efficient insecticide programmes for the growing season are also necessary, especially with active ingredients effective against immature stages and selective in favour of natural enemies (Qureshi *et al.*, 2014).

- Frequent application of insecticides is the primary management tool for *D. citri*. However, insecticide efficacy varies by time depending on the route of entry. Residual life of contact insecticides is short (Chen and Stelinski 2017), whereas soil-drench applied, systemic insecticides, such as neonicotinoids, require up to 2 wk of uptake by plants to become fully effective (Sétamou et al. 2010, Langdon and Rogers 2017).

Dispersal of psyllids

Distributions of *D. citri* (and HLB), when transmitted into initially HLB-free orchards, tend to be clumped around points of entry into orchards and then around subsequent points of dispersal, and influenced by movement of people and equipment within orchards, wind intensity and direction, and the proximity of adjacent plants (for hedgerows, spread tends to be greater within rows than between rows).

In 2008, *D*. *citri* first began expanding northward from Mexico into parts of Southern California. Since 2008, the cumulative abundance of *D*. *citri* in Southern California increased dramatically, particularly in the urban environment. After first appearing on residential citrus in San Diego County, *D*. *citri* positive sites were subsequently identified in parts of other southern California counties. Within these areas, the geographic focus of *D*. *citri* was primarily urban, especially in the first 5 years of the invasion, with the first detections in commercial citrus groves in 2011. For urban areas, Bayles et al (2017) found equivocal evidence of seasonality, with a general increase in the frequency of *D*. *citri* detections during the autumn months; likely attributable to onset of autumn citrus flush. Neither urban nor commercial citrus showed clear evidence of a spring peak in *D*. *citri* positive traps, a time of year that is typically associated with the largest peak in citrus flushing. Bayles et al (2017) found clear evidence that

1. the spatial and temporal distribution of *D*. *citri* in Southern California is non-random.
2. the existence of statistically significant hotspots of *D*. *citri* occurrence strongly associated with certain urbanized regions and suggestive of more frequent introduction events in these areas, perhaps due to the transportation of plant material, equipment and fruit via road networks. Urbanization may also be correlated with the amount of suitable habitat available for establishment and spread. Subsequent spread of *D*. *citri* was likely the result of natural dispersal of the psyllid throughout areas with a high density of residential citrus trees, coupled with some continuing longer distance movement via unregulated or illegal movement of plant material.

1. anisotropic dispersion across the Southern California landscape. The dynamic nature of *D*. *citri* geographic distribution and evidence of anisotropic spread suggests that hotspots are likely a function of not only anthropogenic factors (e.g., transportation associated with trade and commerce), but also differences in environmental suitability (e.g., resource availability, temperature, wind speed, elevation) that exist across urbanization gradients

Information in the section on ACP above suggests that the psyllid:

- is not a strong flyer and dispersal over considerable distance is wind assisted or occurs via the movement of infested plants;
- jumping/landing behaviour such as when disturbed is likely to be less 8 m;
- *D. citri* adults were able to traverse potential geographic barriers such as roads and fallow fields and disperse at least 2 km within 12 d. (Rosenblum et al. 2015);
- dispersal is prompted by high populations;
- may move at least several kilometres during normal air current assisted dispersal flights;
- may move far greater distances during high wind events;

- nymphs are not active walkers and it is not expected that they will contribute significantly to dispersal;
- distribution of eggs and nymphs in naturally occurring psyllid populations is highly aggregated, resulting from initially aggregated migration of adults and a contagious dispersion of them on flushes as the population density increases (Leong et al 2018).
- more psyllids are present on the exterior edges of individual orchard blocks associated with roads, canals, ponds compared with the interior of the same block.This is described as an 'edge effect' related to psyllid movement and migration (Gottwald & Irey 2008; Bartels et al. 2010[166], Luo et al. 2012, Stansly et al 2013);
- *D. citri* has been found capable of moving 100m in 3 days (Boina et al 2011), 400m in 4 days (Tiwari et al. 2010), 2 km in 12 days (Lewis-Rosenblum 2011);
- peak movement occurs at the end of the spring flush (Hall & Hentz 2011);
- adults are initially attracted to '*C*Las'-infected plants (Mann et al. 2012);
- citrus groves that are within 2 km of any other citrus plantings are at risk for *D. citri* infestation and HLB disease introduction from those areas (Rosenblum et al. 2015);
- 'CLas' appears to manipulate inclination for dispersal, flight capacity, and sexual attraction of *D. citri.* Ultimately, these effects increased the movement of those vectors that harbored the pathogen as compared with uninfected counterparts, which may promote spread of '*C*Las' by *D. citri* following pathogen acquisition (Martini et al. 2015).
- apparent absence of significant dispersal by *D. citri* during winter (Hall & Hentz 2011; Lewis-Rosenblum et al. 2015),

Surveillance for psyllids[167]
General:

Identification of volatiles of ACP (Coutinho-Abreu et al., 2014) for surveillance and management has not yet led to standardization of trap selection and optimization of trapping approaches. Populations of ACP continue to be monitored with standard yellow sticky cards, which are not ideally suited for recovery of ACP DNA for CLas detection, and which continue to be collected and replaced according to protocols that differ in different regions.

- how an organism disperses affects surveillance. Frequency of sampling and location and movement of traps are important;
- orchard maps and orchard ownership records must be up-to-date;
- surveys should use trapping and visual, GIS-based data capture and web-based data management;

[166] http://www.aphis.usda.gov/plant_health/plant_pest_info/citrus/downloads/symposium/Abstract-Bartels-a-pilot.pdf
[167] Multi-pest surveillance (MPS) method developed for Huanglongbing (HLB). ARS scientists in Fort Pierce, Florida, developed a multi-pest surveillance method for statewide sweeps for HLB, its vector and several other diseases including citrus black spot (CBS) and it has been very successful, is continuously adapted yearly to new disease priorities as requested by USDA APHIS, and is re-deployed yearly. California regulatory agencies have asked that we develop a MPS specifically for California. Risk-based residential and commercial survey methods for Asian Citrus Psyllid (ACP) and HLB are in the 4th year of deployment in California, Texas and Arizona, and validation indicates they are successful in detecting new HLB outbreaks. Surveys are being used by regulatory agencies (CDFA and APHIS) and commodity groups to target disease/vector hotspots for existing HLB and predict new outbreaks locations. Survey data and model risk predictions provide the empirical evidence on which management and regulatory decisions are being made. Multi-pest surveillance (MPS) method developed for Huanglongbing (HLB). ARS scientists in Fort Pierce, Florida, developed a multi-pest surveillance method for statewide sweeps for HLB, its vector and several other diseases including citrus black spot (CBS) and it has been very successful, is continuously adapted yearly to new disease priorities as requested by USDA APHIS, and is re-deployed yearly. California regulatory agencies have asked that we develop a MPS specifically for California. Risk-based residential and commercial survey methods for Asian Citrus Psyllid (ACP) and HLB are in the 4th year of deployment in California, Texas and Arizona, and validation indicates they are successful in detecting new HLB outbreaks. Surveys are being used by regulatory agencies (CDFA and APHIS) and commodity groups to target disease/vector hotspots for existing HLB and predict new outbreaks locations. Survey data and model risk predictions provide the empirical evidence on which management and regulatory decisions are being made. https://www.ars.usda.gov/research/programs-projects/project/?accnNo=423073&fy=2016

- Surveillance may be improved by following the leading edge of hotspots along a trajectory (Bayles et al 2017). Distribution of *D. citri* may ultimately be more useful for enhanced HLB risk prediction and less for psyllid eradication.
- a training program on identification of ACP should be developed for growers and consultants;
- trap placement in a non-quarantine area should be along highways and in commercial groves (focusing on the urban interface), packing houses and juice plants, and along every 1 km of orchard perimeter to cover the edge effect;
- in a quarantine area trap placement is every 0.5 km (or every 10 ha). NB Texas surveyed 5 km radius around infected tree and suspended fruit harvest during survey period;
- an effective public outreach program to create awareness and cooperation with a focus on homeowners and public officials in infested areas, retail nurseries and outlets such as Bunnings, gardening clubs, and traditional and social media.

Survey Precautions

- **Pesticide applications:** before starting a survey, always determine if there have been recent pesticide applications that would require additional safety precautions.
- **Private property:** obtain permission from the landowner before entering a property.
- **Sanitation:** other exotic pests and pathogens may be present. Most countries with ACP and '*Ca*. L. spp.' also have citrus canker. Wash bottles containing soap and disinfectant must be provided to disinfect hands and small articles. Care must also be taken with larger articles such as shoes, clothing and vehicles to be certain that these do not become a means for contamination or spreading pathogens (e.g., citrus canker) if present (see QDPI&F Work Instruction ST-W-006[168]).
- ACP is attracted to bright colors, such as yellow. Yellow is a common color for most safety vests and jackets, this creates an issue because most people that own one of these pieces of clothing are unaware that they can very well be unknowingly transporting this pest to different locations . Basic measures such as rolling up vehicle windows, shaking off clothing prior to getting in vehicle can help prevent the spread of ACP.
- **Suspend fruit harvest and any orchard activity in 5 km vicinity that involves movement of citrus material or machinery.**

Survey Methodology for Psyllids

Arevalo HA, Qureshi JA, Stansly PA. 2011. Sampling for Asian citrus psyllid in Florida groves. Field Sheet. SWFREC- University of Florida[169].

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Setamou, M., D. Flores, J. V. French, and D. G. Hall. 2008. Dispersion patterns and sampling plans for *Diaphorina citri* (Hemiptera: Psyllidae) in Citrus. J. Econ. Entomol. 101: 1478-1487.

[168] Telford G. 2007 Decontamination and Hygiene Requirements When Conducting a Survey for Citrus Canker. QDPI&F Work Instruction ST-W-006.

[169] http://edis.ifas.ufl.edu/in867.

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Psyllid Area Wide Control Program Technical Working Group Recommendations[171]. 2009.

CDFA Action Plan for Asian Citrus Psyllid and Huanglongbing (Citrus Greening) in California. August 2016. https://www.cdfa.ca.gov/citruscommittee/docs/ACP-ActionPlan-Rev-8-17-16-web.pdf; . https://extension.arizona.edu/sites/extension.arizona.edu/files/resources/acp-hlb-california-situation-citrus.pdf

Setamou, M.; and Bartels, D. W. 2015. Living on the edges: spatial niche occupation of Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), in citrus groves. PLOS ONE 10 p.e0131917.

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UC Pest Management Guidelines[172] Citrus *Diaphorina citri.* Asian Citrus Psyllid: Provisional Treatment Guidelines for Citrus in Quarantine Areas

USDA/APHIS **Technical Working Group Report National Surveillance Strategies for Asian Citrus Psyllid, (*Diaphorina citri*) and Huanglongbing (associated with *Candidatus* Liberibacter spp.)** https://www.aphis.usda.gov/plant_health/plant_pest_info/citrus_greening/downloads/pdf_files/twg/TWG-NationalSurvey.pdf

http://ucanr.edu/sites/ACP/Grower_Options/Grower_Management/Monitoring_15/

An effective trapping system is paramount for quarantine programs which rely heavily on detection of pests at very low population levels to successfully eradicate targeted pests such as ACP with insecticides.

Survey methodologies for ACP can comprise:

- visual inspections for adults on mature leaves, particularly on the underside of leaves in between flush cycles, particularly in regions with distinct winters;
- visual inspections for eggs and nymphs on flush growth from 5 mm to 50 mm long, particularly in spring, within 14 days of buds opening;
- visual inspections of young flush growth (5 mm to 50 mm long) for honeydew, particularly in spring, within 14 days of buds opening;
- tapping (beating) foliage, particularly young flush growth, to dislodge adults into, for example,a 300 mm-diameter white pan containing a shallow film of mineral oil, a shallow 20 cm 20 cm white pan, or a piece of A4 paper on a clipboard (see Hall et al. 2007, Qureshi 2009, Qureshi et al. 2009);

[170] http://www.aphis.usda.gov/plant_health/plant_pest_info/citrus_greening/downloads/pdf_files/twg/TWG-NationalSurvey.pdf

[171] http://www.aphis.usda.gov/plant_health/plant_pest_info/citrus_greening/downloads/pdf_files/twg/Psyllid%20Area%20Wide%20Control2.09.09.pdf

[172] http://www.ipm.ucdavis.edu/PMG/r107304411.html

- use of portable air suction machines (e.g., D-Vac or Dietrick-vacuum insect collectors: http://www.rinconvitova.com/d-vac.htm) to suck psyllid adults from within canopies; and
- use of yellow sticky traps to trap flying adults (see Samways 1987, 1990, Aubert & Xia 1990, Hall 2009; Hall et al. 2007, 2010; Hall & Hentz 2010; Setamou et al. 2014).
- Flush has been found to be a strong attractant for *D. citri* in the field (Lewis-Rosenblum et al. 2015).
- Monzo et al. (2015) determined that vacuum sampling was the most effective sampling method for *D. citri* compared with tap sampling or visual examination, but it was also the most time consuming.
- Martini et al (2016) found no significant pattern in the distribution of *D. citri* adults with respect to canopy height but found significantly more psyllids located on canopy with southern exposure than northern exposure in Florida.
- use of lime green traps baited with blends of plant volatile and pheromone compounds[173] (known as Alpha Scent® traps[174])[175] (Czokaljo et al. 2015).
- vector surveys have been routinely conducted to maintain 'disease-free' and 'disease- and vector-free' areas in Japan. To improve methods that can detect *D. citri* in native insect populations, Fujiwara et al (2016) developed a method of using conventional and real-time PCR to detect *D. citri* among bulk insects captured in sticky traps without the need for preliminary differentiation steps based on morphology. They used a D. citri -specific molecular marker (Boykin et al. 2012) to differentiate D. citri from other psyllid species, important citrus pests, and native insect populations present in areas of Japan

Each method has limitations governed by:

- the extent of an infestation and distributions of psyllids on hosts within the vicinity of the incursion (e.g., contagious (aggregated) distributions of infestations within urban areas or within an orchard where psyllids may be present on some trees but not on others);
- availability of personnel to undertake surveys, their experience, training and knowledge;
- visual sampling was efficient for detecting and monitoring ACP at low densities. Suction sampling was time consuming and taxing but the most sensitive of all methods for detection of sparse populations (Monzo et al. 2015);
- visual color-based traps are only attractive to ACP for a short distance;
- the usefulness of sticky traps in urban situations and orchards is limited by the distances *D. citri* can fly, and on their need to disperse from a host plant. Their use in nurseries (open or screened) and small urban areas (e.g., in the coastal regions of northern Australia) would be appropriate if inspected every 7 days;
- in Texas and California, green-sticky traps were more efficient than yellow-sticky traps to attract adult psyllids (Sétamou et al. 2008, Czokaljo et al. 2015);
- yellow traps caught significantly more *D. citri* adults than the other four traps; red and green traps caught significantly more *D. citri* than blue and white traps, which were not significantly different (Setamou et al 2014);
- traps must be placed against an open background (Aubert & Xia 1990);

[173] ACP exhibits strong preference for citrus volatiles and aggregate and lays eggs exclusively on young unexpanded leaves. Thus, plant-related chemicals are crucial signals used by adults for plant selection. In addition, there is evidence documenting that mate location in ACP is mediated by a volatile sex pheromone and hydrocarbons emitted from the cuticle or outer surface of the insect (Czokaljo et al. 2015). Visual cues are important in the host finding behavior of adult *D. citri* (Setamou et al. 2014).

[174] http://www.alphascents.com/traps/traps.html

[175] Czolajlo et al. (2015) stated that monitoring ACP with Alpha Scents' ACP trap and lure is six to eight times more effective than the currently used un-baited yellow traps

- trap catches were significantly influenced by the citrus species; traps placed on lemon trees[176] captured more *D. citri* than those placed on sweet orange and grapefruit, suggesting that plant preference exhibited by *D. citri* may influence the performance of traps (Setamou et al. 2014); thinner canopies may have also contributed;
- captures of adults by yellow sticky card (panel) traps were not reduced by rain and usually only to a minor extent by wind (Hall 2009);
- increasing the number of yellow traps per tree from one to three improved detection of adults in trees when adult population levels were low (Hall 2009);
- yellow sticky traps deployed in citrus trees are inconsistent as indicators of absolute densities (Hall 2009);
- Halbert & Manjunath (2004) cite research that yellow sticky traps worked best on sunny days whereas 'brown yellow' traps worked best on cloudy days;
- the optimum height for capture is 1.5 m (Aubert & Xia 1990);
- YP traps can be useful for detection in commercial situations, for ongoing monitoring programs or to assist in delimiting incursions (Samways 1984, Aubert & Quilici 1988, Aubert & Xia 1990);
- the ability to test psyllids from yellow traps for HLB can be impaired due to degradation of decaying specimens over time;
- YP traps were less effective at detecting ACP during periods of flush growth as the psyllids were more attracted to young flush;
- the benefits of using portable air suction machines machines appears to be outweighed by the cost, maintenance, hygiene requirements to prevent spread of other pests and diseases and the time required to sort samples (Thomas 2012);
- sticky trap deployment, collection and reading took 14 times longer than the tap sample: an average of 7 min per trap (hanging, collecting and reading) compared with 30 s per tap sample (Qureshi et al. (2009)[177]. The sticky traps caught about 10 times more psyllids but this was over a fortnight whereas tapping provided immediate information on psyllid numbers;
- both tap and sweep net seem to be equally effective for psyllid counts and time at both high and low densities. However, the sweep net was reported by Qureshi et al. (2009)[178] to be more work, ACP counts were more difficult at high densities and there was increased risk of spreading citrus canker; and
- usefulness of air suction machines will be limited by the number of trees to be sampled and would not be practical in urban situations, nurseries, or orchards unless used to sample large numbers of trees collectively to determine the presence of adult psyllids in a defined area.

Based on experiences in California and Florida, and early warning surveillance experience in Queensland, the following table illustrates the strengths and weaknesses of each method for detecting *D. citri*.

Comparison of methods used to survey for Asiatic citrus psyllid (ACP) (courtesy of Ceri Pearce, Department of Employment, Economic Development & Innovation**).**

Method	How?	Cost/Time	Advantages	Disadvantages
Visual	Visual examination of foliage, particularly young flush of citrus or other host plants to	Labour & magnifying glass / lens. Visual inspection takes more time than	Can detect all life cycle stages of ACP. Recognised as most accurate method of detection. Can target	Surveillance staff must be competent.

[176] More open tree?
[177] http://www.imok.ufl.edu/entlab/pdf/grant/Sampling.pdf
[178] http://www.imok.ufl.edu/entlab/pdf/grant/Sampling.pdf

Method	How?	Cost/Time	Advantages	Disadvantages
	detect ACP eggs, nymphs or adults. Requires training & magnifying glass / lens.	setting a trap or sweeping a net.	young citrus flush where ACP is more likely to be found. Normal surveillance hygiene to manage the risk of disease spread on a property. HLB testing of ACP detections possible.	
Sweep net	Operator uses a fine net in a sweeping action over host, targeting young flush. Net contents are examined carefully. Requires a sweep net, spare nets, trained operators & magnifying glass / lens.	About $25 for net. More rapid than visual assessment alone. Added time required to change nets on poles.	Suitable for detecting adults on young flush of citrus or other hosts (e.g., orange jasmine) where ACP is more likely to be found. Permits sampling above head height. Proven effective method of insect capture: routinely used by QLD surveillance staff and entomologists. HLB testing of ACP detections possible.	Surveillance staff must be competently trained. Net can be torn by thorns or broken branches. Net difficult to disinfest, change & wash between properties. Net may become contaminated by contact spread of pathogens (e.g., viroids, canker) or pests (mites). Sweeping may damage tree foliage & dislodge fruit.
Tap method	Operator places a clipboard under a branch and taps the branch firmly 3 times. Surface of clipboard is examined. Requires a clear or white clipboard, tapping stick / PVC pipe, solution to trap insects on the board (spray soap or oil solution), a trained operator & magnifying glass / lens.	About $10 for equipment.	Principally detects adults. Simple, rapid and reliable way to monitor ACP. Equipment is easily cleaned between trees / properties. Potentially quantifiable. HLB testing of ACP detections possible.	Surveillance staff must be competently trained. Sample area targeted is small and only within personal reach. Some insects may jump away if not counted or capture quickly. Tapping may damage foliage and dislodge fruit.
Suction machine	Operator uses machine (D-Vac or similar machine) to 'suck' insects from foliage. Requires a trained operator & magnifying glass / lens.	$1000-$2000 for suction machine. Time required to sort ACP from other insects and debris may reduce efficiency.	Useful for collecting ACP adults. Can target young citrus flush where ACP is more likely to be found. Permits sampling above head height. If samples are sorted within 2 days or preserved in ethanol then HLB testing of psyllids is feasible.	Relatively long sorting times. Potentially heavy & cumbersome. Backup batteries / battery charging required. Cost of machinery maintenance. Possible contamination of machine by plant pathogens. Machine may damage foliage & dislodge fruit.
Yellow sticky trap	Polybutene covered traps (cylindrical or flat) with grid lines are hung near or within host plant, usually 1.5 m above ground height. Requires trained operator & magnifying glass / lens.	About $1 each. Trap deployment, collection & reading may take 14 times longer than tap sampling[179].	Useful for remote monitoring for intervals up to 14 days. Also useful for ongoing grid trapping programs or supplementary surveillance. Can capture other high risk pests or be used to monitor beneficial insects.	Capture is often accidental, particularly in the presence of attractive host flush. Traps catches can be revolting to screen (maggots, decomposing geckos, frogs). If ACP adults are capture, samples may be too old to test for HLB unless removed within 1-2 days of capture.

[179] In California, California Department of Food & Agriculture surveillance staff can set/clear up to 45 traps/day using paper based data capture systems. Citrus Research Board staff can set / clear up to 85 traps / day in commercial orchards using PDA technology and bar-coded tree / trap IDs for data capture.

See also Appendix 4 of USDA Technical Working Group Report National Surveillance Strategies for Asian Citrus Psyllid, (*Diaphorina citri*) and Huanglongbing (associated with '*Candidatus* Liberibacter spp.') 2010[180]. Appendix 6 of the same document has the following recommendations:

Commercial and abandoned orchards: use sticky traps around orchards combined with visual inspections.
Residential: use traps and visual inspections[181]
Nursery: Use perimeter sticky traps, visual inspections and traps within screenhouses
Packing houses: use sticky traps as a monitoring tool.
All psyllids should be tested for HLB-associated liberibacters.

[180] http://www.aphis.usda.gov/plant_health/plant_pest_info/citrus_greening/downloads/pdf_files/twg/TWG-NationalSurvey.pdf
[181] US uses travel and census data to determine high risk areas, trap intensity and density.

Appendix 7. Matrix of methods for Asian citrus psyllid survey

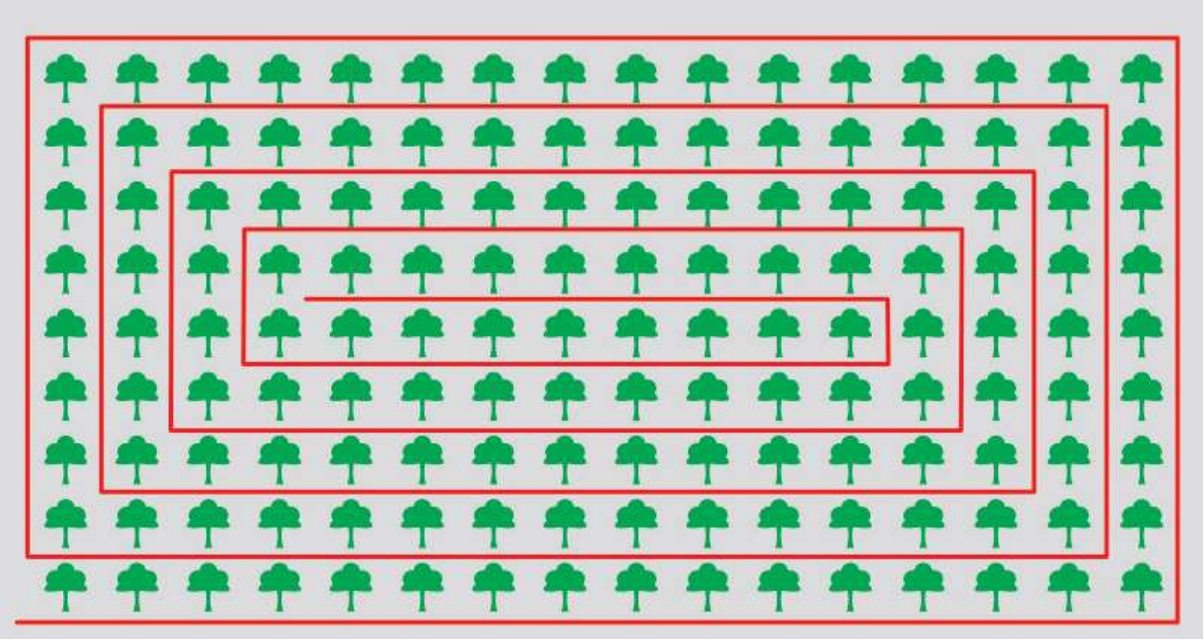
ACP Survey Matrix									
Survey Method			Visual	Sticky Trap	Stem-tap Test	Sweep Heavy	Sweep Light	Dvac	Suction
Class I - Detection									
	Commercial		Y	Y	N	N	N	N	N
		Quarantine	Y	Y	N	N	N	N	N
		Non-quarantine	Y	Y	N	N	N	N	N
	Residential		Y	Y	N	N	N	N	N
	Nursery		Y	Y	N	N	N	?	N
	Packing House		N	Y	N	N	N	N	N
Class II - Delimitation									
	Commercial		Y	N	Y	Y	Y	N	N
	Residential		Y	N	Y	Y	Y	N	N
	Nursery		Y	N	Y	N	Y	?	N
	Packing House		Y	Y	N	N	N	Y	N
	Abandoned Groves		Y	Y	N	N	N	N	N
Class III - Monitoring									
	Commercial		Y	N	Y	Y	Y	N	?
	Residential		Y	N	Y	Y	Y	N	N
	Nursery		Y	N	Y	N	N	N	N
	Packing House		N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Abandoned Groves		Y	N	Y	N	N	N	N

Appendix 7. Matrix of methods for Asian citrus psyllid survey

ACP Survey Matrix								
Survey Method		Visual	Sticky Trap	Stem-tap Test	Sweep Heavy	Sweep Light	Dvac	Suction
Life stage	Egg	Y	N	N	N	N	N	N
	Nymph	Y	N	N	N	N	N	N
	Adult	Y	Y	Y	Y	Y	Y	Y
Season	Spring	Y	Y					
	Summer	Y	Y					
	Winter	Y	Y					
	Fall	Y	Y					
Tree age	Young	Y	?	N	N	Y	Y	Y
	Mature	Y	Y	Y	Y	Y	Y	Y
Tree phenology								
Risk of *Xanthomonas citri* pv. *citri*		?	N	Y	Y	Y	Y	N
Ability to test for HLB-associated Liberibacter		Y	Poor	Y	Y	Y	Y	Y
Population density		Y	Poor	Y	Y	Y	?	Y
Cost	Manpower	$$$$	$$	$	$	$	$$$	$$$$
	Equipment	$	$	$	$	$	$$$	$$$$
	Supplies	$	$$$	$	$	$	$$	$

In Brazil (see Fundecitrus Guide to Monitoring for the psyllid *Diaphorina citri*), numbers of psyllids have been found to be larger at the edges of orchards.

Visual inspections should be done weekly on new shoots and mature leaves. It is necessary to survey 1% of the plants, evaluating three to five new branches, looking for the presence of eggs, nymphs and adults, and in the underside of the leaves in search of adults, especially in border trees. The inspection should be done in spiral form, beginning with the edges of the field and finishing in the centre:



Traps should be placed in the upper third of the canopy, at the end of the branch and facing out of the field, so that they are clearly visible to the insect. Installation should preferably be done on the plants at the edge of the field and at the property's edge.

Trapping sites and trap density

In California trapping is carried out as follows[182]:

Yellow panel traps have proven successful at detecting infestations of ACP. At all locations where traps are placed, the host plant is visually inspected for ACP. If there is evidence that ACP exists, the host will be visually surveyed for ACP samples.

- Trap Density: Five to 16 traps/square mile.
- Trap Servicing Interval: Every two to four weeks.
- Trap Relocation and Replacement: Traps should be replaced and relocated every four to eight weeks to another host at least 500 feet away if other hosts are available.
- Visual surveys and tap sampling are conducted once at each trapping site when the trap is placed or relocated at that site.

Transect Survey

If high or scattered ACP populations are found in the initial inspections, a transect survey may be implemented to rapidly determine the extent of the infestation. This involves inspecting a minimum of 20 properties per square mile and/or placing 20 traps per square mile along eight radii in the cardinal directions (e.g., north, northeast, etc.). Transect surveys extend between five and 20 miles beyond a detection site, depending on the situation.

Commercial Grove Trapping

In counties with substantial commercial citrus production, traps are placed within the groves at the density of one trap per 40 acres. Traps are replaced every month and submitted for screening.

In generally infested areas (currently Orange, western Riverside, San Bernardino, Ventura and San Diego counties), the traps are removed and replaced with HLB survey and ACP collection for HLB testing.

Delimitation Trapping and Visual Survey Outside of the Generally Infested Area

The protocols below are the actions in response to the detection(s) of one or more Asian citrus psyllids (ACP) in the area outside of the generally infested area. The detection of a single ACP remains a quarantine trigger.

Response to a single ACP

Trapping

Density will be 100 traps per square mile in a 1.5 mile radius, to form a nine square mile delimitation area. Traps will be serviced weekly for two months. After that, the traps will be serviced monthly for two years past the identification date. Additional detections may increase

[182] http://citrusinsider.org/wp-content/uploads/2016/08/CDFA-Handout-Final.pdf
CDFA Action Plan for Asian Citrus Psyllid and Huanglongbing (Citrus Greening) in California. August 2016. https://www.cdfa.ca.gov/citruscommittee/docs/ACP-ActionPlan-Rev-8-17-16-web.pdf

the size of the delimitation survey area and will restart the two-year clock on the trap servicing requirement.

Visual Survey

All find sites and adjacent properties will be visually surveyed for ACP and HLB. Additional sites may be surveyed as part of the risk-based survey.

Response to 2 or more ACP within 1.5 miles of each other, detected within 6 months of each other, or 1 or more ACP within 1.5 miles of commercial citrus

Trapping

Same as for one ACP, centered on each detection site.

Visual Survey

All properties within 400 meters will be visually surveyed for ACP and HLB.

The following is from 2010 Technical Working Group Report on National Surveillance Strategies for Asian Citrus psyllid (*Diaphorina citri*) and Huanglongbing (associated with *Candidatus* Liberibacter spp.):

a human-mediated incursion (see supplement). If available, local knowledge and expert opinion will help to fine-tune the pest risk estimates. If grown in a high risk area, residential citrus host trees should have frequent visual inspections for ACP eggs, nymphs, and adults when the tree supports new flushes of growth (< two weeks developed). At least one sticky trap should be maintained in the upper canopy of trees from spring through fall to promote detection of ACP adults. Sticky traps do not attract psyllids as well when trees are flushing, so visual detection during that time period will augment survey data. Establishing 'sentinel' plots, or trees, that can be forced to flush at non-synchronous times will function to attract insects to a limited, and more manageable amount of plant tissues for examination. Regularly timed visual examinations of *Murraya* sp. should also be made. If ACPs are identified, plant tissues, as well as psyllids, should be tested for HLB-associated *Candidatus* Liberibacter spp. It is recognized that most of the initial incursions of HLB and ACP have been in residential areas. Residential citrus probably poses the highest risk for establishment of this pest complex.

Surveys for ACP in commercial citrus groves should be systematic, utilizing at least one trap per multi-block in 100% of the commercial blocks. Traps should be placed from spring through fall and serviced (replaced) every 3-5 days during the survey period. The TWG recognizes that servicing one trap per block every 3-5 days may not be practical due to resource limitations. The intent of this recommendation is to maximize the probability of early detection of ACP establishment and HLB occurrence. Trapped insects degrade quickly in warm weather which reduces the likelihood that Liberibacter spp. can be detected from insect samples that are more than three days old. Too few sampling units could result in lost opportunities for control resulting in establishment of ACP and HLB. Sentinel plots should be established where trees can be forced to flush non-synchronously with commercial groves. Visual inspections of flushing materials enhance ACP detection since all insect growth stages can be identified. Foliar material with symptoms similar to those of HLB should be tested. Trapped ACPs should be tested individually. Small commercial groves of specialty fruit, managed by people from high risk parts of the world (e.g. Asia, Florida, etc.) should be targeted initially for these surveys in areas where the pest complex is not established already.

Residential or commercial citrus hosts located in areas with lower risk of human-mediated disease spread can be monitored for ACP less frequently during flush cycles compared with the locations mentioned above.

California surveys for Asiatic citrus psyllid

Pre-incursion:

In California, prior to 2005, surveys for ACP and HLB (and other exotics) were primarily conducted by California Department of Food and Agriculture (CDFA) in commercial citrus groves with 25% of the total commercial acres in each county surveyed annually in spring or fall. Every 5 or 10 rows and the edges of the blocks were inspected. As a result of the detection of HLB in Florida that year, additional efforts were made to look for these pests in nurseries and high risk urban residential surveys.

Post-incursion:

In May 2009 the Californian Asian Citrus Psyllid Technical Working Group reported that '*when ACP is found in California and within ½ mile of the site, detection relies on visual surveys, vacuum trapping and increased yellow panel sticky trap trapping, combined with insecticide treatments within 400 meters of the detection. The trapping density is increased to 100 traps/mi^2 in the ½ mile radius around a find site, and a trapping density of 50 traps/mi^2 is placed out to a radius of 1.5 miles. In the current non-commercial areas, a trap is placed within each host plant or in at least one plant in a group planting, within ½ mile of the detection after an ACP find. In commercial citrus, the normal trapping density is 5 traps/square mile*'.

In October 2009, following detection of the Asiatic citrus psyllid in the Los Angeles area (backyards, non commercial groves or nurseries) of California, Dr Mary Polek[183] reported that the following general survey methodologies were adopted:

- the California Citrus Research Board (CRB) surveyed commercial citrus using standard yellow sticky traps and placed them every 400 m along grove perimeters within the quarantine area and every 800 m in non-quarantined areas;
- trap density in young or newly planted groves was higher, about every 200 m, and additional traps were placed within groves;
- traps were monitored and serviced every two weeks;
- trees with sticky traps were labelled with specific CRB bar codes and photographed so that positive detections could be precisely recorded and located;
- maps showing citrus species, varieties, and rootstocks, along with when leaf flushing, were prepared; and
- trees were visually inspected, especially during flushing.

Godfrey & Kosta (2009) reported that from June 2008 (after *D. citri* was found in Tijuana) survey protocols were based on use of:

- 1-5 traps per 2.6 km^2, depending on housing densities, for surveys in urban and residential areas,
- 1 trap per 16 ha in rural areas (crop land); and
- and 2-5 traps per 4000 m^2 in nurseries.

From August 2008, after *D. citri* was found in southern California the following protocol was adopted:

- for urban and residential areas, 100 traps in the core area and 50 traps in the buffer zone;
- for rural areas, 2-3 traps per 4000 m^2; and
- for nurseries, 5-10 traps per 4000 m^2 (Godfrey & Kosta 2009).

In 2015 in California https://extension.arizona.edu/sites/extension.arizona.edu/files/resources/acp-hlb-california-situation-citrus.pdf

:

[183] International Organisation of Citrus Virologists (IOCV) Newsletter, October 2009.

- in urban areas of California, sticky traps are placed on trees within nine miles of the initial find and are saturated throughout the region as inspectors canvas neighborhoods looking for citrus plants.
- CDFA are conducting what's called a "high risk" survey for the Asiatic citrus psyllid which sweeps every square mile in Los Angeles County twice per year.

The Asian Citrus Psyllid Residential Program was updated (http://citrusinsider.org/2017/03/asian-citrus-psyllid-residential-program-updated/ March 31, 2017) to help focus trapping and pest detection efforts in areas most at risk. The trapping density was reduced in response to Asian citrus psyllid detections in Northern California counties (excluding Kern, Kings, Tulare, Fresno and Madera counties). Specifically, the program moved from placing 100 traps per square mile in the 9-square mile Asian citrus psyllid detection core, to 25 traps per square mile.

Additional changes to the program included raising the residential treatment threshold that accompanies area-wide management treatments in nearby citrus groves. CDFA will treat residential properties located within 400 meters around commercial properties that are participating in an area-wide management program if 90 percent of commercial citrus in the treatment area participate. This is up from 75 percent, making it imperative for growers to work together when treating for the psyllid.

Florida growers have said that their biggest error was not addressing ACP control when the pest was first discovered in Florida in 1998. By the time disease symptoms showed up on trees, the state was infested. Therefore, a major component of the CPDPP is a residential detection and treatment program implemented by the California Department of Food and Agriculture (CDFA). Psyllid control in California has been our number one defense since the pest was discovered here.
Traps are deployed throughout the state, and the public is encouraged to report potential psyllid detections through a free state hotline, which is promoted via the CPDPP outreach program. When the psyllid was first discovered in California and pest populations were minimal, the CPDPP handled every pest detection with aggressive treatments. As populations of the pest increase in the state, the program has diversified its efforts to also include the rearing and release of biological control agents, and has shifted its treatmentstrategy to areas most at risk for the disease or near commercial citrus operations. To maximize the impact of area-wide ACP treatments in orchard areas, the CDFA will treat residential properties located within 400 meters around commercial properties that are participating in an area-wide management program. This only happens, however, if 90 percent of commercial citrus in the treatment area is participating. It is imperative that growers work together to receive a true area-wide effect. (Citrograph Summer 2017).

To protect surrounding commercial citrus groves, the Kern County Pest Control District is offering to remove residential citrus trees for free due to detections of the Asian citrus psyllid in the Arvin/Mettler area. Along with free tree removal, participating residents can receive up to $50 cash per tree as a thank you from local citrus farmers (http://citrusinsider.org/2017/03/residential-tree-removal-offered-in-kern-county/).

In **Brazil** (February 2016, Beattie observations): "Bad neighbours" within 5-8 km who don't control psyllids or eradicate symptomatic trees are the major concern. Consequently due to the "edge effect[184]" good growers attempt to monitor psyllids coming in from neighbours by employing yellow sticky traps at 1 trap every 150m checked weekly, along the orchard perimeter. Windbreaks have little effect in reducing the ingress of citrus psyllids into an orchard.

Quarantine Area

- Delimiting surveys will determine the size of the pest quarantine area to help determine if eradication is feasible. A buffer zone or restricted area around affected sites should be established until the extent of the incursion has been determined. The extent of surveillance activities and the size of quarantine zones will vary depending on many factors eg climate, topography, size and degree of isolation of locations (e.g., towns, cities and orchards) where citrus and/or alternative hosts (e.g., *Murraya,* desert lime, finger limes) of the disease and its vectors occur, and regions where native hosts occur naturally; information on flight activity, particularly potential flight distance and prevailing winds, may be needed to establish the size of a quarantine area.

At January 2016, California's total quarantine area for ACP was 53,087 square miles — nearly one-third of the state's entire land mass[185] requiring that fruit moved from those areas be free of leaves and stems and restricting movement of any nursery stock that isn't grown in a USDA-approved facility. Some growers and officials have attributed the spread of the psyllids to "hitchhikers" on vehicles, crates, equipment or fruit that were brought in from areas where the pest was already established[186]. A proposal[187] for mitigating ACP movement on bulk citrus is as follows:

- All bulk citrus must be run through a wet wash process, this can occur in a field run wash line or in a packinghouse.
- Mandatory tarping of **all** loads no matter where it's coming from or where it's headed.

In 2017 a statewide regulation was put in place that restricts the movement of regulated articles from "or within" a quarantine area. Under the new regulation, all bulk citrus loads must be safeguarded regardless of the origin or the destination. This can be done in several ways, including but not limited to the use of a shipping container, tarp, enclosed vehicle, including curtain van, or another method that completely covers bulk citrus during transport. If using a tarp, tarps must reach the bed of the truck[188].

In March 16, 2018, information for Citrus Growers/Grove Managers in an Asian Citrus Psyllid (ACP) Bulk Citrus Regional Quarantine Zone or Huanglongbing (HLB) Quarantine Area was implemented – see
http://phpps.cdfa.ca.gov/PE/InteriorExclusion/pdf/acpgrowerinformation.pdf

[184] more psyllids are present on the exterior edges of individual orchard blocks associated with roads, canals, ponds compared with the interior of the same block.This is described as an 'edge effect' related to psyllid movement and migration (Gottwald & Irey 2008; Bartels et al. 2010[184], Luo et al. 2012, Stansly et al 2013).

[185] http://www.capitalpress.com/California/20160115/californias-asian-citrus-psyllid-quarantine-keeps-wideninghttp://www.capitalpress.com/California/20160115/californias-asian-citrus-psyllid-quarantine-keeps-widening [185] http://uccemg.com/files/226843.pdf

[186] http://californiaagtoday.com/acp-found-near-juice-plant/

[187] http://citrusinsider.org/2016/01/acp-regional-quarantine-concept/;

[188] http://citrusinsider.org/2017/03/new-bulk-citrus-compliance-agreements-mailed/ http://phpps.cdfa.ca.gov/PE/InteriorExclusion/pdf/acpgrowerinformation.pdf

A list of products and use rates recommended by University of California, Integrated Pest Management Program (UC IPM), and agreed upon by CDFA, are provided in https://citrusinsider.org/wp-content/uploads/2018/03/PEA-10-2018-Citrus-Grove-Pre-Harvest-Treatment-Products.pdf

In November 2018 **ACP-Free and HLB-Pest Risk Mitigated Bulk Citrus Movement from an ACP Bulk Citrus Regional Quarantine Zone or an HLB Quarantine Area was changed again by CDFA (https://citrusinsider.org/wp-content/uploads/2018/11/acpgrowerinformation.pdf):**

Shipping to a Packer/Processor	
Within the Same ACP Bulk Citrus Regional Quarantine Zone or into Regional Quarantine Zone 6	**In a Different ACP Bulk Citrus Regional Quarantine Zone***
Transport Completely Tarped or in a Fully Enclosed Vehicle	Field Clean **OR** Treatment Option **AND** Transport Completely Tarped or in a Fully Enclosed Vehicle **AND** Complete ACP-Free Declaration Form

Shipping to a Packer/Processor	
Within the Same Contiguous HLB Quarantine Area	**Outside of HLB Quarantine Area OR Different HLB Quarantine Area**
Field Clean **OR** Spray & Harvest **AND** Transport Completely Tarped or in a Fully Enclosed Vehicle **AND** Complete HLB Pest Risk Mitigation Form	Wet Wash **OR** Field Clean and Spray & Harvest **AND** Transport Completely Tarped or in a Fully Enclosed Vehicle **AND** Complete HLB Pest Risk Mitigation Form

The following are methods that CDFA agrees would ensure the pest risk has been mitigated in bulk citrus shipments from an ACP Bulk Citrus Regional Quarantine Zone or an HLB quarantine area:

1. Cleaned free of all stems and leaves.

2. All fruit must be transported to the packer/processor in a fully enclosed vehicle or completely covered by a solid or mesh tarp.

In California, treatments in response to Asian citrus psyllid detections are[189]:

- For a single ACP detection not within 1.5 miles of commercial citrus, the find site and adjacent properties are surveyed and treated.
- If the ACP find is within 1.5 miles of commercial citrus, or if multiple ACP are detected within six months of each other, the survey and treatment area expands to 400 meters.
- Homeowners can opt-out of CDFA treatments, however CDFA and the county agricultural commissioner will work with the homeowner to try to get permission to treat.

In California[190] treatment of citrus plants takes place within 400 or 800 meters of the initial find, depending on the region of the state. Cyfluthrin, a contact insecticide of the pyrethrin family, is sprayed directly onto the foliage of host plants. The program used in California also applies imidacloprid, to kill immature psyllids, to the soil beneath the plants[191]. The CDFA 2014 Asian Citrus Psyllid and Huanglongbing Science Advisory Panel Report:

1) What is the appropriate size of treatment areas around ACP find sites in eradication zones under a variety of scenarios?
   a) An urban area where no HLB has been detected – **Treating all urban ACP hosts within 400 m as is currently done seems appropriate as well as any commercial citrus grove that falls within 400 m.**
   b) An urban area where HLB has been detected – **Treat 800 m for urban ACP hosts as well as any commercial citrus groves that falls within 800 m. In addition, background checks should be done to try and determine why HLB was likely present. All infected trees should be removed rapidly and trees in the area should be tested for CLas using the best detection methods available at that time, especially during the spring and fall when titers are highest. Because transovariole transmission of CLas within ACP occurs at low rates, detection of CLas in ACP nymphs from an urban tree is proof that the tree is infected with CLas (i.e. it then falls under category 1b).**
   c) A commercial grove where no HLB has been detected – **Treat that grove and any grove or urban ACP hosts that fall within 400 m.**
   d) A commercial grove where HLB has been detected – **Treat that grove and any grove or urban ACP host that falls within 800 m. In addition, background checks should be done to try and determine why HLB was likely present. All infected trees should be removed rapidly and trees in the area should be tested for CLas using the best detection methods available at that time, especially during the spring and fall when titers are highest. Once the cumulative number of infected trees in that grove has reached 2%, all trees in the grove should be removed. Because transovariole transmission of CLas within ACP occurs at low rates, detection of CLas in ACP nymphs from a commercial tree is proof that the tree is infected with CLas (i.e. it then falls under category 1d).**

- A quarantine zone encompasses a five-mile radius of the discovery. Traps are placed out to a radius of nine miles.
- The quarantines prohibit the movement of citrus (including kaffir lime leaves) and curry leaf, *Citrus, Murraya* and *Choisya* nursery stock, including all plant parts except fruit, out of the quarantine area and requires that all citrus fruit be cleaned of leaves and stems prior to moving out of the quarantine area. An exception may be made for nursery stock and

[189] http://citrusinsider.org/wp-content/uploads/2016/08/CDFA-Handout-Final.pdf

[190] http://www.proag.com/News/Asian-Citrus-Psyllid-Pest-Expands-California-Base-2015-11-02/3984
[191] http://www.independent.com/news/2016/jan/18/psyllid-poison-meeting-announced-tuesday/

budwood grown in USDA-approved structures which are designed to keep ACP and other insects out. Residents with backyard citrus trees in the quarantine area are required not to transport or send citrus fruit or leaves, potted citrus trees, or curry leaves from the quarantine area.

- Regulations (https://www.cdfa.ca.gov/plant/acp/docs/mtgs/CitrusCommodityScopingMtg.pdf) regarding ACP in California in August 2016 were:

- **Texas A & M has been looking at erecting net-like borders around ACP-free orchards because the psyllid tends to go to the edges and treating the netting with yellow strips of sticky paper that would attract and/or apply insecticides to kill the psyllid**.
- A fruit harvest protocol for trees being harvested within the five-mile radius quarantine with trees treated with a pyrethroid one or two days before harvesting for a quick knockdown of any psyllid populations. For orchards in an ACP quarantine area, consideration must be given to in-zone packers to clean and re-bin the fruit before trucking to the packinghouse, or manually or mechanically removing the debris in the field[192]. Fruit bins are loaded onto lorries, covered with tarpaulins and delivered to a packing shed where fruit are washed immediately with high pressure water. a fogging system that utilizes Evergreen[R] to kill ACP in loaded bins in the field before they move to the packinghouse is envisioned to be an alternative to wet washing[193].
- ACP adults have been observed in truck shipments of unprocessed fruit (Halbert et al. 2010; http://californiaagtoday.com/acp-found-near-juice-plant/) and adults may survive on harvested fruit for 10–13 days (Hall & McCollum 2011). In California most of the ACP finds are occurring along the Highway 99 corridor indicating it isn't a natural migration but rather the psyllids are "hitchhikers," aboard truck and cars heading north from southern California. A proposal[194] for mitigating ACP movement on bulk citrus is as follows: 1. All bulk citrus must

[192] The U.S. Department of Agriculture's Animal and Plant Health Inspection Service established a permanent, 5-mile quarantine around a Texas, citrus grove that tested positive for HLB in 2012. The rule, effective Sept. 1, regulates the movement of host plants within or out of the quarantine area (Aug. 9 *Federal Register* notice). The old rule required that leaves and stems be removed from all fruit originating from within the quarantine zone before it could be moved to outside packinghouses or processors. But the requirement was more difficult than it sounds, increasing harvest costs by 50% to 100%. The new leaf-removal requirement only applies to infected groves. Otherwise, the new rule requires packinghouses that receive fruit from the quarantine zone to dispose of leaves and stems in prescribed ways. All groves within the quarantine zone must now be treated with an insecticide a few days before harvest. http://www.thepacker.com/fruit-vegetable-news/Citrus-greening-triggers-Texas-quarantine-167059085.html

[193] http://citrusindustry.net/2017/07/24/field-fruit-fogging-psyllid-control/

[194] http://citrusinsider.org/2016/01/acp-regional-quarantine-concept/

be run through a wet wash process, this can occur in a field run wash line or in a packinghouse. 2. Mandatory tarping of **all** loads leaving one region to final pack in another region.

- It's no coincidence that ACP finds in California have been occurring along major thoroughfares and at juice plants. Almost daily, bulk citrus loads are moving uncovered into the Central Valley from southern California. Packinghouses are sending dirty bins back into the groves. Equipment is being hauled up and down the highway loaded with plant material[195]. Trapping results from residential areas, packinghouses and juice plants in California indicate transportation corridors are major pathways for ACP dispersal (Gautam et al 2018). So requirements for the movement of bulk citrus are necessary[196]. Gautam et al (2018) developed a bin-fogging treatment to disinfest fruit before it leaves the orchard to minimise vehicular transport of ACP. High pressure fogging with 300 gallons of an aqueous mixture containing 0.1% Evergreen* (6% pyrethrins and 60% piperonyl butoxide) and 0.5% (v/v) BreakThru (polysiloxane surfactant) controlled adult ACP infesting a 48 bin truckload of fresh citrus (Gautam et al 2018).

Currently (May 2016) in California[197]the regulations for commodities are:

- Request for full county quarantine must come from County Agricultural Commissioner.
- Citrus commodities are prohibited movement from an ACP quarantine area except under permit.
- Citrus commodity shipments must be free of stems and leaves or moved under "spray and move" option to packinghouse outside of an ACP quarantine area. Asian citrus Psyllids continue to spread north with an increase in findings, often times just off of Highway 99, possibly due to leaf trash off transported fruit even with the spray-and-move option. There is an extra cost to have fruit cleared for shipment and southern California growers who send their crop out of county for packing see that cost doubled. There is the less expensive spray-and-move option for growers but there can be problems with that method as resistance may develop to the same pesticide being used every seven day period during picking[198].

- In Argentina, all fruit from the HLB infected area of Missiones should be packed. As a solution for small producers, the province is acquiring mobile packing plants and funding fixed plants at strategic points[199].
- Cleaning green waste from bins and equipment, including sprayers, tractors and hedging equipment. Procedures are needed for preventing or eliminating the movement of ACP on citrus products and equipment being moved out of a quarantine area.
- In California and Texas when ACP and/or HLB are found, the minimum quarantine radius around a detection site is currently 5 miles (8km)[200]. NB 32.2 km (20 miles) was considered earlier.
- Packinghouses inside the quarantine area will also have to double-bag and dispose of field trash from citrus inside the eradication zone at a designated landfill.

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195 http://westernfarmpress.com/orchard-crops/compliance-failures-could-kill-california-citrus-industry

196 **http://citrusinsider.org/2017/03/new-bulk-citrus-compliance-agreements-mailed/.**
http://phpps.cdfa.ca.gov/PE/InteriorExclusion/pdf/acpgrowerinformation.pdf
https://www.cdfa.ca.gov/plant/pe/InteriorExclusion/grower-packer-hauler-information.html
https://citrusinsider.org/quarantines-restrictions/
https://citrusinsider.org/wp-content/uploads/2018/02/PEA-06-2018-Movement-of-Citrus-Fruit-From-an-HLB-Quarantine-Area.pdf

197 https://www.cdfa.ca.gov/plant/acp/docs/mtgs/CitrusCommodityScopingMtg.pdf

198 http://agnetwest.com/2016/06/09/psyllid-quarantines/

199 http://www.freshplaza.com/article/154210/Argentina-Misiones-adapts-to-avoid-the-HLB

200 https://www.aphis.usda.gov/plant_health/plant_pest_info/citrus_greening/downloads/pdf_files/cg-acp-faqs.pdf

- Fruit that is commercially cleaned, graded, and packed within the quarantine area may move within or from the area (https://citrusinsider.org/wp-content/uploads/2018/02/PEA-06-2018-Movement-of-Citrus-Fruit-From-an-HLB-Quarantine-Area.pdf)[202].
- Trade of *Citrus*, *Murraya*, *Bergera* and *Clausena* nursery stock, budwood and fresh leaves (e.g., leaves of *C. hystrix* and *B. koenigii*[203]) from quarantined areas will need to be prohibited until a protocol can be put in place eg Interstate movement of citrus and other rutaceous nursery stock is prohibited from areas in US quarantined for HLB and ACP (April 2011) unless moved in accordance with a protocol given at http://www.aphis.usda.gov/plant_health/plant_pest_info/citrus/downloads/interstate-mvmnet-protocol.pdf). See also http://phpps.cdfa.ca.gov/PE/InteriorExclusion/pdf/acptreatments.pdf for approved treatment protocol for intra quarantine and interstate movement of regulated nursery stock in California.
- In California all host plants offered for sale at retail establishments within the quarantine area are required to be treated with a systemic and a foliar insecticide every 90 days the plant is for sale. A blue tag with treatment information is placed on each plant and information is posted at the retail nursery explaining that the blue tagged plants cannot be moved out of the quarantine area. Maps are posted with the information. Nurseries are inspected to ensure compliance. Non-compliance results in 'stop-sale' of host plants and/or plant treatment or destruction at nursery expense.
- In Jan 2018, citrus trees in a nursery within the 5 mile quarantine zone from a HLB infected tree were destroyed by CDFA[204][205].
- Any fruit or plants brought into farmers or flea markets within a quarantine area, cannot be taken back to *a* farm *or nursery* outside the quarantine area. This is because of the risk of psyllids hitchhiking a ride on non-hosts.
- All venues where host plants may be exchanged (purchased or otherwise traded) are trapped with yellow panel traps if held at a permanent location, and/or inspected on a regular basis for compliance.
- In a quarantine area in California trap placement is every 0.4 km (¼ mile) (i.e., every 14.2 ha or 35 acres) (http://www.fritolayag.com/public/HLB/MaryLou_Polek_Nov_18_09.pdf).
- Shared responsibility between regulators and industry occurs in California with compliance agreements allowing a packer/processor/grower/transporter to "*self-execute requirements to facilitate the movement of ACP host articles and commodities from ACP quarantine areas*" if they agree to abide by certain rules and regulations. For ACP compliance standards in California see https://www.cdfa.ca.gov/plant/pe/InteriorExclusion/acp_free_compliance.html.

[201] California Department of Food and Agriculture Pest Exclusion Advisory No. 23-2009. Bulk Citrus Shipments from Asian Citrus Psyllid Quarantine Areas

[202] California Department of Food and Agriculture Pest Exclusion Advisory No. 23-2009. Bulk Citrus Shipments from Asian Citrus Psyllid Quarantine Areas https://citrusinsider.org/wp-content/uploads/2018/02/PEA-06-2018-Movement-of-Citrus-Fruit-From-an-HLB-Quarantine-Area.pdf

[203] see: State of California Department of Food and Agriculture, Pest Advisory Advisory 21-2007 - http://www.co.san-diego.ca.us/reusable_components/images/awm/Docs/ipd_asian_citrus_psyllid.pdf.

[204] https://www.dailybulletin.com/2018/01/19/more-than-4000-citrus-trees-destroyed-while-disagreement-between-bloomington-nurseries-state-continues/
https://www.cdfa.ca.gov/plant/acp/nurseries.html

[205] **Section 3639, Huanglongbing Disease Eradication Area:**
This emergency action established HLB as the targeted pest, the entire State as the eradication area, the hosts, means and methods to eradicate or control HLB, and that any HLB host nursery stock which is regulated under the HLB Interior Quarantine, and which does not meet the requirements of its restrictions, cannot be maintained free from HLB and is declared a public nuisance under FAC section 5762 and subject to the provisions of FAC section 5763. https://www.cdfa.ca.gov/plant/regs_hlb_archive.html

Regional Treatment and Area-wide Management

Texas Citrus Greening and Asian Citrus Psyllid Action Plan. A Grower Sponsored Plan to Combat a Serious Disease of Citrus in Collaboration with State, Federal and International Agencies. Texas Citrus Greening Task Force 2009.

California Action Plan https://www.cdfa.ca.gov/citruscommittee/docs/ActionPlan.pdf

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http://ucanr.edu/sites/ACP/Grower_Options/Grower_Management/Monitoring_15/

NAPPO Discussion Document DD05. Management of Huanglongbing and Its Vecor the Asian Citrus Psyllid, *Diaphorina citri.* . http://www.nappo.org/files/9314/4865/6656/DD_05_HLB__Vector_AWM_15-10-2015-e_.pdf

Flores-Sánchez JL, Mora-Aguilera G, Loeza-Kuk E, López-Arroyo JI, Gutiérrez-Espinosa MA, Velázquez-Monreal JJ, Domínguez-Monge S, Bassanezi RB, Acevedo-Sánchez G, Robles-García P. 2017. Diffusion Model for Describing the Regional Spread of Huanglongbing from First-Reported Outbreaks and Basing an Area Wide Disease Management Strategy. Plant Disease 27:PDIS-04.

Singerman A, Lence SH, Useche P. 2017. Is area-wide management useful? The case of citrus greening. Applied Economic Perspectives and Policy, https://doi.org/10.1093/aepp/ppx030

Area-wide management of ACP/HLB is a strategy to reduce ACP populations and lower the risk of HLB in a sustainable manner. AWM should include all settings where citrus is grown and also target riparian habitats where native host plants are present (Setamou et al. 2016). Singerman et al (2017) reviewed the benefits of AWM for HLB control in Florida.

The size and location of area-wide management of *D. citri* can be based on empirical assumptions (Bove 2012), restricted spatial data obtained at plot level (Gottwald et al 2014), operational criteria (Rogers et al 2011) or flexible diffusion modeling and probable risk scenarios (Flores-Sanchez et al 2017).

Citrus health management areas (CHMAs) have been implemented throughout Florida (www.chma.org) initially to provide regional coordination for insecticide sprays to control ACP following a National Research Council (2010) recommendation. The objective is to remove sources of re-infestation as well as subsequent refuge for insecticide tolerant individuals (Jones et al. 2013). CHMAs[206] are groupings of commercial citrus groves in close proximity where growers work cooperatively to manage the spread of HLB. Participants in a CHMA coordinate psyllid control sprays to provide long-lasting effective psyllid control to minimize movement of psyllids between groves and reduce the time needed before additional sprays are required. Grower participation in a CHMA

[206] http://www.crec.ifas.ufl.edu/extension/chmas/chma_overview.shtml

is voluntary; however, growers should encourage their neighboring growers to participate as the level of overall success of the program is dependent on participation of all groves in the area.

Citrus Health Management Areas (CHMAs) were established in Florida with the goal of regional participation in insecticide applications for ACP control. These have been only somewhat effective due to incomplete grower participation in some areas (Singerman et al., 2017). A CHMA-like approach has been effective in Brazil, but a component of their effectiveness has been continuous removal of infected trees and complete eradication of abandoned groves and alternative hosts (Belasque et al., 2010).

The Texas Citrus Greening and Asian Citrus Psyllid Action Plan (2009) provides the following information:

- The timing of the treatment before the insects begin their spring reproductive cycle has a dramatic impact on the overwintering populations. These overwintering adults are responsible for the initial colonization of newly produced flush shoots in spring. In areas where HLB is present, a high proportion of these overwintering adults carry '*C*Las' (Chen et al. 2009). Growers should apply a dormant season insecticide prior to the spring flush cycle. In case orchard management strategies such as irrigation and/or hedging that stimulate flush growth are implemented in winter, a dormant spray application should follow within two to three weeks to protect the subsequent flush shoots.
- A robust monitoring program following this initial treatment is critical in following the impact on vector populations to ascertain the timing of additional treatments when necessary.
- Backyard trees or ornamental plants that serve as hosts for an insect vector and the disease it can transmit are always a challenge to area wide pest management programs. A very extensive educational program is essential in providing the necessary information to the general public regarding the impacts of backyard plantings left unattended and not included in the management of the pest.
- Public lands must also be incorporated in the AWM program
- Organically grown citrus groves will factor into an area-wide treatment program. Fortunately there are compounds with insecticidal properties for organically grown citrus that can be used in an area-wide strategy. These strategies involve not only ground applications but include aerial applications. Fortunately, ACP adults and nymphs are typically found on the new flush on the outside of the canopy of the tree making them susceptible to lower volume aerial applications. The benefit of an aerial application of an insecticide is the ability to cover large areas in a minimal amount of time.
- A local committee comprised of growers, state and federal personnel with experience in ACP survey, control and outreach efforts will be formed to develop a detailed plan for the area-wide ACP control program.

Area-wide ACP control is achieved with effective communication and coordination of treatments among local citrus growers and grove managers. ACP can be successfully controlled with coordinated treatments because the insect population will have fewer individuals left from which to reestablish.
Specific recommendations (https://www.cdfa.ca.gov/citruscommittee/docs/ACP-ActionPlan-Rev-8-17-16-web.pdf; https://www.cdfa.ca.gov/citruscommittee/docs/ActionPlan.pdf) include:
 Treat as much citrus acreage as possible during each spray cycle to maximize coverage and prevent the establishment of pest refuge areas.
 Coordinated area-wide treatment applications should be completed within a two to three week time frame.

 Mode of Action (MOA) use should be coordinated and rotated within management areas to prevent development of insect resistance.
 Dormant season applications are most critical overall in maintaining ACP population reductions.
 Application methods (aerial, ground) should be tailored to fit each management area by considering geographical or environmental influences as well as unique location characteristics such as residential, organic production, or critical habitat interfaces.
 Management areas should be as large as possible, taking advantage of any natural geographic separations and existing cooperative efforts among producers.
 Management practices which promote flushing should ideally be coordinated within a management unit.
 Scouting emphasis for ACP detection should be placed on grove block perimeters. Scouting method(s) (sticky trap, visual, stem tap) should be tailored to the specific area and circumstance.
 Organic growers within a management area should utilize the most efficacious product available during the coordinated treatment window.
 Extension, outreach and communication groups should be engaged to assist with education, communication, and public awareness in citrus growing states.

In California CDFA treats residential properties 400 meters around commercial properties. Residential properties are only treated if 75 percent of commercial citrus is treated as part of an area-wide management program. • There are two applications per year.

The CDFA (2018) requirements[207] for mitigating ACP movement on bulk citrus is as follows:

Growers in an HLB quarantine sending their fruit for packing outside of the quarantine area or in a non-contiguous HLB quarantine area will be required to meet either option (1) or both options (2) and (3) below to achieve the pest risk mitigation performance standard:
1. The fruit must be run through a wet wash which includes thoroughly wetting the fruit by spraying/dunking/drenching with water and brushing/cleaning.

If the wet wash option is not chosen, both of the following mitigations must be used:
2. The grove must be treated with a CDFA agreed upon pre-harvest product effective against psyllids within 14 days of harvest.

3. Fruit must be field cleaned and be practically free from all stems, leaves, and other extraneous host material prior to leaving the origin grove for packing/processing. In October 2018 CDFA clarified the requirement that any field cleaning of citrus fruit must be done with a field cleaning machine. Cleaning citrus fruit by hand is not one of the approved mitigation measures. The alternative to the "field cleaning by machine," is "spray and harvest," or "wet wash" methods (https://citrusinsider.org/2018/11/clarification-on-field-cleaning-requirements-for-movement-of-bulk-citrus/).

Packers/processors located outside of the HLB quarantine area that receive citrus fruit grown in an HLB quarantine area shall give priority to such shipments for receiving, unloading, and cleaning.

The requirements for growers located within an HLB quarantine area sending citrus fruit for final packing within the same contiguous quarantine area have not changed. These growers only need to comply with option (2) or (3) listed above.

207 https://citrusinsider.org/wp-content/uploads/2018/02/PEA-06-2018-Movement-of-Citrus-Fruit-From-an-HLB-Quarantine-Area.pdf

Mandatory tarping of **all** loads leaving one region to final pack in another region.

Farm workers to keep vehicle windows and doors shut in the groves and to clean bags at the beginning and the end of the day.

Starting Feb. 1, 2016, some 25,000 acres of citrus crops in the Central Valley of California will be sprayed for the Asian citrus psyllid[208],[209]. For growers who oppose the use of chemicals on their crops, other treatments e.g. pyrethrum will be made available. Local packinghouses will not be allowed to handle citrus from untreated orchards.
Beth Grafton –Cardwell in a submission to CDFA (http://citrusinsider.org/wp-content/uploads/2016/08/ACPScopingResponsetoComments.pdf) wrote:

9. It is critical to shift from spraying orchards with insecticides before harvest to washing fruit within a quarantine region before shipping to other regions. Data shows that insecticide treatments do not kill all psyllids. Under current regulations, a sprayed orchard can be harvested with fruit and leafy material into bins and shipped to other regions of the state. This practice has resulted in psyllids being found at packinghouses and juice plants – an indication that it is ineffective in preventing psyllid spread. Wet washing will remove psyllids from fruit and eliminate leaves and stems that the psyllids could be infesting. This practice will greatly lower the risk of moving psyllids that could harbor HLB in their bodies.

demonstrates that psyllids are being transported. Recent research by my post doc in Riverside is documenting that insecticide treatments do not kill all the psyllids in an orchard. Therefore, allowing orchards to be treated and shipping the fruit without washing is allowing psyllids to move.

fruit and provide that information to the regulatory agencies. It is clear that simply spraying fruit with water is not sufficient, because psyllids can survive in areas between fruit that are not reached by the spray. But fruit dunk tanks may be a very good option.

Movement Of Citrus Nursery Stock From Areas Quarantined For Asian Citrus Psyllid

USDA & APHIS September 2017. INTERSTATE MOVEMENT OF CITRUS NURSERY STOCK FROM AREAS QUARANTINED FOR CITRUS CANKER, CITRUS GREENING, AND/OR ASIAN CITRUS PSYLLID ttps://www.aphis.usda.gov/plant_health/plant_pest_info/citrus/downloads/Citrus-Nursery-Stock-Protocol.pdf

III. Requirements For Interstate Movement From Asian Citrus Psyllid (ACP) Quarantined Areas To All U.S. States:

A. Structure. Citrus nursery stock must be grown in an approved structure that meets the requirements of Section I. D. of this protocol.

B. Visual Inspection, Trapping, and Detection.

1. Visual Inspection: Plants in the approved structure(s) must be visually inspected by inspectors for the presence of ACP using methods approved by APHIS and stipulated in the compliance agreement. The interval between inspections must not exceed 30 calendar days.

2. Inspection methods may include, but not be limited to: a. Yellow sticky panels

[208] http://www.vcstar.com/news/local/oxnard/county-program-laid-out-to-combat-citrus-disease-2955b83b-4ca8-022f-e053-0100007fae03-365443051.html

[209] At January 2016, California's total quarantine area for ACP was 53,087 square miles — nearly one-third of the state's entire land mass. http://www.capitalpress.com/California/20160115/californias-asian-citrus-psyllid-quarantine-keeps-wideninghttp://www.capitalpress.com/California/20160115/californias-asian-citrus-psyllid-quarantine-keeps-widening

b. Vacuum suction of plants
c. Tapping of plants
d. Other methods approved by APHIS

3. If ACP is detected in an approved structure: a. APHIS must be notified immediately of the findings; and
b. No plants are eligible for interstate movement until an official identification is completed.

4. If ACP is confirmed in any approved structure: a. All plants from the affected structure(s) are ineligible for interstate movement; and
b. Affected structure(s) and contents must undergo APHIS approved and verified mitigation measures before plants eligible for interstate movement can be placed in the structure(s); and
c. APHIS will complete a risk assessment as needed.

C. Treatment For Shipment
1. All citrus nursery stock must be treated with an APHIS-approved systemic insecticide (soil drench) at least 30 days but no more than 3 months (90 days) before shipment. This must be followed by an APHIS-approved foliar spray no more than 10 days before shipment. Treatment must be with an APHIS- and EPA-approved product labeled for use in nurseries. Persons applying treatments must follow the product label, its applicable directions, and all restrictions and precautions, including statements pertaining to Worker Protection Standards.
2. Treatments must be verified by APHIS or State personnel.
D. Eligibility for Shipment. Citrus nursery stock for interstate movement must be subjected to at least 3 consecutive 30 day negative inspection cycles.
First inspection (1), then 30 day inspection (2) then 30 day inspection (3) for 60 days total.

Best Management Practices for Nursery and Garden Centres[210]:
See Minimizing the impact of Asian citrus psyllid in Nurseries
http://ucanr.edu/sites/ucipmretaildocs/files/232553.pdf

- Citrus trees should be treated with insecticides when they leave wholesale nurseries. However, these treatments remain effective for only about three months. Ensure fast movement and turn around of citrus stock before trees become unprotected.
- Excessive watering can leach out soil-applied insecticides, so only water enough to wet the soil in pots.
- If possible, place trees inside a screened-in structure to protect them against psyllids. If not an option, take advantage of the psyllids' preference for sunny, warm conditions by keeping citrus and other hosts under shade structures or even inside the store.
- As you are caring for and handling citrus stock, carefully check the leaves and stems for psyllids.
- Be sure your garden centre or nursery sells or buys disease-free trees from a reputable source.

Morse et al (2016) http://citrusinsider.org/wp-content/uploads/2016/08/ACPScopingResponsetoComments.pdf wrote:

[210] http://uccemg.com/files/226843.pdf;

3. We support the concept of treating with the systemic pesticide near the time of shipment to retail nurseries and a 150 day limit on the period of time post-treatment that potted citrus can reside in a retail nursery, at which time it must be retreated or disposed of. These measures will help ensure that the retail nurseries do not become a source of psyllids or the CLas bacterium which causes HLB.

4. A problem with the current regulations is that they specify the maximum label rate of the systemic pesticide be used rather than specifying a specific effective use rate. With imidacloprid, this is a significant problem because many labels do not allow a sufficiently high use rate on potted citrus to justify the 150-day retention period that is proposed (example – Admire Pro [4.6 lb AI/gal], maximum use rate is 0.5 ml per 0.1 cu ft = 2.76 g AI per cu ft; labels with many of the 2 lb AI/gal formulations [Advise 2FL, Alias 2F, Couraze 2F, Montana 2F, etc.], list a maximum use rate of 0.75 ml/ cu ft = 0.18 g AI per cu ft; a rate that is 15.3X lower). We cannot support this 150-day period (increased over the current 90-day period) UNLESS a sufficiently high (effective) use rate is specified.

To resolve this, we suggest that a specific use rate be associated with 150-day post treatment interval before either retreatment (if the label allows this; a label modification is needed) or crop destruct is needed. We suggest this use rate should be 3.3 ml/cubic foot of the 4.6 lb ai [active ingredient]/gallon material (1.82 g ai/cu ft = 0.004 lb ai/cu ft). In order to harmonize use rates between products with different formulations, we suggest that the use rate recommendation be expressed in grams ai/cu ft.

5. An issue with the current regulations is that the treatments at the wholesale citrus nurseries are often occurring long before (up to 90 days before) the potted plants are shipped to the retail nurseries. It is critical that the period before shipment be shortened to 14 days (research is underway to see if this time period can be shortened further), so that the pesticide treatments have maximum residual life while the plants reside at the retail nursery. This will minimize the risk of psyllids establishing on and/or transmitting HLB to retail nursery citrus plants. Research has clearly shown that 14 days is adequate to establish effective doses in potted citrus leaf tissue with imidacloprid.

Eradication/Management

http://ucanr.edu/sites/ACP/

Five main features characterize most successful eradications (Simberloff 2009):
a) *Detecting an invasion early, and acting quickly to eradicate it.*
b) Sufficient resources allocated at the start to finish
the project, including post-eradication surveys and follow-up, if necessary.
c) Existence of a person or agency with the authority to enforce cooperation. Eradication cannot succeed even if the great majority of stakeholders cooperate in the campaign so long as a small minority allow the invader to persist on property they control.
d) The target species must be studied well enough to suggest vulnerabilities. Often basic natural history suffices.
e) Project leaders must be energetic, optimistic, and persistent in the face of occasional setbacks.

Diaphorina citri was recorded in Australia, in the Northern Territory in 1915, but it was eradicated by chance during the 1916-1922 eradication campaign for citrus canker (*Xanthomonas citri* subsp. *citri)* when all cultivated species and hybrids of *Citrus* trees north of 19°S were destroyed (Mertin 1952, Hollis 2004, Bellis et al. 2005).

D. citri and HLB are far more serious threats to the Australian citrus industry than citrus canker. We have been prepared to eradicate citrus canker through the eradication of all citrus in an area. We should be prepared to do the same if *D. citri* is identified in an area provided the incursion has been detected early and is in a restricted area. NB It is highly likely that HLB already occurs in Australia in a tree produced from illegally imported budwood as was found to be the case in Florida and California after the introduction of *D. citri*.

When eradication of *D. citri* is considered, whether carrying '*Ca.* L. asiaticus' or not, it is recommended that such action should be based on:

- quarantining the property or properties within 5 miles[211] of where the psyllid is found so as to prevent the removal of any HLB host plants, budwood, or psyllid vector hosts;
- ACP is not a strong flyer and may move at least several kilometres during normal air current assisted dispersal flights or occurs via the movement of infested plants; jumping/landing behaviour such as when disturbed is likely to be less 8 m; *D. citri* were able to traverse potential geographic barriers such as roads and fallow fields and disperse at least 2 km within 12 d. (Rosenblum et al 2015);
- dispersal is prompted by high populations; more psyllids are present on the exterior edges of individual orchard blocks associated with roads, canals, ponds compared with the interior of the same block.
- adults have been found capable of moving 100m in 3 days (Boina et al. 2011), 400m in 4 days (Tiwari et al 2010) and 2 km in 12 days (Lewis-Rosenblum 2011).
- citrus groves that are within 2 km of any other citrus plantings are at risk for *D. citri* infestation and HLB disease introduction from those areas (Rosenblum et al. 2015).

Treatment with insecticide:

- when Asian citrus psyllid first appears in a region, numbers are low and the population can potentially be eradicated locally, if treated aggressively with insecticide over an 800 meter area[212].
- the size of the treatment area should be defined by the geography and continuity of citrus trees, but at a minimum, all orchards intersecting 800 meters of the find site are treated. If part of an orchard is within the 800-meter radius, the entire orchard should be treated.
- there should be immediate application of insecticides to all host plants (including *Murraya* and *Choisya* etc) as foliar sprays using backpack applicators, by employees or agents of the commercial plant nursery or orchard, other ground equipment, or through soil drench by watering or dipping)[213].
- two insecticides should be used, preferably a foliar insecticide or oil for knockdown and a systemic for a more persistent effect.

The most effective foliar treatments[214] are: pyrethroids, thiamethoxam alone, or mixtures of neonicotinoids with other products because they are broad-spectrum, have the longest residual activity, and are toxic to all stages they contact (Byrne et al 2014).

Imidacloprid persists for several months (depending on tree size and irrigation system) and moves into the new leaves to kill the hard-to-reach immature stages. Residues of the 3 neonicotinoids in

[211] 20 miles has been considered in California

[212] UC Pest Management Guidelines. Citrus. Asian Citrus Psyllid. http://www.ipm.ucdavis.edu/PMG/r107304411.html

[213] The potential environmental effects of these pesticides is summarised in the 'USDA Citrus Greening Control Program in Florida Nurseries. Environmental Assessment January, 2006 http://www.aphis.usda.gov/plant_health/ea/downloads/citrusgreening1-06ea.pdf.

[214] UC Pest Management Guidelines. Citrus. Asian Citrus Psyllid. http://www.ipm.ucdavis.edu/PMG/r107304411.html

potted citrus nursery plants treated at standard label rates were detected in leaf tissues within 1 week after treatment. Peak concentrations established at 1 week for imidacloprid and dinotefuran and at 2 weeks for thiamethoxam. Imidacloprid and thiamethoxam outperformed the control and dinotefuran treatments at protecting trees from infestations by ACP eggs and nymphs. For a given insecticide concentration in leaf tissue, thiamethoxam induced the highest mortality of the 3 insecticides, and dinotefuran was the least toxic (Byrne et al 2016).

- Apply when root growth is occurring for best root uptake.
- Apply to soil; it remains effective for 2 to 3 months.
- Imidacloprid requires 3 to 4 weeks for uptake into mature citrus to begin to kill pests.
- Pre-wet soil before treatment is applied. For optimum uptake, apply to newly planted trees or trees irrigated by drip, microsprinkler, low-pressure irrigation systems. Emitters must provide even, uniform distribution of water. Lightly pre-wet soil for several hours before application to break soil surface tension. Once the irrigation system reaches operating pressure, inject the treatment into the system over a calculated time interval (generally 2 hours) to allow uniform distribution throughout the system. The use of a dye marker in the treatment solution is recommended to determine when lines are clear of the treatment. Once the solution has cleared all irrigation lines and emitters, continue irrigation to move the insecticide into the active root zone but do not overirrigate or cause runoff. Wait 24 hours before subsequent irrigations.
- Imidacloprid is toxic to bees.

Tree removal or skeletonising

Chiyaka et al. (2012) modelled HLB development within a tree and showed that the effect of spraying of psyllids depends on time of initial spraying, frequency, and efficacy of insecticides. Lee et al. (2015) presented experimental evidence showing that young flush becomes infectious within 15 d after receiving psyllid transmitted '*C*Las' inoculum, but may not show symptoms for months or even years!

Given these findings, following an insecticide spray, consideration should be given to skeletonising all *Citrus* trees in orchards, home gardens and elsewhere. Skeletonising should optimise the prospect of eradication, as the psyllid cannot survive in the absence of host canopies. Skeletonising, as an alternative to tree removal, will minimise costs and allow orchards and nurseries to return to full production sooner while maximising the chance of eradication. It may also prevent '*C*Las' establishing and moving within the tree.

Australia undertook tree removals within 600m of a citrus canker infected tree in the incursion at Emerald (Barkley et al 2014). ACP and HLB are more devastating so why not consider tree removal as was successful in the Northern Territory in the early 1900's!

OTHER *DIAPHORINA* SPP. ON CITRUS

The presence of '*Candidatus* Liberibacter asiaticus' associated with the Asiatic form of HLB, has been confirmed in adults of the black psyllid, *Diaphorina communis* collected in Bhutan (Donovan et al. 2011). Nymphs of the pomelo psyllid *Cacophylla (Psylla) citrisuga* collected from HLB symptomatic lemon trees in Yunnan province, China were '*C*Las' positive (Cen et al. 2012a, 2012b). These results suggest that *Diaphorina communis* and *Cacophylla (Psylla) citrisuga* are carriers of '*Ca*. L. asiaticus'. Transmission has yet to be demonstrated for *D. communis*, but has been tentatively demonstrated

for *C. citrisuga* (Cen et al. 2012b). Other psyllids have been recorded on citrus in Asia but are not known as vectors of citrus liberibacters: e.g., *Cac. citricola, Cac. heterogena, Cac. murrayi, Cac*. (*P.*) *evodiae.*

Populations of *D. citri* and *D. communis* decline with increasing altitude. In contrast, *Cac. citrisuga* is a high altitude species – with incidence falling with declining altitude (Cen et al. 2012a).

D. communis has been recorded on orange jasmine, curry leaf and occasionally from *Citrus* (Mathur 1975). *D. murrayi* has been recorded in Asia on *Murraya* (Mathur 1975).

Diaphorina communis adult on a mandarin leaf at Kamichu, Bhutan, May 2009 (left); adult of (possibly) *Cac. citrisuga* on pomelo flush at Dura Sakalgre, Garo Hills, Meghalaya, India (right) (GAC Beattie).

Species in Africa and Indian Ocean islands include *D. auberti* Hollis in the Comores Archipelago (Hollis 1987), and *D. punctulata* Pettey and *D. zebrana* Capener in Swaziland (Catling & Atkinson 1974, Aubert 1987c). *Diaphorina auberti* can develop on citrus, with the larvae concentrating on the upper surfaces of young leaves, near the midribs, causing the lateral leaf margins of the leaves to curl upwards and inwards, often forming an enclosed roll (Hollis 1987, citing a personal communication from Bernard Aubert). *Diaphorina punctulata* and *D. zebrana* feed on citrus, but are not vectors of '*Ca*. L. africanus' (Catling 1970, Catling & Atkinson 1974).

AFRICAN CITRUS PSYLLID (*TRIOZA ERYTREAE*)

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[215] http://www.cabi.org/isc/datasheet/54914

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'*Ca.* L. africanus' and its natural vector, *T. erytreae (*McClean & Oberholzer 1965a), represent significant threats to commercial citrus production in Australia and native *Citrus* species, though, if introduced, their impact is likely to be greatest in the temperate southern regions of the mainland rather than in the subtropical and tropical northern regions. *Trioza erytreae* cannot establish in hot and dry areas where midday temperatures and RH regularly reach 32°C or more combined with 30% RH (Catling 1969b, Aubert 1987c).

Distribution of *Trioza erytreae*

T. erytreae is native to, and occurs widely in, sub-Saharan Africa. It also occurs in Saudi Arabia, Yemen, restricted occurrence in Spain[216] (Galicia)[217], and few occurrences in Portugal[218] the Indian Ocean islands of Madagascar, Mauritius, and Réunion, and the Atlantic Ocean islands of Saint Helena, Madeira, Porto Santo, Tenerife and Gomera (Aubert 1987c, OEPP/EPPO 2005b, Bové 2006a). *Trioza erytreae* is well established in the Portuguese Madeira Island and the Spanish Canary Islands[219], not far from the Moroccan coast (Bove, 2006).

[216] The recent detection in Galicia and Portugal of the African psyllid (*Triozaerytreae*), one of the vectors of HLB, has raised the alarm among producers in Spain.In early 2016, as an urgent measure, the Ministry of Agriculture, Food and Environment published a Royal Decree against HLB that includes a program for the control and eradication of the Triozaerytrae and a National Plan for the prevention of *Diaphorinacitri* and *Candidatus Liberibacters* pp in citrus. http://www.freshplaza.com/article/166451/Researchers-warn-that-HLB-could-put-an-end-to-Spanish-citrus

[217] http://www.freshplaza.com/article/135096/Citrus-greening-vector-detected-in-Spain

[218] http://planthealth.org/article/experts-believe-impossible-prevent-arrival-citrus-pest-castellon-el-mediterraneo-newspaper

[219] http://www.freshplaza.com/news_detail.asp?id=110780

Hosts of *Trioza erytreae*

Known hosts of *T. erytreae* are listed in Appendix VII of Beattie & Barkley (2009)**.** Its preferred and possibly original rutaceous host is possibly *Vepris lanceolata* (Moran 1968)[220]. Other native hosts in South Africa include *Clausena anisata* and *Zanthoxylum capense*.

The results of Khamis et al (2017) clearly call for a more comprehensive documentation of *T. erytreae* host plants range, since this has potential implications for managing this important vector pest and associating plants outside of the family Rutaceae that could have serious consequences for its control and/ or containment. Although *T. erytreae* is known to develop exclusively on host plants of Rutaceae family (Aubert 1987), the Maximum Likelihood model-based phylogenetic analysis results showed that the triozid found on *S. abyssinica*, Menispermaceae is also identified as *T. erytreae*. These results are conformed with a previous study by Kalyebi et al. (2015) which stipulated that *Ficus* spp. Moraceae),
Diospyros mespiliformis Hochst. ex A. DC (Ebenaceae) and *S. abyssinica* were host plants for *T. erytreae* though based on only pit gall formation.

The survival, reproduction and morphometry of *T. erytreae* in Kenya were supported by five main rutaceous host plants, namely, *C. anisata, V. bilocularis, M. koenigii, T. nobilis and C. capense. Murraya koenigii* proved to be the most suitable host plant species in terms of African citrus triozid reproduction but also in several morphometric traits. The results of Aidoo et al (2018) suggest that the five studied rutaceous host plants can possibly influence the population dynamics of African citrus triozid as well as the epidemiology of African citrus greening by acting as reservoirs of the CLaf pathogen.

Description of *Trioza erytreae*

OEPP/EPPO 2005. EPPO Standards. Diagnostics PM 7/57 *Trioza erytreae*. EPPO/EPPO, Bulletin OEPP/EPPO Bulletin 35: 271–273.

A Guide for Diagnosis and Detection of Quarantine Pests. African Citrus Psyllid. *Trioza erytreae* (Del Guercio) Hemiptera Triozidae. 2013[221].

Khamis FM, Rwomushana I, Ombura LO, Cook G, Mohamed SA, Tanga CM, Nderitu PW, Borgemeister C, Sétamou M, Grout TG, Ekesi S. 2017. DNA Barcode Reference Library for the African Citrus Triozid, Trioza erytreae (Hemiptera: Triozidae): Vector of African Citrus Greening. Journal of Economic Entomology. 2017 Oct 16:tox283.

There are five nymphal instars. The nymphs are dorso-ventrally flattened with a distinct marginal fringe of white, waxy filaments and vary in colour from yellow, olive-green to dark grey. They are largely sedentary and form conspicuous colonies, settling on the underside of young leaves where, after a few days of feeding, they produce distinctive cup-shaped or pit-like, open galls. *T. erytreae* can cause severe leaf distortion, curling, stunting, galling and chlorosis. The leaves may also be dusted with faecal pellets.

[220] Observations reported by Evers & Grisoni (1991), and reports by Temu & Andrew (2008) and Burgess et al. (2002), suggest that two other species of *Vepris*, *V. mildbraediana* G.M. Schulze and *V. morogorensis* var *subalata* (Kokwaro) W. Mziray, may be also be hosts of *T. erytreae*, and possibly '*Ca*. L. africanus' (and other liberbacters?) in Morogoro, Tanzania.
[221] http://ppo.ir/Uploads/English/Articles/insect/African-citrus-psyllid-Trioza-erytreae.pdf

Trioza erytreae fifth instar nymph and egg (left) and an adult female *T. erytreae* (right) (Peter Stephen: Citrus Research International, South Africa).

Khamis et al (2017) have shown that DNA barcoding based on the 5' end of the mitochondrial COI gene is a suitable tool for identification of *T. erytreae* attacking citrus and other alternative host plants.

Damage caused by *Trioza erytreae*

The presence of T. erytreae can easily be detected in the field because their nymphs are only found on the underside of the leaves, where their activity promotes the formation of typical galls, each corresponding to a nymph nest. Each nest consists of a globular distortion on the upper side of the leaf corresponding to a concave hollow on the lower side, where the nymph inhabits until its development is completed. Then, the adults leave the nests, but the empty hollows remain clearly visible, representing a hint for the early diagnosis of T. erytreae presence (Bové and Duran-Vila 2016).

In addition to galling, infestations can cause leaf distortion, curling, stunting and chlorosis (OEPP/EPPO 2005b). Small flush points may be so densely packed with eggs that they may shrivel and fall off (Catling 1972). Chlorosis disappears after nymphs reach maturity (van den Berg 1990). The pellets of excrement (honeydew) voided by this psyllid have the appearance of minute white eggs, and the ground or vegetation under a badly-infested tree may appear as if dusted with white powder (van der Merwe 1941). The severity of these symptoms is related to levels of infestations.

Pit-gall damage caused by *T. erytreae* nymphs (Pat Barkley)

Important points about *Trioza erytreae*

The following is a brief summary of the studies on the biology and ecology of *T. erytreae*:

- The larvae of this psyllid are nesting in galls on the underside of leaves and their transport on long distances will occur on rooted planting material. Then adults, with fairly good flight capability, will spread the species in the newly contaminated territories (Aubert 2009).
- Green & Catling (1971) defined a saturation deficit index (SDI) based on a regression curve that depicted the combined effects of temperature and humidity on the mortality of eggs and first instar nymphs in the field and explained the known geographic distribution of *T. erytreae*[222].
- A provisional mapping of expected vector spread may be obtained by computing temperature and relative humidity data:

Temperature and relative humidity

Mapping potential *T. erytreae* spread is obtained by calculating peaks of daily saturation deficits, whence:

Ds = Pvtm x (100-rh/100)

Ds = maximum daily saturation deficit

Pvtm = saturation vapour pressure at peak daily temperature

rh = relative humidity observed at 13.00

Pvtm is taken from psychrometric tables. The death rate of *T. erytreae* eggs and first instar nymphs is 90% from 40 millibars.

Aubert (2009)

For example, *Trioza erytreae* would be able to colonise Mediterranean coastal areas, with egg laying and larval development periods in the spring time, and adults over-surviving the other seasons (Aubert 2009).

- A correlation analysis for egg to first instar survival showed that maximum daily saturation deficit, and maximum daily temperature combined with vapour pressure were both highly significant and convenient predictors of mortality. Catling (1969b) gave a regression equation for survival against the mean saturation deficit for the three severest days during the egg and first instar stages. He showed that, because of the occurrence of distinct field generations and the extreme sensitivity of the young stages, actual mortality depended largely on the age distribution in the population. Frequency and timing of daily saturation deficits above 3.453 kPa[223] were found valuable in explaining differences in population densities.
- Extreme weather plays a predominant role in regulating populations of *T. erytreae* by inducing, through desiccation, heavy mortality in the developmental stages of the psyllid (Catling 1969b);
- *Trioza erytreae* cannot establish itself in hot dry areas where midday temperatures regularly reach 32C or more combined with 30% RH (Aubert 1984).

[222]For more on SDIs and Trioza erytreae see Samways (1987; 1990), Aubert (1984); Schwarz & Green (1970), Aubert & Quilici (9th IOCV); van den Berg et al. (1991); Catling et al. (1972); van den Berg (1990); Tamesse & Messe (2004).

[223] 25.9 mm Hg

- Evers & Grisoni (1991) noted that eggs and first instars are extremely vulnerable to temperatures above 25°C. Field observations showed that 100% mortality of eggs and first instar nymphs occurred at maximum saturation deficit (msd[224]) of ≥ 45 mbars; 70% mortality occurred at 35 mbars and 10% at 15 mbars.
- the host range in Africa includes citrus, orange jasmine, *Triphasia trifolia* and *Cl. anisata* (all introduced) and native Rutoideae, *V. lanceolata* (the favoured and possibly original host), possibly *Vepris* spp., *Z. capense* and *Toddalia asiatica*[225] (see Appendix VII of Beattie & Barkley, 2009);
- host preference is influenced by season, variety, flush morphology, abundance, frequency and duration of flushing;
- psyllid populations can increase rapidly, particularly in spring when the N content of usually abundant flush growth is high and when competition between females for flush growth is low (Catling 1971);
- *Trioza erytreae* populations multiply rapidly in spring and early summer and less rapidly in autumn and winter (Catling 1971);
- *Trioza erytreae* reproduces sexually (Catling 1973), and eggs of unmated females are infertile (Catling 1973);
- sex ratios fluctuate but females were always predominant (Catling 1973);
- mating takes place as soon as the teneral adult hardens and occurs at all times of the day (Catling 1973);
- adult longevity is 17-50 d depending on season (shorter in summer, longer in winter) (Catling 1973);
- the pre-oviposition is usually 3-5 d in summer (mean temperature 24°C-26°C) and 6-7 d in winter (14°C-l6°C) (Catling 1973);
- mated females may lay 217-1305 eggs (Catling 1973: under insectary conditions);
- eggs are laid on the shoot tips of the young flush growth (van der Merwe 1923, Annecke & Cilliers 1963; Moran & Blowers 1967, van den Berg 1990);
- eggs hatch in 6-15 days and nymphal development takes 17-43 days, both strongly correlated with mean temperature (Catling 1973);
- there are five nymphal instars;
- the threshold temperature for nymphal development is probably between 10°C and 12°C (Catling 1973);
- nymphs form open 'pit' galls, mostly on the lower (abaxial) surfacces of immature leaves (van den Berg et al. 1991c);
- *T. erytreae* did not appear to possess strong dispersal powers (Catling (1973). Adults can disperse, with the aid of prevailing winds, at least 1.5 km (van den Berg & Deacon 1988);
- most flight activity of occurs in two peaks, the first between 10:00 and 11:00 and the second and larger peak shortly before sunset (van den Berg & Deacon 1989);
- in the absence of host plants adults live about 85 days and die from desiccation rather than starvation (van den Berg & Deacon 1988);
- adults disperse more frequently as numbers of eggs, nymphs or adults on the citrus increase and when flush growth declines (van den Berg et al. 1991a); and

[224] msd = svp × (100 – rh)/100, where msd = maximum saturation deficit in mbars, svp = saturation vapour pressure (derived from tables) in mbars, and rh = relative humidity with temperature taken at midday and relative humidity at 15:00.

[225] Each rutaceous genus appears to harbour different specific Laf-like liberibacters. Those found in *Xanthoxylum* appear to be most closely related to Laf from *Citrus*. *Vepris* and *Clausena* harbour liberibacters more closely related to the LafC subspecies found in *Calodendrum.* This very recent suggestion of a further three subspecies of Laf (Roberts et al., 2014) can be resolved within this proposal of haplotypes rather than subspecies within Laf by giving them a biotype designation, recognising the current host plant differences. Laf subspecies vepridis is a biotype ofLafA, while Laf subspecies zanthoxyli and Laf subspecies clausenae are biotypes of LafC (Nelson et al 2015).

[225] For example, orange jasmine, *M. paniculata*/*M. exotica*.

- movement of adults between citrus orchards and stands of alternative hosts is influenced by season (van den Berg et al. 1991b).
- Catling (1971) reported that nymphs underwent prolonged development on poorly nourished citrus leaves, with poor condition in the field causing high rates of mortality.
- the incubation period in the field varied from 6-15 d and nymphal development from 17-43 d, both being strongly correlated with mean temperature (Catling (1973).
- McClean (1974) reported observations that indicated that the number of adult *T. erytreae* carrying the African HLB pathogen was relatively small in relation to the total adult population found on trees in South Africa in a region where the incidence of the disease was high. He reported evidence of seasonal fluctuations in the number of carriers: more occurring among adults emerging from the summer flushes on citrus trees, and few, if any, among those emerging during winter and early spring. However, he noted that the efficiency of the vector was not seriously impaired by the small number of carriers; it was offset by the large numbers of adults that emerged during favourable conditions, thus insuring spread of the disease to many healthy trees, and to healthy sectors of partially diseased trees.
- there was no evidence that the causal organism of greening is passed through eggs laid by infective adults (McClean 1974).

Surveillance

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Other *Trioza* spp. on Citrus

A second species of *Trioza*, *T. litseae* Bordage (syn. *T. eastopi* Orian) has been recorded feeding occasionally on *Citrus* (Aubert & Quilici 1984, Aubert 1987b), and a third, *T. citroimpura* Yang & Li, was collected from mandarin trees in Yunnan, China (Yang & Li 1984). Nelson (2012) reported the New Zealand native psyllid *Trioza vitreoradiata* completing its life cycle from egg to adult on grapefruit in a garden next to a psyllid-infested *Pittosporum.*

'Trioza citroimpura'. Left specimen kept in 80% ethanol (A. Beattie).

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Field Tolerance:

Scion varieties:

Field symptomatology depends on:

- tolerance/resistance of the variety
- whether a tree flushed during a period of psyllid activity (de Lange et al. 1985, Koizumi et al. 1994);
- resistance[226] to the Asian citrus psyllid (Westbrook et al. 2011, Hall 2013).
- the presence and severity of CTV strains also present; and
- the form of '*Ca.* Liberibacter'
- the strain of '*Ca*. L. asiaticus' (Tsai et al. 2008).

Different isolates of '*Ca.* L. asiaticus' can cause different levels of disease in citrus cultivars. For example, inoculation of different citrus hosts with '*Ca.* L. asiaticus' conducted in Taiwan resulted in significant differences between several isolates of '*Ca.* L. asiaticus' in their ability to induce disease in cultivars like Eureka lemon and pomelo ranging from the absence of bacteria and no symptoms to high concentrations of '*Ca.* L. asiaticus' and the development of severe symptoms (Tsai et al. 2008). At the same time, most isolates were similar in the degree of disease induced in other citrus cultivars such as mandarin and sweet orange.

Theoretically, rate of development of HLB/CLas could be due to attractiveness of trees to ACP, CLas establishment at ACP feeding, CLas proliferation following ACP inoculation, systemic movement of CLas with subsequent further proliferation, and development of plant responses observed as HLB symptoms. Reduction or slowing of any of these steps may slow disease development and spread (Stover et al. 2010a).

As stated by Aubert (1990b), among the commercial varieties of citrus, HLB causes a wide range of reactions from relatively mild to extremely severe: *'The group of acid citrus (limes and lemons) is much less sensitive than the group of sweet citrus (mandarins, oranges, tangors, tangelos) and Poncirus[227] is tolerant but not immune.'*

Because a variety is tolerant does not reduce its significance as a reservoir of infection e.g., lemons are important reservoirs of infection, in part because of their more frequent flushes, which are attractive to the vector. In South Africa, the highest percentage of HLB fruit symptoms occurred where *Citrus* (*Poncirus) trifoliata* was used as rootstock. The effect was attributed to an influence of *C. trifoliata* on extending the flushing rhythm of the tree, and thus, extending the vector breeding and feeding periods, increasing the chances of transmitting the pathogen (van Vuuren and Moll 1985).

Keremane et al (2012) tested seedlings of 96 cultivars (8 replications of each) of 18 genera of the subfamily Aurantioideae and family Rutaceae by PCR over 3 years in a field site in Florida where HLB is endemic. While most cultivars were found to be susceptible to '*Ca.* L. asiaticus' , the bacterium was not detectable in many trifoliate and trifoliate hybrids, some species of *Bergera, Casimiroa, Clausena, 'Eremocitrus', Glycosmis, 'Microcitrus', Murraya, Naringi* and *Zanthoxylum.* In the genus *Citrus* only two species showed either no or very low levels of '*Ca.* L. asiaticus'. Partial resistance was observed in some clonal populations of *C. latipes* (Ramadugu et al 2013). Ramadugu et al (2015,

[226] Ammar et al (2013) suggested that thickness of the fibrous ring may be a barrier to stylet penetration into the vascular bundle
[227] *Poncirus trifoliata* (L.) Raf. is, as originally described, *Citrus trifoliata* L.

2016[228]) claim there is HLB tolerance in the Australian native finger limes (*Microcitrus*) and desert lime (*Eremocitrus*)[229].

This trial was further examined by Miles et al (2017). The healthiest trees with low or no HLB symptoms were distant citrus relatives: *Balsamocitrus dawei, Bergera koenigii, Casimiroa edulis, Clausena excavata, Murraya paniculata*, and one accession of *Severinia buxifolia*. Within *Citrus*, most of the healthiest trees with densest canopies, little leaf loss, and greater growth were those with pedigrees that included Citrus medica (citron). These included progenies of Citrus hybrid ('Limon Real'), *Citrus limetta, Citrus limettioides, Citrus limonia, C. medica, Citrus volkameriana*, and some *Citrus limon* accessions. Trees in this category exhibited distinct leaf-mottle characteristic of HLB and substantial pathogen titers, but maintained dense canopies and exhibited good growth. Trees from seed-source accessions in the genus *Citrus* without citron in their background were generally among the least healthy overall with less dense canopies. The exceptions were progenies of two *Citrus aurantium* accessions, which were markedly healthier than progenies of other *Citrus* seed-source accessions not derived from citron.

Stover & McCollum (2011a) examined '*Ca.* L. asiaticus' levels in flowers, anthers and leaves of 30 citrus genotypes. Mandarin hybrids had the highest levels of '*Ca.* L. asiaticus' in all leaf samples while some trifoliate hybrids had fewer '*Ca.* L. asiaticus' genomes per nanogram nucleic acid sample. Further work by Stover & McCollum (2011b) showed that across groves in the Indian River area of Florida, Temple tangor showed the most consistently low incidence of HLB symptoms and '*Ca.* L. asiaticus' titre; in contrast Murcott tangor and Minneola tangelo had the highest incidence of HLB symptoms and highest '*Ca.* L. asiaticus' titre. Sweet orange had an intermediate '*Ca.* L. asiaticus' titre. Along with Temple tangor, Fallglo, Sunburst and grapefruit showed markedly lower '*Ca.* L. asiaticus' titres (higher relative Ct) and low HLB incidence. Stover & McCollum (2011b) pointed out that differences in tree age, tree size, flushing pattern and coincidence among flushes, pest control and ACP outbreaks may have influenced results and contributed to cultivar responses, although this was unlikely across groves in a region.

See McCollum G, Hilf M, Irey M, Luo W, Gottwald T. 2016. Susceptibility of sixteen Citrus genotypes to 'Candidatus Liberibacter asiaticus'. Plant Disease. 100 (6):1080-6.

Table 1. Field tolerance of *Citrus* and *Citrus* relatives to HLB.

Pathogen Country	Reference	Highly Intolerant (highly sensitive)	Intolerant	Fairly Tolerant (tolerant)	Tolerant (moderate)	Susceptible (severe)
'*Ca*. L. asiaticus'						
Thailand	Koizumi et al. 1997				rough lemon, calamondin, Som-pan and Ladu mandarins, Sour lime (but a major source of inoculum as a backyard tree), Tahiti lime	Large numbers of sweet orange (e.g., Neck orange, Washington navel); Murcott tangor, mandarin (Fremont, Ponkan, Queen, Som-keowan, Shogun) and tangelo
Thailand	Koizumi et al. 1993, 1994			Queen mandarin, Avon Ever Bearing	Ladu and Som-pan mandarins[230]	Ortanique, Wilking, Ellendale, Pet-yala,

[228] Ramadugu et al. **Citrograph Vol. 7, No. 2** | Spring 2016, pp. 46-51.

[229] Both are now classified as *Citrus.* Mabberley. 1998. Australian Citreae with notes on other Aurantioideae (Rutaceae). Telopea 7: 333-44.

[230] Koizumi et al. (1994) claim that *D. citri* undergoes 'rapid extinction' on Ladu and Som-pan mandarins

Pathogen Country	Reference	Highly Intolerant (highly sensitive)	Intolerant	Fairly Tolerant (tolerant)	Tolerant (moderate)	Susceptible (severe)
				calamondin, rough lemon		Beauty, Onesco, Tankan, Nian-ju, sweet orange were most susceptible, followed by the mandarins Fairchild, Murcott, Kinnow, Clementine, Fremont, Ponkan, King, Som-keo-wan, Satsuma, Som-keaw and Ba-yue-ju.
Thailand	Miyakawa & Zhao 1990 Schwarz et al. 1973a,b				som-o pomelo	Som-keowan mandarin, Som-kleang sweet orange, Manao lime (symptoms masked by CTV)
Malaysia	Aubert 1992	limau kupas langkat (mandarin)				
India	Fraser & Singh 1969	All sweet oranges, blood oranges and Mosambi deteriorate quicker than Jaffa, Valencia, Hamlin and Sathgudi; all tangelos especially Yalaha and Orlando, mandarin hybrids (e.g., Kinnow) and some native mandarins	Nagpur and Coorg mandarins, Clementine, Ladu and Emperor when infected as nursery trees, but less affected than sweet orange when infected as mature trees. Differences in susceptibility between local strains of mandarins. Grapefruit and sour orange when infected as nursery tree, but tree deterioration of adult trees takes longer than sweet orange.	trifoliate orange and its hybrids show prominent symptoms but less tree deterioration. Satsuma mandarin, kaffir lime, Ichang papeda, citron, Meyer lemon	pomelo, Cleopatra mandarin, Some native mandarins, Guntur sour orange, acid lime in northern and central India, but not in southern India, rough lemon, *C. karma*, sweet lime, kaffir lime, a native lemon, Tahiti lime, adajamir, Rangpur lime, sweet lime and some rough lemons, as rootstocks, may improve performance of some susceptible scion varieties	
India	Fraser & Singh 1966			some varieties of mandarin; some rough lemons	sweet lime; Rangpur lime	All varieties of sweet orange, grapefruit (onset may be later than sweet orange); some varieties of mandarin; some rough lemons
India	Nariani et al. 1973				sweet lime, pomelo (chakotra), kaffir lime (wild in Assam, no symptoms, pathogen not isolated on indexing). Adajamir in glasshouse trials	
India	Nariani 1981			sweet lime (resistant), Italian, Eureka and Lisbon lemons (tolerant)	trifoliate orange and citranges	7 cultivars of sweet oranges, 2 mandarins (Dancy and Santra) highly susceptible
India	Cheema et al. 1982			5 of 25 rough lemon strains, (Milam, Miri, South Africa-I, South		

Pathogen Country	Reference	Highly Intolerant (highly sensitive)	Intolerant	Fairly Tolerant (tolerant)	Tolerant (moderate)	Susceptible (severe)
				Africa-II and Volkamar in inoculation trials		
Philippines	Altamirano et al. 1976				Szinkom x Ladu hybrid mandarin, calamondin, lime, lemon	
Philippines	Salibe & Cortez 1966, Miyakawa & Zhao 1990				Ladu mandarin, sweet and sour orange, citron	Szuwuikom, Ponkan, Tankan, Batanges, calamandarin, Szibat and Szinkom mandarins, Mazoe rough lemon
Philippines	Martinez & Wallace 1969			grapefruit, sour orange, Sunki mandarin, rough lemon, Rangpur lime, Borneo red lime, Eureka lemon, Key lime, Palestine sweet lime, Shekwasha, native and Siamese pommeloes, citron showed mild-moderate leaf symptoms and slight stunting. Calamondin, alemow, and Chinese box orange showed slight leaf symptoms and stunting.	trifoliate orange, Troyer and Carrizo citranges, Chevalier's aeglopsis, and a misnamed plant ('*Atalantia trifolia*'), showed no leaf symptoms. Tolerant rootstocks conferred no resistance to the scion.	Szinkom, Sziwuikom, Sun wuikom, Batangas, Ladu, calamandarin, Ransas, Cleopatra, Ponkan, Oneco, King, Avana, Malvasio, Murcott mandarins, Madam Vinous, Pera, Hamlin, Koethen, Shamouti, Caipira, Campbell Valencia, Washington navel oranges; Orlando tangelo, Szinkom x Batangas, Szinkom x Ladu, Szinkom x King, Shekwasha x calamondin
Philippines	Gonzales & Viñas 1981			Bearss lime	sweet orange less susceptible than mandarins	Mandarins and mandarin hybrids highly susceptible
Philippines	Gonzales et al. 1972				trifoliate orange and its hybrids, lemon and sweet lime	In decreasing order of susceptibility: sweet orange, pomelo, grapefruit, hybrids of mandarin and sweet orange, mandarin
China (Taiwan)	Miyakawa 1980 (greenhouse tests, graft inoculations)				Eureka lemon, sour orange grapefruit, Sexton tangelo, trifoliate orange, jasmine orange	sweet orange, mandarins e.g., Ponkan and Orlando tangelo; kumquats
China (Taiwan)	Su & Huang 1990				Wentan and Peiyu pomelos tolerant pre-1970 but not post 1970	most cultivars
China (Taiwan)	Su & Matsumoto 1972 (glasshouse inoculations)					Valencia orange, Rangpur lime, Etrog citron, Szinkom mandarin, calamandarin, Ponkan/ Sunki, Tankan/ Sunki, Sunki
China	Lin 1956					Nearly all species and varieties of citrus in affected area are susceptible. Order of degree of susceptibility

Pathogen Country	Reference	Highly Intolerant (highly sensitive)	Intolerant	Fairly Tolerant (tolerant)	Tolerant (moderate)	Susceptible (severe)
						is: Ponkan, Chiaokan, Chachihkan, Lukan, and Tungkan mandarins, various varieties of sweet orange, Hungninmeng (a lemon x mandarin hybrid), Nienchu mandarin, Shangmayu pomelo and Shihchicha.
China	Zhao 1981, Miyakawa & Zhao 1990				trifoliate orange (Zhao 1981)	All stock/scion combinations including Mauritius papeda (kaffir lime), Ichang papeda, honglinmon (a lemon), Shantianyou pomelo, Xuegan orange, Shinsan sour orange and Suanju. Citrange seedlings (Zhao 1981)
China	Zhao 1987				Satsuma	
China	Deng et al. 2008					HLB in pomelo potentially as severe as in sweet orange and mandarin
Indonesia	Tirtawidjaja et al. 1965 (glasshouse inoculations)					West Indian lime, grapefruit, Djeruk Siem mandarin, Palestine sweet lime, sweet orange, rough lemon, Japanese citron, and Djeruk Grant mandarin
Indonesia	Aubert et al. 1985				Pomelos more tolerant than orange or mandarin	symptoms of lopsided fruit and seed abortion on rough lemon and Rangpur lime
Brazil	Lopes et al. 2006)					sweet orange (Valencia, Pera, Natal, Hamlin, Westin, Lima verde, Bahia, Bahianinha, Folha murcha, Shamouti), mandarins (Ponkan and Cravo), Persian and Tahiti limes, Murcott tangor; jasmine orange
'*Ca*. L. africanus'						
South Africa	McClean & Schwarz 1970			well defined symptoms not seen in trifoliate orange or Troyer citrange	grapefruit, lemons, rough lemon	The more susceptible varieties of tangerine and mandarin e.g., Satsuma are more sensitive than sweet orange. Tangeloes react similarly to mandarins e.g., Orlando and Minneola are very sensitive. Most varieties of sweet

Pathogen Country	Reference	Highly Intolerant (highly sensitive)	Intolerant	Fairly Tolerant (tolerant)	Tolerant (moderate)	Susceptible (severe)
						orange including Valencia and Washington navel. (Hamlin and Tomango oranges appear more tolerant).
South Africa	de Lange et al. 1985			true lemon, rough lemon, and trifoliate orange and its hybrids; lime group highly tolerant.	Gold Seal sweet orange	sweet oranges, mandarin and grapefruit, citron and shaddock
South Africa	Schwarz 1968a			Palestine sweet lime, West Indian lime, rough lemon, trifoliate orange and its hybrids	lemon	Severe symptoms on sweet orange, many mandarin, tangelo and grapefruit varieties
South Africa	Manicom & van Vuuren 1990			lime, pomelo, trifoliate orange and citranges	grapefruit, lemon, sour orange,	All cultivars of sweet orange (especially blood oranges), tangelo (especially Orlando and Minneola). Mandarins vary in susceptibility and symptoms severe if infected as young trees, but less severe tree deterioration than sweet orange if infected later.
South Africa	Oberholzer 1965, von Standen & Basson 1965					All major commercial varieties equally susceptible but Valencia orange shows more pronounced foliar symptoms than Washington navel
Réunion	Aubert (cited as pers. comm. in de Lange et al. 1985)				sweet orange cultivar 'Gold Seal'	

A survey conducted by Castle (2013) collected grower observations on the incidence of HLB among groves of commercial scion varieties: see below. See also Stover et al (2016).

Table 1. Order of incidence of HLB from lowest [top] to highest [bottom] among scion varieties in commercial groves.*

North Ridge-1**	Central Ridge-1	CR-2	CR-3	CR-4	CR-5	CR-6 & Flatwoods	South Ridge-1	SR-2	SR-3A	SR-3B	SR-4	SR-5	SR-6	SR-7	Indian River-1
								Grapefruit							
Temple***	Temple	Temple	Temple	Temple	Vernia	Temple		Temple			***Temple***	***Temple***		Temple	
	Navel	Navel	Navel	Navel	Navel	Navel						Parson Brown			
	Grapefruit	Grapefruit	Grapefruit	Grapefruit	Fallglo	Grapefruit			Vernia			Vernia		Fallglo	Grapefruit
	Fallglo	Sunburst		Fallglo	Grapefruit	Fallglo	Valencia				Grapefruit	Valencia			
Hamlin	Hamlin	Fallglo???		Hamlin		Hamlin	Hamlin	Valencia	Hamlin	Hamlin	Valencia	Pineapple	Hamlin	Hamlin	Hamlin
Valencia	Valencia	Hamlin	Hamlin	Valencia		Valencia		Hamlin	Valencia	Valencia	Hamlin	Hamlin	Valencia	Valencia	
Sunburst	Minneola	Valencia	Valencia	Minneola	Midsweet	Minneola	Pineapple	Minneola							
Minneola	Sunburst	Midsweet	Midsweet	Sunburst		Sunburst	Midsweet	Sunburst	Pineapple	Murcott	Midsweet		Sunburst	Sunburst	Sunburst
W. Murcott	Murcott	Pineapple	Pineapple	Murcott	Sunburst	Murcott		Murcott	Murcott	Pineapple	Pineapple		Murcott	Murcott	Murcott
Murcott		Minneola	Minneola		Murcott	Pineapple			Midsweet	Midsweet	Murcott			Pineapple	
		Murcott	Murcott			Midsweet				Queens				Midsweet	

IR-2	IR-3	IR-4	IR-5	IR-6	IR-7	IR-8	IR-9	IR-10	IR-11	IR-12	Peace River Valley-1	PRV-2	Gulf Coast-1	GC-2	GC-3
	Nova			Nova					***Nova***		Nova				
Temple	Temple	Temple	***Temple***	***Temple***	Temple		Temple		***Temple***	Temple	Navel	Temple	Temple		Temple
Navel	Navel	Navel	Navel	Navel	Fallglo	***Navel***	Navel	Navel		Navel	Vernia	Navel	Fallglo		
Grapefruit	Grapefruit	Grapefruit	Grapefruit	Grapefruit	Navel		Fallglo	Red grpft			Earlygold	Grapefruit	Grapefruit		Grapefruit
Fallglo		Fallglo	Fallglo	Fallglo	Grapefruit	***Grapefruit***	Grapefruit	White grpft	***Grapefruit***			Sunburst	Sunburst		Navel
Hamlin	Hamlin	Hamlin	Hamlin	Hamlin	Hamlin		Sunburst	Hamlin	***Earlygold***	Grapefruit	Valencia	Hamlin	Navel	Valencia	
Valencia	Sunburst	Valencia	Valencia	Valencia	Valencia	Earlygold	Hamlin	Midsweet			Hamlin	Valencia	Orlando	Hamlin	
Minneola	Valencia	Minneola	Minneola	Minneola	Vernia		Valencia	Valencia				Minneola	Hamlin		Murcott
Sunburst		Sunburst	Sunburst	Sunburst	Sunburst		Minneola		***Minneola***		Midsweet	Murcott	Valencia		Hamlin
Murcott	Murcott	Murcott	Murcott	Murcott	Minneola		Murcott		***Murcott***		Pineapple		Pineapple	Pineapple	Valencia
	Orlanique				Murcott						Murcott		Midsweet		
													Murcott		

* The information contained in this table results from an informal survey of citrus growers. Additional ***SCION*** and ***ROOTSTOCK*** comments are presented in Table 2.

** North Ridge [NR] is Lake County; Central Ridge [CR] is Polk County; South Ridge [SR] is Highlands County; Indian River [IR] is the east coast; Peace River Valley [PRV] is mostly the area covered by Hardee and Desoto Counties; and, the Gulf Coast [GC] is the southwest region consisting of Charlotte County and south to Collier County.

Rootstocks

A particular citrus rootstock-scion combination when infected with *Candidatus* Liberibacter asiaticus (*C*Las) would be considered tolerant if infected trees had no more than slight reductions in performance, sometimes accompanied by a reduced level of the pathogen. That definition implies that the plant can cope with infection and continue to be productive and profitable. A practical definition of tolerance is: the ability of a *C*Las-infected tree to sustainably produce profitable quantities of fruit of acceptable quality over time. Such a definition should be viewed as "economic tolerance" (Castle 2016; Castle et al 2016, Albrecht 2017)

In South Africa, the highest percentage of HLB fruit symptoms occurred where *C. trifoliata* was used as the rootstock. The effect was attributed to an influence of *C. trifoliata* on extending the flushing rhythm of the tree, and thus, extending the vector breeding and feeding periods, increasing the chances of transmitting the pathogen (van Vuuren and Moll 1985).

C. (Poncirus) trifoliata hybrids grown by Stover et al. (2010a) in the USHRL variety block on Sun Chu Sha mandarin were tested for '*C*Las' 16S rDNA and citrus dehydrin by qPCR, assessing random quadrant samples, a diagnostic 'worst' sample, and rootstock suckers (November 2009). The two *P. trifoliata* had non-detectable or low CLas abundance, as did two citranges, except that citrange diagnostic samples and rootstock samples had very high CLas (20-24 CLas rDNA/Citrus dehydrin). Variability was observed in relative CLas abundance among the ten citranges tested with most showing high abundance in quadrants (20 CLas/citrus gene), and all showed high CLas in rootstock suckers. The data suggest that *Poncirus* and some *Poncirus* hybrids "tolerate" and/or suppress CLas even when grafted onto a high-titer source.

Trifoliate orange and some of its hybrids reportedly lack distinct disease symptoms despite infection with the pathogen. US-897 is a hybrid of trifoliate orange and 'Cleopatra' mandarin, the latter being highly susceptible to HLB. Naturally infected US-897 trees exhibited no distinct disease symptoms commonly associated with HLB, except for the occurrence of few mottled leaves in a small percentage of trees. Graft-inoculated US-897 seedlings became PCR-positive for the pathogen but exhibited a superior performance compared with 'Cleopatra' mandarin seedlings, which displayed severe disease symptoms soon after inoculation. Despite infection, most US-897 seedlings did not develop leaf symptoms typical for HLB. The superior performance of US-897 plants in greenhouse and field locations suggest tolerance of this genotype to '*Ca.* L. asiaticus' (Albrecht & Bowman 2011).

Stover & McCollum (2011) also found US-119 and US-812 to have low levels of '*Ca.* L. asiaticus' in leaf samples. Hybrids of *Poncirus* beyond F1s continue to display significantly lower Las levels even after chronic infection (Stover et al 2014).

Fruit of *P. trifoliata* hybrids typically have an unpleasant flavour. The juice of *P. trifoliata* was characterized by having a large amount of 20 esters and 32 sesquiterpene hydrocarbons, in addition to alcohols, monoterpenes, and aldehydes. The diversity of volatiles produced by *P. trifoliata*, and the fact that only a sub set of these volatiles were found in Citrus × *P. trifoliata* hybrids suggest that *P. trifoliata* characteristics might be transmitted at different levels through subsequent generations (Deterre et al 2013). Among the non-volatile compounds, the bitter compounds are limonin, nomilin, naringin, neohesperidin and poncirin (Horowitz and Gentili, 1963; Nagy and Attaway, 1980).

The presence of a thick, well-developed fibrous ring around phloem tissues of mature leaves acts as a barrier to frequent or prolonged phloem ingestion by *D. citri* from citrus leaves. This may have an

important role in limiting or preventing CLas acquisition and/or transmission by *D. citri*, and could be used for identification and development of resistant citrus cultivars. (George et al 2017).

HLB symptom expression of trees on different rootstocks is ranked Swingle citrumelo > Carrizo citrange > sour orange > Cleopatra mandarin which follows rootstock intolerance of bicarbonate. Orchards under high bicarbonate stress declined 20% in yield compared to orchards with low bicarbonate stress with a 6% increase in production. Yield loss was correlated with reduced fibrous root density (Graham et al 2014).

Albrecht & Bowman (2012) hypothesised that tolerance of US-897 to '*Ca.* L. asiaticus' is associated with "the constitutively higher expression of defense-related or other genes rather than with an induced expression in response to bacterial infection" and that "expressing these genes using biotechnology approaches may be one strategy to counteract the detrimental effects of HLB". PP2 is characteristic for the late symptomatic stage of HLB produced in an attempt to restrict further spread of the pathogen (Albrecht & Bowman 2008). Although not significantly induced in US-897 seedlings in response to infection, transcript levels for PP2 were generally higher in this genotype compared with uninfected Cleopatra seedlings suggesting PP2 involvement in tolerance to HLB.

Albrecht & Bowman (2012) first recognised the potential roles of constitutive disease resistance (CDR) 1 genes in HLB resistance/tolerance in a microarray-based genome-wide gene expression study and Rawat et al (2017) analyzed the expression of the entire CDR gene family in HLB-susceptible and HLB-tolerant genotypes after CLas infection and obtained information about the response of each copy of CDR genes to HLB. They established the potential roles of (CDR) genes in mediating citrus responses to CLas infection and HLB development. PtCDR2 and PtCDR8 were in abundance in the leaf transcriptomes of two HLB-tolerant Poncirus genotypes and were also upregulated in HLB-tolerant, Poncirus hybrids as revealed by real-time PCR analysis.

Compared to the mock-inoculated plants, higher bacterial titers and greater accumulation of callose and starch were found in *C. sinensis, C. sunki* and 10 of the hybrid (*C. sunki x P. trifoliata*)plants. Lower titer and fewer metabolic changes due to Las infection were observed in *P. trifoliata* and in two Las-positive hybrids while three hybrids were Las-negative. Callose accumulation was associated with genes involved in phloem functionality while starch accumulation was associated to up-regulation of genes involved in starch biosynthesis and repression of those related to starch breakdown. Lower expression of genes involved in phloem functionality in resistant and tolerant plants can partially explain the absence of distinct disease symptoms associated with starch accumulation that are usually observed in HLB-susceptible genotypes (Boava et al 2017).

Valencia orange grown on 17 rootstocks through seven years of ageand the first four harvest seasons in a central Florida field trial severely affected by HLB. All trees in the trial had HLB symptoms and were shown by PCR to be infected with '*C*Las'. Large differences were noted between rootstocks for yield, fruit quality, and tree size. Highest yields in the trial were on US-942 rootstock, whichwas significantly more productive than trees on the common commercial rootstocks Carrizo, Kuharske,Cleopatra, and Kinkoji. Other new hybrid rootstocks also performed well in this trial strongly affected byHLB, including the rootstock US-1516, which had the second highest cumulative yield, best tree healthrating, and lowest number of trees lost due to HLB damage. Comparison of tree performance in this trialwith a similar trial conducted prior to the HLB epidemic, allows us to estimate that the disease resultedin a 33% reduction in yield and 21% reduction in tree growth through seven years of age (Bowman et al. 2016). Use of a tolerant rootstock is suggested as an effective means of ameliorating crop losses to HLB.The U.S. Department of Agriculture has released

five citrus rootstocks that show improved tolerance to HLB for use on the Florida flatwoods[231]. They include: US-1279; US-1281, US-1282.

Rootstock performance may be dependent on the scion variety (see Castle, 2016; castle et al 2016).

[231] http://planthealth.org/article/usda-releases-five-hlb-tolerant-citrus-rootstocks-thegrower#sthash.zJPNmAOp.dpuf;

Symptomatology of HLB caused by 'C*a*. L. asiaticus' in citrus

Key HLB symptoms are given in Lin 1956, Schneider 1968a, McClean & Schwarz 1970, Tirtawidjaja 1980, Zhao 1981, da Graça 1991, Gottwald et al. 2007, Bové 2006a; also see http://www.imok.ufl.edu/events/field_days/0605/rouse.pdf:

- leaves with asymmetric, sometimes dull, blotchy-mottling that crosses leaf veins;
- mottled or complete yellowing of leaves and growing shoots (yellow shoots standing out from an otherwise normally green canopy);
- small upright, thickened, chlorotic leaves (sometimes resembling mineral deficiencies, particularly Zn);
- flushing of severely greened trees out of phase with healthy trees;
- dieback of branches;
- reduced fibrous root density (Graham et al. 2013); Las preferentially colonizes roots before leaves, where it multiplies and quickly invades leaves when new foliar flush became a sink tissue for phloem flow. This led to the discovery that roots were damaged by root infection prior to development of visible foliar symptoms and was not associated with carbohydrate starvation caused by phloem-plugging as previously hypothesized (Johnson et al 2014).
- symptomatic HLB-infected trees are much more affected by the extremes of temperature and moisture than trees without HLB with excessive leaf loss and premature fruit drop. This stress intolerance may be due to a loss of fibrous roots (Graham et al. 2013).
- vein-corking associated with ultrastructural changes to phloem (but note that CTV and boron deficiency can also cause vein-corking;
- unseasonal and heavy flowering on diseased branches;
- small, lopsided, bitter tasting fruit with small, dark and aborted seeds;
- 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;
- 'silver imprint' when finger pressure is applied to the fruit.
- underlying the multitude of symptoms for HLB disease, there are dramatic physiological and anatomical changes, which may influence the susceptibility of the host eg to Diplodia fruit rot (Zhao et al 2014, 2016) and to Phytophthora root rot (Wu et al 2014) and other pathogens;

Symptom development may be delayed for several months, or as long as 2 to 3 years after infection (Lin 1956, Zhao 1981, Capoor et al. 1974, Hung et al. 2001, Gottwald et al. 2007). Zhao (1981) noted that while some symptoms vary between different citrus species and varieties (see Appendix xx), it is not significant in a diagnostic sense.

Photographs of some of these key symptoms are presented below.

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

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

Mottled leaves left, pomelo leaves in Hong Kong (Pat Barkley, 1969); right, sweet orange leaves in Florida (GAC Beattie, 2007) with small upright, thickened, chlorotic leaves, some with green islands, and some resembling mineral deficiencies, particularly Zn.

Yellow veins (left, sweet orange leaves in Brazil (Pat Barkley, 2006); right, sweet orange leaves in Florida (GAC Beattie, 2007)

Unseasonal flowering on diseased sweet orange branches in Brazil (Pat Barkley 2006).

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

Vein-corking of pomelo leaves associated with ultrastructural changes to phloem (GAC Beattie: Việt Nam 2007).

Small, lopsided, bitter tasting fruit (left & right) with small dark and aborted seeds (right) (Pat Barkley: Brazil 2006)

'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) (Pat Barkley: Brazil 2006).

As disease severity increases, yield is reduced, mainly by the early drop of fruit from affected branches. The yield reduction can reach 30 to 100%, depending on proportion of affected canopy (Aubert et al. 1984, Bassanezi et al. 2006a) and makes the orchard economically unviable 7 to 10 years after planting (Gottwald et al. 1991, Roistacher 1996).

Compared to normal fruit, symptomatic fruit are small, light, more acidic, and have lower juice percentage, °Brix, total soluble solids per box, total soluble solids per fruit, and °Brix/acidity ratio. These effects of fruit quality were less pronounced on early and mid season sweet orange cultivars than on late season cv. Valencia (Bassanezi et al. 2009). Baldwin et al. (2010) showed that asymptomatic fruit from symptomatic trees were similar to healthy fruit for many of the quality factors measured, but that juice from asymptomatic, and especially symptomatic fruits, were often higher in the bitter compounds limonin and nomilin. However, values were generally below reported taste threshold levels, and only symptomatic fruit seemed likely to cause flavour problems. HLB is associated with pre-harvest fruit drop. Fruit that were loose on the tree and fell upon shaking the tree exhibited fungal infection of the abscission zone and altered flavor compounds indicating that the fruit and juice would be less sweet and more bitter and astringent with altered aroma compared to HLB fruit that were retained on the tree (held tightly to the tree) or compared to healthy fruit (later confirmed by sensory analyses) (Baldwin et al 2017)[232].
Baldwin et al. (2010) and Plotto et al (2010) considered it likely that the detrimental flavour attributes of symptomatic fruit (which often drop off the tree) will be largely diluted in commercial juice blends in Florida that include juice from fruit of several varieties, locations, and seasons.

Symptomatology of HLB caused by '*Ca*. L. africanus' in Citrus

Schneider (1968a) distinguished symptoms as primary and secondary. The primary symptoms appear on leaves after they mature normally, whereas the secondary symptoms appear on leafy shoots as they grow from branches with primary symptoms. Primary leaf symptoms 'do not result from immediate anatomical changes caused by the virus in the plant, but rather from secondary anatomical changes. The latter are responsible for a yellow, angular blotching, leatheriness, vein clearing, and vein yellowing. The angular blotching has been considered specific for the disease and consists of blotches of yellow on dark greenish-gray leaves. The margins of the blotches are sometimes straight when they follow veins and angular when they follow intersecting veins. Blotching occurs only on mature leaves, the symptom being found by looking back into the tree at older leaves' (Schneider 1968a).

Secondary leaf symptoms '*of greening are further removed from initial effects of 'virus' than are the symptoms designated as primary, and they occur on shoots that grow from virus-weakened branches exhibiting primary symptoms. Secondary symptoms appear during morphogenesis of leaves and consist of several chlorotic leaf patterns that are similar to evidences of mineral deficiencies in citrus, namely Zn, Fe, Mn, Ca, S, and B. Symptoms resembling those of zinc deficiency*[233] *are the most common, e.g., the leaf blade along the mid and main lateral veins is green, but the inter-veinal areas are yellow; in extreme cases, leaves remain small, erect, and yellow with green spots, as with acute zinc deficiency. Chlorotic leaves usually abscise, and the twigs that bear them die.*

On the same tree, some branches may be free from symptoms, others may show primary symptoms, and still others, secondary symptoms. The 'virus' moves slowly or not at all from one side of a mature tree to another, and systemic infection seems to depend on multiple insect inoculations' (Schneider 1968a).

McClean & Schwarz (1970) also noted two types of leaf symptoms for African HLB. '*Some leaves turn yellow along their main and secondary veins. The "yellow-vein" appearance later changes to a blotchy-mottle as the discoloration spreads away from the veins. The leaves showing this symptom*

[232] Abstract **3.b.2** **Citrus greening disease or huanglongbing (HLB) impacts on flavor compounds of oranges with compromised abscission zones with secondary infection by the fungus, *Lasiodiploida theobromae, HLB Conf Florida 2017.***

[233] Although HLB-affected citrus appear zinc deficient, zinc amendments increased the pathogen levels and shifted the microbiome (Zhang et al 2016).

are mostly the larger ones on the lower parts of branches, those on short branches back in the tree, and those on the more vigorous shoots that develop during the late summer flush. The yellow discoloration usually develops at some stage after the leaves mature.

Leaves on weak terminal twigs are small, upright and show a variety of chlorotic patterns, superficially suggestive of zinc and iron deficiencies. The small leaves are pale at first and develop secondary chlorotic patterns as they mature. They sometimes turn yellow but more often remain pale and turn green along the main and lateral veins. Sometimes green spots and green blotches develop. The intensity of these symptoms varies a lot. They may be well defined, or only slight with the small terminal leaves mostly green and only a few of them showing a slight interveinal chlorosis.'

Symptomatology varies with season (McClean & Schwarz 1970). McClean & Schwarz (1970) published an extensive collection of photographs of African HLB symptoms.

'*Ca*. L. africanus' is not as aggressive as '*Ca*. L. asiaticus' and symptoms of African greening, caused by '*Ca*. L. africanus', are less severe than Asiatic HLB, caused by '*Ca*. L. asiaticus', and the two forms can be distinguished on the basis of temperature tolerance (le Roux et al. 2006a). Leaf symptoms of African HLB are more pronounced in the cool areas, than in the low-lying hot areas and are more pronounced in winter (Schwarz 1968a).

HLB Symptoms in *Murraya*

Lopes et al. (2006a, 2009) reported that *M. paniculata,* as street trees in Brazil, show yellow leaves and shoot dieback, principally due to '*Ca*. L. americanus', and occasionally by '*Ca.* L. asiaticus'. Symptoms were present in one or more sectors of the tree canopy. Contrary to what happens in citrus, mottled leaves or fruit symptoms were not found in *Murraya*, which makes field diagnosis more difficult.

Graft inoculated *Murraya* test plants showed mineral deficiency-like patterns initially (Fig. 29), but almost disappeared after 6 months after transferring the infected plants to larger pots and applying fertilisers frequently (Lopes et al. 2010). 'Ca. L. americanus' transmission from orange jasmine to citrus was demonstrated experimentally (Gasparoto et al., 2010).

M. paniculata var. *exotica* with HLB by graft transmission in Brazil (Pat Barkley).

M. paniculata var. *exotica* with HLB following transmission by *D. citri* from a nearby HLB-infected citrus tree at South China Agricultural University in studies conducted by Deng Xiaoling: the plants were initially PCR positive for HLB when symptoms developed, but PCR negative 6 months later (GAC Beattie, 13 April 2007).

When considering host-plant specificity, distinction should be made between nymphs and adults. The latter are more catholic in their feeding habits and appear to feed on plant species unsuitable for nymphal development. A host plant is thus here defined as a plant species on which a psyllid is able to complete its development (Hodkinson 1974).

Recently the term host has also been applied by some authors to any plant on which immature or adults feed. Burckhardt et al (2014) has proposed a terminology to clarify associated plant definitions, and suggest restricting the use of the term *host-plant* to plants on which a psyllid species completes its immature to adult life cycle. For the other plant associations we suggest the terms *overwintering* or *shelter plant* (plants on which adult psyllids overwinter and on which they may feed), *food plant* (plants on which adult psyllids feed, but do not breed and do not spend an extended period of time) and *casual plant* (plants on which adult psyllids land but do not feed).

Like most insects, particularly in the rapid expansion phase after initial colonization of an area, *D. citri* occasionally might colonize extraneous hosts. Given that in truly thousands of samples of *D. citri* over the past nearly 14 years, Halbert et al. found only one or two credible samples (with nymphs) from outside Rutaceae. Halbert believes that regulating Rutaceae is sufficient (Halbert pers. comm.).

Hitchhikers are another matter entirely. Clearly, *D. citri* adults can be found in many places, both on plants, and on inanimate objects. *D. citri* came in to California from Hawaii on non-host herb shipments of malungai *(Moringa oleifera* Lam. [Brassicales: Moringaceae]) in November 2008[234], and sweet basil leaf *(Ocimum basilicum* L. [Lamiales: Lamiaceae]) January 2009); and coriander *(Coriandrum sativum* L. [Apiales: Umbelliferae] from Mexico into USA[235] (2009)).

Assuming a host relationship from the presence of one, or even hundreds, of adults is commonplace among entomologists. But such assumptions are often invalid, with many published "host records" of species being no more than "finding places" of adults with little biological significance. One questionable record was fig as a breeding host for ACP (Thomas & de Leon, 2011[236]); but CDFA deemed fig to be a host[237]. ACP is also reported to feed and survive on gallberry (*Ilex glabra*) (Martini et al 2013).

Hoffmann (1936) rated hosts, in order of preference, as lemon, huangpi or wampee, Kam, Kat (*C. reticulata*), sweet orange and pomelo.

[234] California Department of Food and Agriculture, Pest Exclusion Advisory 32-2008 – Asian Citrus Psyllid Found in Hawaiian Herb Shipment.
[235] http://www.themonitor.com/articles/pharr-32961-shipment-bridge.html
[236] Thomas D. B. & De Leon J. H. 2011 - Is the Old World Fig, Ficus carica L. (Moraceae), an Alternative Host for the Asian Citrus Psyllid, Diaphorina citri (Kuwayama) (Homoptera: Psyllidae)?. Florida entomologist 94(4): 1081-1083.
[237] http://www.cdfa.ca.gov/plant/docs/3591-21-FOE-4122012.pdf)

Table 3. Classification of Host Plants of *D. citri* the Asian Citrus Psylla (after Aubert 1987)

Perferred Host Plants	Murraya paniculata Citrus Aurantifolia	Leaf Sucking	Egg Laying	Nymphal Development
Common Host Plants	Citrus lemon	+ +	+ +	+ +
	Citrus sinensis	+ +	+ +	+ +
	Citrus medica	+ +	+ +	+ +
	Citrus nobilis	+ +	+ +	+ +
	Citrus reticulata	+ +	+ +	+ +
	Citrus deliciosa	+ +	+ +	+ +
	Microcitrus Australisiaca	+ +	+ +	+ +
	Citrus paradisi	+ +	+ +	+ +
Occasional Host Plants	Citrus hystrix	+	+	+
	Citrus grandis	+	+	+
	Triphasia trifoliata	+	+	+
	Fortunella sp.	+	+	+
	Poncirus trifoliata	+	+	+
	Murray koenigii	+	-* +	-* +
	Toddalia asictica	+	-	-
	Vepris lanceolata	+	-	-
	Corica sp.	+		
	Atalantia sp.	+	unknow	unknow
	Clausena lansium	+		

\+ occasional
\+ + usual
\+ + + very common
\- not observed in natural nor experimental conditions
*+ recently observed in Malaysia only

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From Batra et al (1970):

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Aubert B. 1987. Trioza erytrea Del Guercio and *Diaphorina citri* Kuwayama (Homoptera: Psylloidea), the two vectors of Citrus Greening Disease : Biological aspects and possible control strategies.

TABLE 1

Population incidence of leaf miner and citrus psylla in different species of citrus

Completely resistant (0-0%)	Commercially resistant (0-10%)	Slightly resistant (10-20%)	Moderately susceptible (20-40%)	Highly susceptible (40-100%)
		Citrus psylla		
—	Cleopatra Rubidoux	Orlando Kara Honey Wilking Kinnow Rangpur lime Coorg lime	Blood Red Campbell Valencia Sweet lime Pearl Dancy Savage Carrizo Severinia King Jatti Khatti	Minneola Frost Marsh Troyer Citrumello Kharna Khatta

From Chakravarthi et al 1998:

Table 1 Screening of citrus germplasm against citrus psylla

Resistant (0-5)	Moderately Resistant (>5-10)	Susceptible (>10-25)	Highly susceptible (>25)
Enter prise - 8718, Sour Orange-8751, Rangpur lime (California), Rangtra mandarin, Mandarin, Temple orange, Calamondin, Ichange lemon, Citrumelos-4475, Umatilla fangelo, *Trifista, trifoliata, on lime karna, C. hystrix, C. Karna*	Jaffa malta, Blook red mutation, Mediterranean sweet, Pera orange, Rough lemon - 8780, Rangpur lime-8744, Rangapur lime (Abohar), Marmalade orange, Sunki mandarin, Unshiu, Kinnow orange, Pummelo 21-III-10, Kara tangerine, *E. depressa, C. Wilsonii.*	Queen, Dulcis malta, Pine apple sweet orange, Homosassa Brown, Parson Brown, Cadenara fine, Egypt sweet, Carlton, Australia Sour Orange, Willow leaf sour orange, Jambheri (poona), Shomyndong, Rough lemon 8779, 8781, 8782, Limonaria, Pant lemon-I, Rangpur lime Florida, Australia, Brazilean & california, 71-64-Lee, Sunkokan, Texas ruby red, Nicholas Roy, grapefruit, Grapefruit small, Grapefruit Punjab, Japanese summer orange	Jaffa 8761, Campbell valencia sweet orange, Valenca sweet orange, Valencia late, Paper rind sweet orange, Sours dig, Rajkaipuli sour orange, Acid lime, Sweet lime, Lisbon lemon, Kunembo mandarin, Marsh white grapefruit, *C. macrophylla, C. taiwanica, C. moi, C. nakoor*

From Patil (1972):

TABLE — 2

POPULATION INCIDENCE OF CITRUS PSYLLA IN DIFFERENT VARIETIES OF CITRUS

Completely resistant i.e. completely free under natural conditions 0.0% infestation.	Completely resistant 0-10% infestation	Slightly resistant 10-25% infestation	Moderately susceptible 24-40% infestation	Highly susceptible 40-160% infestation
1	2	3	4	5
Gajanimma	Sylhet.- Citrange Morton	Nepal round	Galgal	Jamberi Kodur
Rusk Citrange	Carrizo citrange	Rangpur lime	Malta lemon	Rough lemon
Mexican lime	Rusk citrange	King of Sami	Sweet soyman lime	Pat lemon
Citron coorg	Citrange Rusk Morocco	Cleopatra	Tengelo malta	Karna Khatta
Lisbon lemon	Poin-cirus Trifoliata	Trifosta Trifiliata	Herale	Tonmyndong
Agle	Saverina	Salem Orange	Sour orange	Adajamir
	Satuma Micon Coorg	Jetta Orange	Panijamir	Lime Karna
	Rinnow Sett Malta	Italian large	Inosambi	Sweet lime
	Clive Grova sweet lime	Sadaphal	Fara Malta	Scarlet
	Orange michal	Limao Camargo	Watson pummelo	Philippine Red lime
	Palestine lime	Barnapur Nagpur	Gabbu chinoe	Baramshi lime
	White sapota	Smooth Sour Orange	Jathi Khatti	Scarlet
	Nakur-lemon	Newton Valencia	Excellior Malta	Coorg orange
	Baramashi lime	Jallindri Khatti	Blood Malta	Seville
	Grope fruit foster	Domolosis Malta	Orange East India	Mojiphal Sweet
	Martenga	Belalladikeitnulli	Assam Lemon	Pineapple
	Huilidikithulli	Sangumiro Gro	Coorg Satgudi	Sweet orange
	L-5	Long Sport Valencia	Kithilli Chethilli	Vemekapulli
	Thompson Grape Fruit	Lucknow lemon	Moogy Nimbe	Chethilli
	Grape Fruit Deshndo	Kara	Trifoliata orange	Mahlung
	Eartrong Citron	Generutenga	Watson pummelo	
	Pummelo pink flesh	Feronia Limonia	Gabbu-Chinee	
	L-8	Majorica Malta	Rajmhendri lime	
	Limao Scifameo		Mandarin Beauty of	
	Declabre Malta		Glen Retreat	
	Kodur Satgudi		Valencia orange	
	Rough lemon. Athur			
	Tengalo Wekewa			
	Coorg lemon			
	Malta hamlin			

(On page 52)

Westbrook et al. (2011) assessed 87 Rutaceae in the field for their propensity in a free choice stuation for infestations of populations of *D. citri.* The majority of test populations surveyed hosted all 3 life stages. *Casimiroa edulis* Llave et Lex (white sapote, subfamily Toddalioideae) was completely avoided by all life stages. Germplasm in the following major groups of Rutaceae did not have any significant resistance to infestations of the Asian citrus psyllid (ACP): sweet oranges, citrons, pomelos, limes, lemons, sour oranges, papedas, mandarins and hybrids among these groups including grapefruit. However, within the trifoliate group, most (but not all) accessions of *Poncirus trifoliata* as well as a number of its hybrids (×Citroncirus) were shown to have natural resistance to ACP. Two types of resistance have been identified. One of these resistance types (antixenosis) greatly reduces infestation levels of the psyllid, a resistance trait that may be related to differences in volatiles used by the psyllid to find and infest plants or the presence of a volatile that repels the psyllid. The other type of resistance (antibiosis) results in reduced longevity of psyllids, possibly related to the presence of toxic secondary plant metabolites.

Westbrook et al (2011) found that Australian native seedlings of C. (E*remocitrus) glauca, C. (Microcitrus) inodora, C. australasica* were avoided by adults as food and resting hosts; *M. inodora* had a moderately high ranking for nymphs and *M. australasica* had a moderately high rank for eggs. Seedling test populations of *Glycosmis pentaphylla* and *Clausena hernandina* were colonised less by all three life stages of ACP (Westbrook 2011).

Laboratory and greenhouse investigations confirmed that *P.trifoliata* cultivars usually are colonized less by the psyllid than are *Citrus* cultivars (Richardson & Hall 2013; Hall et al. 2015). Results of a field

survey revealed that relatively large infestation densities of the psyllid developed on conventional *Citrus* and 4 citrange (including Carrizo and C-35) cultivars but not on any of 6 *Poncirus trifoliata* cultivars. . Reduced colonization by the psyllid on *P. trifoliata* was largely a result of reduced rates of oviposition. *Poncirus trifoliata* resistance to oviposition was not observed in 4 citrange cultivars (Hall et al 2017).

Based on reduced oviposition and delays in development, *P. trifoliata* exhibits a combination of antixenosis and antibiosis host-plant resistance to*D. citri*.Acompanionplant assay showedthat thepresence of *C.macrophylla* stimulated higher oviposition rates on *P. trifoliata*, but nymph development remained retarded on *P. trifoliata* (George and Lapointe 2018*)*.

Table 2. Test populations colonized least by each life stage of *D. citri* among seedlings of 87 seed-source genotypes of *Citrus* and *Citrus* relatives surveyed in Ft. Pierce, FL.[a]

Botanical name of seed parent	Common name of seed parent	CRC	Mean rank	Mean count (0–3)
Adults				
Casimiroa edulis Llave et Lex	White Sapote	—	158.9	0.00
Poncirus trifoliata L.	"Little-Leaf" trifoliate	4007	175.4	0.06
Poncirus trifoliata L.	Simmons trifoliate	3549	179.8	0.07
Glycosmis pentaphylla (Retz.) Corr.	Orangeberry/Gin berry	3285	203.0	0.16
Microcitrus inodora (F.M. Bail) Swing.	Large leaf Australian wild lime	3785	203.2	0.14
Clausena harmandiana (Pierre) Guillaumin	*Clausena harmandiana*	4034	206.4	0.19
Eremocitrus glauca (Lindley) Swing. hybrid	Australian desert lime hybrid	4105	208.4	0.20
Citrus halimii B.C. Stone	*Citrus halimii*	3780	214.3	0.27
Microcitrus australasica (F.J. Muell.) Swing.	Australian finger lime var. Sanguinea	1484	216.2	0.24
Zanthoxylum ailanthoides L.	Japanese prickly-ash	—	216.5	0.29
Microcitrus hybrid (*M. australis* × *M. australasica*)	Sydney Hybrid	1485	224.6	0.26
Citrus leiocarpa hort ex Tan.	Koji mandarin	3147	226.0	0.25
Citrus aurantium L.	Standard sour orange	628	226.7	0.29
Nymphs				
Zanthoxylum ailanthoides L.	Japanese prickly-ash	—	91.3	0.00
Casimiroa edulis Llave et Lex	White Sapote	—	92.0	0.00
Poncirus trifoliata L.	Simmons trifoliate	3549	95.7	0.04
Glycosmis pentaphylla (Retz.) Corr.	Orangeberry/Gin berry	3285	96.9	0.03
Poncirus trifoliata L.	"Little-Leaf" trifoliate	4007	100.4	0.03
Clausena harmandiana (Pierre) Guillaumin	*Clausena harmandiana*	4034	111.2	0.15
Severinia buxifolia (Poiret) Tan.	Chinese box orange (brachytic form)	1497	159.3	0.48
Microcitrus australasica (F.J. Muell.) Swing.	Australian finger lime var. Sanguinea	1484	173.5	0.48
Eggs				
Zanthoxylum ailanthoides L.	Japanese prickly-ash	—	153.2	0.00
Casimiroa edulis Llave et Lex	White Sapote	—	154.1	0.00
Poncirus trifoliata L.	Simmons trifoliate	3549	160.3	0.04
Glycosmis pentaphylla (Retz.) Corr.	Orangeberry/Gin berry	3285	162.2	0.03
Clausena harmandiana (Pierre) Guillaumin	*Clausena harmandiana*	4034	181.3	0.23
Poncirus trifoliata L.	"Little-Leaf" trifoliate	4007	189.1	0.16
Aegle marmelos (L.) Corr.	Indian Bael fruit	3140	228.8	0.46
Microcitrus inodora (F.M. Bail) Swing.	Large leaf Australian wild lime	3785	236.3	0.64
Eremocitrus glauca (Lindley) Swing. hybrid	Australian desert lime hybrid	4105	236.4	0.50
Citrus medica L.	Indian citron hybrid	661	246.5	0.46
Citrus aurantium L.	Sour orange var. salicifolia	3289	249.7	0.43

[a]For each life stage, seed-source genotypes are listed in order of increasing colonization of their seedling test populations. Members of the Rutaceae vary greatly in their incidence of nucellar embryony (reviewed in Frost and Soost, 1968) and so some of the plants tested were essentially genetically identical to the seed parent, whereas others represent half-sib families with only the seed parent known.

Westbrook et al 2011

Hu et al (2013) found differences in susceptibility to ACP among the broad citrus groups: lemons and pommeloes were highly susceptible hosts and not significantly different to *Murraya paniculata* which was the most suitable host; mandarins, sweet oranges and tangerines were moderately susceptible hosts and cumquats and sour oranges less susceptible.

Alves et al (2014) studied the biology of *D. citri* on different varieties of juicing oranges. Valencia and orange jasmine were the most suitable hosts, whereas Hamlin was least suitable for the development of *D. citri*. Borgoni et al 2014 concluded that cultivars of sweet orange are the most susceptible genotypes to D. citri, mainly cultivar 'Pera'.

Choisya ternata and *C. arizonica* all were found to be feeding hosts for the psyllid. Egg laying was found on torchwood and egg laying and nymphal development were found on C. ternata. http://research.citrusrdf.org/reports/2013/01/18/progress-report-final_Alternative_host_16.pdf

Beloti VH, Alves G, Coletta-Filho H, Yamamoto P. The Asian citrus psyllid host *Murraya koenigii* is immune to citrus Huanglongbing pathogen 'Candidatus Liberibacter asiaticus'. Phytopathology. 2018 Apr 12(ja).

Table 1. Known Aurantioideae: Aurantieae species on which the Asiatic citrus psyllid, *Diaphorina citri*, has been observed to rest, feed or complete its development[239].

Species or hybrid	Cited as	Common name	Original citations	Summary of observations
Aegle marmelos (L.) Corr.	*Aegle marmelos* (L.) Corr.	bael	Khan & Borle 1989; Viraktamath & Bhumannavar 2001	feeding (Khan & Borle)
Aeglopsis chevalieri Swingle	*Aeglopsis chevalieri* Swingle		Koizumi et al. 1996	survival for more than 5 weeks but no increase in numbers
Afraegle gabonensis (Swingle) Engl.	*Afraegle gabonensis* Engl.	Gabon powder-flask-fruit	Halbert & Manjunath 2004; Halbert pers. comm.	nymphs found in survey in Florida arboretum
Afraegle paniculata (Schumach. & Thonn.) Engl.	*Afraegle paniculata* (Schaum.) Engl.	Nigerian powder-flask-fruit, citron d'éléphant	Halbert & Manjunath 2004; Halbert pers. comm.	nymphs and eggs found in survey in Florida arboretum
Atalantia buxifolia (Poir.) Oliv.	*Atalantia buxifolia* (Poiret) D. Oliver	Chinese box-orange	Xu et al. (1988b)	survives and propagates normally
	Severinia buxifolia (Poiret) Ten.	Chinese box-orange	Koizumi et al. 1996; Hung et al. 2001; Halbert & Manjunath 2004; Halbert pers. comm. Hu & Brlansky 2014	survival for more than 7 weeks but no increase in numbers (Koizumi); normal development in the laboratory (Xu et al.); cited as suitable host (Hung et al.); damage evident, eggs, nymphs and adults observed in Florida surveys (Halbert & Manjunath)
Atalantia monophylla (L.) Corr. Serr.	*Atalantia monophylla* (L.) Corr.	Indian atalantia	Halbert & Manjunath 2004; Halbert pers. comm.	adult observed in survey in Florida arboretum
Balsamocitrus dawei Stapf	*Balsamocitrus dawei* Stapf.	Uganda powder-flask-fruit	Koizumi et al. 1996	survival for more than 7 weeks but no increase in numbers
Citropsis articulata (Spreng.) Swingle & M. Kellerman	*Citropsis schweinfurthii* (Engl.) Swingle & Kellerm.	West African cherry-orange	Capoor et al. 1967; Chavan & Summanwar 1993[240]; Chavan 2004	abundant source (Capoor et al. 1967); good host (Chavan & Summanwar); completes development; moderate host (Chavan)

[239] Good photographs of some Australasian species, excluding those from New Caledonia, can be viewed on Mike Saalfeld's website (http://www.saalfelds.freeserve.co.uk/HobbyCitrusGrowers.htm).

[240] The Chavan & Summanwar 1967 paper is not about Citropsis but rather is about Triphasia. They misidentified the plant that they were working with (Smith pers. comm. 24 March 2016).

Citropsis gilletiana Swingle & M. Kellerman	*Citropsis gilletiana* Swingle & M. Kellerman	Gillet's cherry-orange	Halbert & Manjunath 2004; Halbert pers. comm.	eggs, nymphs and adults observed in survey in Florida arboretum; high populations prevented bud development
Citrus amblycarpa (Hassk.) Ochse	*amblycarpa* (Hassk.) Ochse (a possible hybrid)	nasnaran	Halbert pers. comm.	eggs observed in Florida arboretum survey
Citrus australasica F. Muell.	*Microcitrus australasica* (F.J. Muell.) Swingle	Australian finger-lime	Koizumi et al. 1996; Halbert & Manjunath 2004; Halbert pers. comm.	no multiplication, but adult survival for 5 weeks (Koizumi); damage evident, adults only present, in survey in Florida arboretum (Halbert & Manjunath)
	Microcitrus australisiaca (sic)	Australian finger-lime	Aubert 1987a, b, 1990a;	full development in laboratory (Aubert)
Citrus australis (Mudie) Planch.	*Microcitrus australis* (Planch.) Swingle	Australian round lime, dooja	Halbert & Manjunath 2004; halbert pers. comm.	damage evident, and eggs and nymphs observed, in survey in Florida arboretum
Citrus cavaleriei H. Léveillé ex Cavalerie	*Citrus ichangensis* Swingle	Ichang (Yichang) papeda	Halbert pers. comm.	slight damage observed in Florida arboretum survey
Citrus glauca (Lindl.) Burkill	*Eremocitrus glauca* (Lindley) Swingle	Australian desert lime	Koizumi et al. 1996	variable adult survival but no multiplication and death after 4 weeks
Citrus glauca × Shakura *Citrus reticulata*	*Eremocitrus* hybrid		Halbert & Manjunath 2004; Halbert pers. comm.	eggs present and damage evident in survey in Florida arboretum
Citrus hystrix DC	*Citrus hystrix* DC.	leech lime, limau purut, limau hantu, kaffir lime, Mauritius papeda	Aubert 1987a, b, 1990a, 1992; Lim et al. 1989, 1990, Osman & Lim 1992	complete development and an occasional or common field host in Asia (Aubert); common (Lim et al. 1989, 1990); good field host in Malaysia (Osman & Lim 1992)
Citrus inodora F. M. Bailey		Russell River lime, large-leaf Australian wild lime	Halbert pers. comm.	slight damage in Florida arboretum survey
Citrus japonica Thunb.	*Fortunella crassifolia* Swingle	kumquat (Meiwa)	Halbert & Manjunath 2004; Halbert pers. comm.	good host, with eggs, nymphs and adults observed in survey in Florida arboretum
	Fortunella hindsii (Champ. ex Benth.) Swingle	Hong Kong kumquat	Halbert & Manjunath 2004, Li et al. 2007	some psyllid damage and some adults in Florida arboretum surveys (Halbert & Manjunath): host (Li et al.)
	Fortunella japonica		Li et al. 2007	host
	Fortunella margarita (Lour.) Swingle	kumquat	Halbert & Manjunath 2004; Halbert pers. comm.	nymphs in survey in Florida arboretum
	Citrus japonica Thunb.	kumquat	Halbert pers. comm.	few nymphs and adults with little

				damage in Florida arboretum
	Fortunella polyandra (Ridley) Tanaka	kumquat	Halbert & Manjunath 2004; Halbert pers.comm.	eggs, nymphs and adults observed in survey in Florida arboretum
	Fortunella sp.	kumquat	Aubert 1987a, b, 1990a	complete development in laboratory cage studies and an occasional field host in Asia
Citrus maxima (Burm.) Merr.	pomelo	pomelo	Flecther 1919	In India (North-West Frontier Province, Lyallapur, Pusa, Poona and Coimbatore), usually a minor pest, sometimes occurring in large numbers and doing considerable damage
	Citrus decumana L.	pomelo, pummelo	Husain & Nath 1927	attacked
	Citrus grandis (L.) Osbeck	pomelo, pummelo	Hoffmann 1936; Aubert 1987a, b; Rao & Pathak 2001	least most favoured of 6 hosts (Hoffmann); complete development and an occasional field host in Asia (Aubert); moderate incidence (Rao & Pathak)
	Citrus maxima (Burm.) Merr.	pomelo, pummelo	Catling 1968; Aubert 1990a	attacked (Catling); complete development and an occasional or common field host in Asia (Aubert)
	Citrus obovoidea Hort. ex Tanaka cv 'Kinkoji'	pomelo kinkoji	Halbert & Manjunath 2004	survey in Florida
Citrus medica L.	*Citrus medica* L.	citron	Aubert 1987a, b, 1990a; Rao & Pathak 2001	complete development and a common field host in Asia (Aubert); some on 'Gandharaj' (Rao & Pathak)
	Citrus medica medica	citron	Husain & Nath 1927	attacked
Citrus reticulata Blanco	*Citrus deliciosa* Tenore	mandarin	Aubert 1987a, b	complete development and a common field host in Asia
	Citrus depressa Hayata	flat lemon	Yasuda et al. 2005	host in Okinawa
	Citrus nobilis Loureiro	kam	Hoffmann 1936	third most favoured of 6 hosts
	Citrus nobilis Loureiro var. *deliciosa* (Ten.) Swingle[241]	kat	Hoffmann 1936	fourth most favoured of 6 hosts
	Citrus reticulata Blanco	mandarin	Aubert 1987a, b, 1990a; Catling 1968; Koizumi et al. 1996; Rao & Pathak 2001;	complete development and a common field host in Asia (Aubert); attacked (Catling);

[241] But possibly *C.* × *aurantium* 'King orange', often called, incorrectly, 'King mandarin'.

			Halbert & Manjunath 2004	survival for more than 7 weeks but no increase in numbers on 'Som-pan' (Koizumi); good on 'Khasi' (Rao & Pathak); common in surveys in Florida (Halbert & Manjunath)
Citrus trifoliata L.	*Poncirus trifoliata* (L.) Raf.	trifoliate orange	Aubert 1987a, b, 1990a, 1992; Koizumi et al. 1996 Westbrook et al (2011)	occasional host with oviposition but no nymphal development in laboratory cages (Aubert); survival for more than 7 weeks but no increase in numbers (Koizumi); very low levels on 2 clones under field conditions in Florida (Westbrook)
Citrus wintersii Mabb.	*Microcitrus papuana* H.F. Winters	Brown River finger lime	Halbert & Manjunath 2004; Halbert pers. comm.	damage evident, nymphs and adulys observed, in survey in Florida arboretum
Citrus sp.	*Citrus limmettoides*	sweet lime	Rao & Pathak 2001	moderate
Citrus spp.	*Citrus* spp.		Kuwayama 1908; Crawford 1912, 1913; Lal 1917, 1918, Miyatake 1965; Aubert 1990a; Viraktamath & Bhumannavar 2001; Halbert & Manjunath 2004	host (Kuwayama); common host (Crawford); Spraying with ½ pint of crude oil in 4 gals. of water proved effective against a Psyllid, *Euphalerus citri*, on *Citrus* (Lal 1917); Spraying in March and June with crude oil and tobacco decoction was successfully tried against the Psyllid, *Euphalerus citri*, which has been causing great damage to *Citrus* (Lal 1918); uncommon host (Miyatake); common host (Aubert, Viraktamath & Bhumannavar, Halbert & Manjunath)
Citrus × *aurantiifolia* (Christm.) Swingle	lime	lime	Fletcher 1919	In India (North-West Frontier Province, Lyallapur, Pusa, Poona and Coimbatore), usually a minor pest, sometimes occurring in large numbers and doing considerable damage
	Citrus aurantifolia (Christm.) Swingle	lime, kagzi lime	Catling 1968; Aubert 1987a, b, 1990a; Rao & Pathak 2001; Halbert & Manjunath	attacked (Catling); complete development and a preferred field host in Asia (Aubert); zero population (Rao

			2004; Halbert pers. comm.	& Pathak); preferred host in Florida surveys (Halbert & Manjunath); heavy psyllid damage
	Citrus pennivesiculata (Lush.) Tanaka	moi		moderate damage in Florida arboretum survey
Citrus × *aurantium* L.	orange	orange	Fletcher 1917, 1919	'*Euphalerus citri* is psyllid found commonly on orange plants in India. It is usually a minor pest, occasionally occurring in large numbers' (Fletcher 1917; In India (North-West Frontier Province, Lyallapur, Pusa, Poona and Coimbatore), usually a minor pest, sometimes occurring in large numbers and doing considerable damage (Fletcher 1919)
	Citrus aurantium L.	sour orange, karun jamir	Rao & Pathak 2001	moderate (Rao & Pathak);
	Citrus aurantium L.	Chinotto	Halbert & Manjunath 2004; Halbert pers. comm.	eggs, nymphs and adults, with damage evident, in Florida surveys (Halbert & Manjunath)
	Citrus maxima var *racemosa*		Aubert 1990	occasional in field, with complete development
	Citrus nobilis Lour.	not cited	Aubert 1987a, b	common
	Citrus × *nobilis* Lour.	not cited	Halbert & Manjunath 2004	common in Floida surveys (Halbert & Manjunath)
	Citrus × *paradisi* Macfad.	grapefruit	Aubert 1987a, b;; Tsai & Liu 2000; Halbert & Manjunath 2004	occasional in field, with complete development (Aubert), best host in laboratory (Tsai & Liu); common and a preferred host in Florida (Halbert & Manjunath)
	Citrus sinensis (L.) Osbeck	sweet orange (navel & Valencia); soh nariang	Husain & Nath 1927; Hoffmann 1936, Catling 1968; Aubert 1987a, b, 1990a; Rao & Pathak 2001; Halbert & Manjunath 2004	attacked (Husain & Nath); fifth most favoured of 6 hosts (Hoffmann); attacked (Catling); complete development and a common field host in Asia (Aubert) moderate (Rao & Pathak); common in Florida surveys (Halbert & Manjunath)

	Citrus sulcata hort. ex I. Takahashi	sanbokan	Halbert pers. comm.	heavy psyllid damage in Florida arboretum survey
	Citrus tamurana hort. ex Tanaka	Hyuganatsu pomelo	Halbert pers. comm.	a host
Citrus × *junos* Siebold ex Tanaka (possibly a *C. cavaleriei* H. Léveillé ex Cavalerie (syn. *C. ichangensis* Swingle) x *Citrus reticulata* Blanco hybrid)		yuzu	Halbert pers. comm.	some psyllid damage in Florida arboretum survey
Citrus × *limon* (L.) Osbeck	lemon	lemon	Fletcher 1919	In India (North-West Frontier Province, Lyallapur, Pusa, Poona and Coimbatore), usually a minor pest, sometimes occurring in large numbers and doing considerable damage
	Citrus assamensis S. Dutta & S.C. Bhattach.	adajamir	Rao & Pathak 2001	good host
	Citrus limon (L.) Burm. f.	lemon	Catling 1968; Aubert 1987a, b; 1990a	attacked (Catling); complete development and a common field host in Asia (Aubert)
	Citrus limonia Osbeck	lemon	Hoffmann 1936	first most favoured of 6 hosts (Hoffmann)
	Citrus medica var. *acida* Hook. f.	sour lime, khatta	Husain & Nath 1927	attacked
	Citrus medica var. *limetta*	sweet lime, mitha	Husain & Nath 1927	attacked
	Citrus medica var *limonum* Hook. f.	lemon, limu	Husain & Nath 1927	attacked
	Citrus meyeri U. Tan	Meyer lemon	Halbert & Manjunath 2004	survey in Florida
Citrus × *microcarpa* Bunge	*Citrus madurensis* Lour.	calamondin	Aubert 1990a; Osman & Lim 1992	complete development and a common host plant in Malaysia (Aubert; Osman & Lim)
Citrus × *taitensis* Risso	*Citrus jambhiri* Lushington	rough lemon; east (Estes?) rough lemon	Rao & Pathak 2001; Halbert & Manjunath 2004	some to moderate (Rao & Pathak); surveys in Florida (Halbert & Manjunath)
Citrus × *virgata* Mabb.	*Microcitrus* sp. 'Sydney'	Sydney hybrid	Halbert & Manjunath 2004; Halbert pers. comm.	damage evident, eggs and nymphs observed, in survey in Florida arboretum
Limonia acidissima L.	*Limonia acidissima* L.	Indian wood apple, elephant apple, wood apple	Khan & Borle 1989; Koizumi et al. 1996; Hung et al. 2000	all stages present (Khan & Borle); marked in increase in populations (Koizumi); suitable host (Hung)
Merrillia caloxylon (Ridley) Swingle	*Merrillia caloxylon* (Ridley) Swingle	kamuning, katinga, ketengah, Malay lemon	Lim et al. 1990a,b	cage in laboratory (Lim et al.)

Murraya elongata/intermedia			Beattie, Namgay	Field at Baychu, Bhutan
Murraya paniculata (L.) Jack (*sensu lato*)	*Murraya exotica* L.[242]	jasmine orange, orange-jessamine	Catling 1968; Khan & Borle 1989; Aubert 1990a, Koizumi et al. 1996; Yasuda et al. 2005	breeding field populations in the Philippines (Catling); all stages present on 'Kunti or China box' (Khan & Borle) adult feeding in laboratory cages (Aubert); marked increases in populations (Koizumi); a preferred host in southern China (Beattie pers. observation, Guangzhou, 2006) where, probably erroneously, only '*M. paniculata*' has been reported as a host (see Yang et al. 2006); a host in Okinawa (Yasuda et al.)
	Murraya paniculata (L.) Jack	jasmine orange, orange-jessamine	Maki 1915; Kuwayama 1931; He (= Hoffmann) & Zhou 1935; Miyatake 1965; Cheema & Kapur 1975; Singh & Nimbalkar 1977; Catling et al. 1978; Tirtawidjaja 1981; Aubert & Quilici 1984; Tsai et al. 1984; Aubert 1987a, b, 1990a; Osman & Lim 1992; Koizumi et al. 1996; Tsai et al. 2000, 2002; Halbert & Manjunath 2004	enumerated as one of the food-plants in Tawian (Maki, Kuwayama); noted as an alternative host (He & Zhou); many nymphs and adults observed on plants over several years (Miyatake); new host (Cheema & Kapur); preferred host (Singh & Nimbalkar); all stages frequently observed in Bangladesh (Catling et al.); vector in transmission studies (Tirtawidjaja); favourable host on 'green hedge plant' (Tsai et al.); suitable host in Reunion with up to 11 generations in a laboratory and 10 in the field (Aubert & Quilici); complete development and a preferred field host in Asia (Aubert); all stages (Osman & Lim); marked in increase in populations (Koizumi); host on which most abundant (Tsai et al.

[242] As noted previously, the common cultivated ornamental form of orange jasmine is considered to be *Murraya paniculata* (L.) Jack var. *exotica* (*sensu* Huang), unless otherwise stated. The status of the species is complex and resolution of this uncertainty is the objective of a PhD being undertaken at the University of Western Sydney by Nguyen Huy Chung.

				2000); present in all seasons in Florida (Tsai et al. 2002); preferred host in Florida surveys (Halbert & Manjunath)
Naringi crenulata (Roxb.) Nicolson	*Naringi crenulata* (Roxb.) Nicolson	hesperethusa	Halbert & Manjunath 2004; Halbert pers. comm.	heavy infestation in survey in Florida arboretum
Pamburus missionis (Wight) Swingle	*Atalantia missionis* (Wall. ex Wight) Oliv.		Tirtawidjaja 1981	successful feeding and transmission
	Pamburus missionis (Wall. ex Wight) Swingle		Halbert & Manjunath 2004; Halbert pers. comm.	eggs, nymphs and adults observed in survey in Florida arboretum
Swinglea glutinosa (Blanco) Merr.	*Swinglea glutinesa*	tabog	Aubert 1990a; Waterhouse 1998	feeding in laboratory cage studies
	Swinglea glutinosa (Blanco) Merr.	tabog	Tirtawidjaja 1981; Halbert & Manjunath 2004; Halbert pers. comm.; ACIAR/UWS/UGM field observations	successful feeding and transmission (Tirtawidjaja); damage evident, eggs and nymphs observed, in Florida surveys (Halbert & Manjunath); feeding, oviposition and development (ACIAR/UWS/UGM)
Triphasia trifolia (Burm. f.) P. Wilson	*Triphasia trifoliata*	limeberry, triphasia	Aubert 1987a, b, 1990a; Osman & Quilici 1991; Koizumi et al. 1996; Waterhouse 1998	complete development in laboratory cage studies and an occasional field host in Asia (Aubert); complete development in cage studies (Osman & Quilici); no increase in populations but survival for 5 weeks (Koizumi et al.)
	Triphasia trifolia (Burm. f.) P. Wilson	limeberry, triphasia	Aubert 1987a; Koizumi et al. 1996; Halbert & Manjunath 2004; Halbert pers. comm.	occasional host (Aubert), adult survival for several weeks but no increase in numbers (Koizumi et al.); adults plentiful, all stages present, and damage evident in Florida surveys (Halbert & Manjunath)
Uncertain (possibly a species or species of *Atalantia*, *Citrus*, *Pamburus*)	*Atalantia* sp.		Koizumi et al. 1996; Aubert 1978 a, b, 1990a; 1992	marked increase in populations (Koizumi); adult feeding but oviposition and development not known (Aubert) — record based on comments by Zhao Xueyuan as reported by Barkley et al. (1979)

Table 2. Known Aurantioideae: Clauseneae species on which the Asiatic citrus psyllid, *Diaphorina citri*, has been observed to rest, feed or complete its development.

Species or hybrid	Cited as	Common name	Original citations	Summary of observations
Bergera koenigii L.	*Murraya euchrestifolia* Hayata	curry leaf	Hung et al. 2000	suitable host
	Murraya koenigii (L.) Spreng.	curry leaf	Fletcher 1917, 1919; Husain & Nath 1927; Chakraborty et al. 1976; Singh & Nimbalkar 1977; Aubert 1987a, b,, 1990a; Osman & Lim 1989; 1990; 1992; Osman & Quilici 1991; Lim et al. 1990a; Chavan & Summanwar 1993; Koizumi et al. 1996; Halbert & Manjunath 2004	alternative food plant (Fletcher; Husain & Nath); found on shoots (Fletcher 1919); when given a choice all adult psyllids migrated from citrus seedlings to curry leaf seedlings on which successful breeding colonies were established and maintained (Chakraborty et al.); preferred host (Singh & Nimbalkar) variable host with no or limited development (Aubert 1987a, b); a good host (Aubert 1990a); an important alternative host (Osman & Lim); an important alternative host (Lim et al.); good population growth (Osman & Quilici); infests and breeds throughout the year (Chavan & Summanwar); some increase in population (Koizumi); not an excellent host in Florida surveys but will support a small population (Halbert & Manjunath)
Clausena anisumolens Merrill	*Clausena anisum-olens* Merrill	kayumanis (Tagalog)	Gavarra & Mercado 1988; Aubert 1990a	feeding, and seemingly more attractive than *M. paniculata*, but **no** oviposition in cage tests (Gavarra & Mercado 1988; Aubert 1990a)
Clausena excavata Burm f.	*Clausena excavata* Burm. f.		Aubert 1990a; Lim et al. 1990a; Osman & Lim 1992	complete development (Aubert); important

				alternative host (Lim et al.; Osman & Lim)
Clausena indica Oliv.	*Clausena indica* Oliver		Tirtawidjaja 1981	a successful feeding and transmission (Tirtawidjaja)
Clausena lansium (Lour.) Skeels	*Clausena lansium* (Lour.) Skeels	wong p'ei, wampee, huangpi	Hoffmann 1936, Xu et al. 1988b; Koizumi et al. 1996; Aubert 1990a; Halbert & Manjunath 2004; Halbert pers. comm.	second most favoured of 6 hosts (Hoffmann); survives and propagates normally (Xu et al.); variable adult survival but no multiplication (Koizumi et al.); complete development and a common field host in Asia (Aubert); eggs, nymphs and adults with populations highly variable in Florida surveys (Halbert & Manjunath; Halbert pers. comm.)
Clausena harmandiana(Pierre)Guillaumin			Westbrook et al 2011	Lowest susceptibility for all 3 life stages

Seedling test populations of *Glycosmis pentaphylla* and *Clausena harmandiana*, members of Swingle's "remote citroid fruits," also were colonized less by all three life stages of *D. citri*. These species are sexually incompatible

Westbrook et al 2011

Table 3. Known species of Rutoideae on which the Asiatic citrus psyllid, *Diaphorina citri*, has been observed to rest, feed or complete its development.

Species or hybrid	Cited as	Common name	Original citations	Summary of observations
Amyris madrensis S. Wats.	*Amyris madrensis*	mountain torchwood	Sandoval 2009[243]	feeding, oviposition, and partial development
			Setamou et al 2016	adult psyllids laid eggs which hatched, but no successful nymphal development
Amyris texana			Setamou et al 2016	adult psyllids laid eggs which hatched, but no successful nymphal development
Choisya dumosa var. *arizonica* (Standl.) L. Benson	*Choisya arizonica*	Arizona orange	Sandoval 2009[244]	feeding, oviposition, development of nymphs to adults

[243] pers. comm. January 2010 and http://www.tamiu.edu/pathways/documents/PATHWAYSONLINEPROGRAM_000.pdf.
[244] pers. comm. January 2010 and http://www.tamiu.edu/pathways/documents/PATHWAYSONLINEPROGRAM_000.pdf. See also, da Graça 2009: https://www.fritolayag.com/public/HLB/John_DaGraca_2009_2.pdf.

Choisya arizonica			Setamou et al 2016	can serve host plants
Choisya ternata Kunth	*Choisya ternata*	Mexicn orange blossom	Sandoval 2009[245]	feeding, oviposition, development of nymphs to adults
			Setamou et al 2016	can serve host plants
Esenbeckia berlandieri Baill. ex Hemsl.	*Esenbeckia berlandieri* Baill. ex Hemsl.	Berlandier's jopoy	Sandoval 2009[246] Setamou et al 2016	feeding but no oviposition
Ravenia spectabilis (Lindl.) Planch. ex Grisbeg. (syn. *Lemonia spectabilis* Lindl.)			Richard Lee and Susan Halbert (pers. comm., November 2008)	adults relatively easy to find on plants in Fairchild Botanic Gardens, Miami, Florida
Tetradium ruticarpum (A. Juss.) T. G. Hartley[247]	*Evodia rutaecarpa* (A. Juss.) Benth.	evodia, wu zhu yu	He (2000) cited by Yang et al. (2006)	Record not verified and *Evodia* misspelt as *Euodia*
Toddalia asiatica (L.) Lamarck	*Toddalia asiatica* (L.) Lam.	orange-climber, forest pepper	Aubert 1987a, b, 1990a, 1992	feeding but no oviposition in cage studies
Vepris lanceolata (Lam.) G. Don	*Vepris lanceolata* G. Don	white ironwood	Aubert 1987a, b, 1990a, 1992	feeding but no oviposition in cage studies
Zanthoxylum fagara (L.) Sarg.	*Zanthoxylum fagara* (L.) Sarg.	lime prickly-ash	Halbert & Manjunath 2004	plenty of suitable new shoots in Florida arboretum survey; very few *D. citri* found; possible non-host.
			Setamou et al 2016	adult psyllids laid eggs which hatched, but no successful nymphal development
Zanthoxylum ailanthoides			Westbrook et al 2011	Only a host to adults

Sandoval et al[248] and Setamou et al (2016) using no-choice and choice experiments. *D. citri* was found to successfully colonize and reproduce on *Choisya ternatea*, *C. arizonica*, and *Helietta baretata* in no-choice tests, but reverted back to its preferred hosts, orange jasmine and curry leaf, in choice tests. On some of the other plant species (*Amyris madrensis*, *A. texana*, and *Zanthoxylum fagara*), adult psyllids laid eggs which hatched but no nymphal development was recorded beyond

http://oal.ca.gov/res/docs/pdf/emergency_postings/2010-0720-01E.pdf; da Graca JV. 2010. Etiology, history and world situation of citrus huanglongbing at http://www.cesavecol.com.mx/archivos/2010/taller%20hlb%20merida/John%20Da%20Graca%20Merida_paper%5B1%5D.pdf

[245] pers. comm. January 2010 and http://www.tamiu.edu/pathways/documents/PATHWAYSONLINEPROGRAM_000.pdf. See also, da Graça 2009: https://www.fritolayag.com/public/HLB/John_DaGraca_2009_2.pdf

[246] pers. comm. January 2010 and http://www.tamiu.edu/pathways/documents/PATHWAYSONLINEPROGRAM_000.pdf

[247] Inoue et al. (2006) recorded *Psylla evodiae* Miyatake feeding on *M. paniculata* in Japan, noting that it also occurs on *Tetradium glabrifolium* (Champ. ex Benth.) T. G. Hartley (cited as *Euodia meliifolia* (Hance) Benth.) and feeds on '*Zanthoxylum beechyanum* var. *alatum* (Nakai) Hara'.

[248] https://www.plantmanagementnetwork.org/proceedings/irchlb/2011/presentations/IRCHLB_2011_11.2.pdf

the first instar. No reproduction occurred on *Esenbeckia berlandieri*, *Ptelea trifoliate,* nor *Casimiroa edulis*, although adult psyllids were able to survive on these species for several days.

Yellow chapote (Casimiroa greggii (S. Watson) F. Chiang) (Rutaceae)) is NOT a host for ACP. Setamou tested it in choice (with citrus) and no choice tests, and although adult feeding may occur and survival on that plant, there is no reproduction of ACP on yellow chapote (Setamou, pers. comm., Setamou et al 2016).

An ACP adult from a native rutaceae, turpentine broom (*Thamnosma montana*) in California (Brian Taylor CRB)

Arredondo Valdés R, Delgado Ortiz JC, Beltrán Beache M, Anguiano Cabello J, Cerna Chávez E, Rodríguez Pagaza Y, Ochoa Fuentes YM. 2016. A review of techniques for detecting Huanglongbing (greening) in citrus. Canadian Journal of Microbiology 62(10):803-11.

As the National Academies of Sciences, Engineering, and Medicine (2018) wrote in *A Review of the Citrus Greening Research and Development Efforts:*
Molecular and serological diagnostic technologies for CLas are ultrasensitive but, on their own, are not ideal for epidemiological and regulatory purposes because of uneven pathogen distribution in the tree.
No single diagnostic method will be sufficient to identify recently infected trees.
Detection of infection prior to symptom development is possible through detection of changes in host metabolites and volatiles.

DNA Hybridisation and PCR
"A quantitative PCR-(qPCR)-based assay (Li et al., 2006) for amplifying CLas 16S RNA has become the standard assay accepted by many laboratories and, more importantly, by regulatory agencies to provide an initial determination of CLas infection. This is followed by conventional PCR assays and DNA sequencing for final verification. Although many reports have been published in the past decade on other methods to detect CLas in plant and insect tissues (Valdes et al., 2016; Ghosh et al., 2017), none of the mechanistically similar technologies (e.g., digital PCR, immunoblots, LAMP, CANARY) have proven to be more sensitive than qPCR. Furthermore, since qPCR can detect as little as one copy of bacterial DNA the issue for CLas detection is not the sensitivity of bacterial detection but rather the uneven spatial and temporal distribution of the pathogen in trees and insects (Tatineni et al., 2008; Li et al., 2009; Kunta et al., 2014; Louzada et al., 2016)" (NAS ***Review of the Citrus Greening Research and Development Efforts)***.

With the advent of DNA hybridisation and PCR, pathogens could be detected, and 'strains' distinguished (Jagoueix et al. 1997). Cloned HLB-DNA fragments In-2.6 and As-1.7, used as DNA probes, were able to specifically detect the '*Ca*. L. asiaticus' and '*Ca*. L. africanus', respectively (Villechanoux et al. 1992, Planet et al. 1995). Dot-blot hybridisation was thus the first molecular technique used to detect the HLB bacteria in citrus. Finally, In-2.6 and As-1.7 (Hocquellet et al. 1999) were used to define a pair of PCR primers, *rpl* A2 and *rpl* J5, which yield a 667bp amplicon with '*Ca*. L. africanus', and a 701bp amplicon with the '*Ca*. L. asiaticus' (Bové 2005). An additional primer set specific to '*Ca*. L. asiaticus' was developed, based on partial sequence of the h-operon (Hung et al. 1999a,b, 2004).

Because '*Ca*. Liberibacter' has not been cultured, only a few fragments of their genomic DNA have been cloned and sequenced (Jagoueix et al. 1994, 1997, Hung et al. 1999a,b, Subandiyah et al. 2000a, Villechanoux et al. 1993, Coletta-Filho et al. 2005, Teixeira et al. 2005c). The best characterised regions are the 16S rDNA and the 16S/23S intergenic regions (Jagoueix et al. 1994, 1997, Subandiyah et al. 2000a, Coletta-Filho et al. 2005, Teixeira et al. 2005c). The sequences of the 16S rDNA are highly conserved among the species of '*Ca*. Liberibacter spp.', but variation is sufficient to design primers capable of detection and identification of the bacterium in conventional PCR assays (Jagoueix et al. 1996, Coletta-Filho et al. 2005, Teixeira et al. 2005c).

'Molecular characterisation of the Asiatic and African HLB bacteria was reported in 1994 and 1997 (Jagouiex et al. 1994, 1997). The gene coding for 16S ribosomal RNA (16S rDNA) was obtained by

PCR amplification with universal primers fD1/rP1, and sequenced. The HLB 16S rDNA sequences were used for the phylogenetic and taxonomical characterizations. The HLB bacteria were found to represent a new bacterial genus within the Gram negative α-Proteobacteria: the genus '*Candidatus* Liberibacter' ('*Ca*. L.'), with two forms: '*Ca*. L. asiaticus' for the Asiatic HLB form, and '*Ca*. L. africanus' for the African form. Forward primers OIn1 + 0Af1, and reverse primer OIn2c were designed from the HLB 16S rDNA sequences. With both '*Ca*. L. asiaticus' and '*Ca*. L. africanus', the same 1160bp amplicons are obtained. However, the amplicon from '*Ca*. L. asiaticus' has one *Xba* 1 restriction site and yields two fragments upon digestion, while '*Ca*. L. africanus' has two such sites and yields three fragments, making liberibacter identification straight forward. With either one of the two primer pairs, *rpl* A2 / *rpl* J5 or [OIn1 + 0Af1] / OI2c, '*Ca*. L. asiaticus' and '*Ca*. L. africanus' can be detected most reliably in leaves showing HLB blotchy mottle, but not in symptomless leaves. These PCR methods make it possible to confirm HLB in suspicious trees, but they should not be used for indexing purposes' (Bové 2005).

Primers f-GB1 and r-GB3 were developed from the 16S rDNA sequence of the new form '*Ca.* L. americanus' (Teixeira et al. 2005a,b,c). These primers are specific for '*Ca*. L. americanus', as they do not detect '*Ca*. L. asiaticus' and '*Ca*. L. africanus'. A duplex PCR method, with primers GB1/GB3 and *rpl*A2/*rpl*J5 for the simultaneous detection of '*Ca*. L. americanus' and '*Ca*. L. asiaticus' in the same PCR tube, has been developed and used routinely (Bové 2005).

Loop-mediated isothermal amplification (LAMP) was developed for '*Ca*. L. asiaticus' detection in laboratories that lack thermocyclers (Okuda et al. 2005). LAMP was adapted for the detection of Candidatus Liberibacter asiaticus by Rigano et al (2014). This methodology was combined with a Lateral Flow Dipstick (LFD) device for visual detection of the resulting amplicons, eliminating the need for gel electrophoresis. The assay was highly specific for the targeted bacterium. No cross-reaction was observed with DNA from any of the other phytopathogenic bacteria or fungi assayed. This sensitivity level was proven to be similar to the values obtained running a real time PCR in parallel. This methodology was able to detect '*Ca*. L. asiaticus' from different kinds of samples including infected citrus plants and psyllids.

A RealAmp assay developed in China is claimed to be low sensitive compared to real-time PCR, no expensive reagents and equipments are required in the assay compared to conventional real-time PCR. A portable fluorescent reader (ESE-Quant Tube Scanner) is sufficient to run a RealAmp assay. Futhermore, the developed closed-tube visual inspection is a qualitative detection technique, which could judge the results by naked eyes. Accordingly, the risk of cross-contamination is minimized in a closed tube detection system, which facilitates high-throughput application. Therefore, the technique is an alternative quantitative detection method, which will be used for a routine detection service for the Las. Taking into account the uneven distribution of HLB in plant tissue and the advantages of RealAmp assay, Wu et al (2016) suggested multiple-spot sampling around a citrus tree used for RealAmp assay detection to avoid missing detections in field surveys.

Although conventional PCR and LAMP methods are sensitive and specific, consistent detection of HLB pathogens in infected plants or vectors remained problematic (Halbert & Manjunath 2004, Okuda et al. 2005). Li et al. (2005) developed quantitative TaqMan PCR using 16S rDNA-based TaqMan primer–probe sets specific to the different '*Ca*. Liberibacter' forms. An additional primer–probe set based on plant cytochrome oxidase (COX) was used as a positive internal control to assess the quality of the DNA extracts. Initial results by Irey (2006a,b) using the real time PCR method of Li et al. (2005), indicated that the incidence of infection in Southern Florida based on PCR testing may be up to two times the incidence of infection estimated by visible symptoms alone.

At the Southern Gardens Laboratory of US Sugar, where 1600 samples have been run per week for HLB, Irey et al. (2008) has used the following protocols: Real Time PCR Taqman, 16S Li primers,

SDS/K acetate extractions for petioles, bark (best for budwood), and fruit peduncles. Receiver operating characteristic (ROC) curve analysis (see Turechek et al. 2008[249]) was used to provide guidance on threshold selection.

Li et al. (2007) compared and validated four PCR-based protocols, one-loop mediated isothermal amplification (LAMP) protocol and three Taqman real-time PCR protocols. The detection sensitivity of the validated conventional PCR assays are improved compared to the original protocols. All methods were reliable for confirmatory tests for the presence of liberibacters in symptomatic samples. There were no differences in assay specificity among the standard format PCR-based methods evaluated. The TaqMan real-time PCR was 10 to 100-fold more sensitive than conventional PCR and LAMP.

Irey et al. (2008) and Wang et al. (2009) reported that when 276 DNA extractions were sent to 13 different laboratories that were using a variety of methods and equipment for HLB diagnosis, the results from the laboratories varied. The methods used included conventional PCR with 16S rDNA primers and qPCR using 16S rDNA, 3-operon primers, and unpublished putative DNA polymerase primers. Detection methodologies included ethidium bromide, TaqMan probes, and other fluorescent dye technologies. The samples included known HLB-positive and HLB-negative samples, and a variety of field samples of unknown status. The results confirmed that the qPCR testing methodology was more sensitive than the conventional PCR. With the exception of laboratories that had specific reagent or equipment problems, all of the laboratories correctly identified the samples >93% of the time. However, most of the laboratories missed one or more of the positive samples and several of the laboratories had what were considered to be false positive results (i.e., positive results from known negative samples). Thus, both false positive or false negative were observed among the laboratories. Within the laboratories, using RT-PCR systems and the same primers, there did not appear to be any difference between machines, reagents or detection systems. Similarly, there did not appear to be any differences in sensitivity for tests using primers based on different genome regions (16S, 3-operon, or DNA polymerase nucleic acid sequences) (M. Irey et al, US Sugar Corporation, Southern Gardens, Florida, unpublished). In summary, the current PCR methods detected '*Ca*. L. asiaticus'. However, it should be noted that all the reported primers/probes are based on the very limited sequence data that were available at the time (16S rDNA, beta-operon, and DNA polymerase) (Wang et al. 2010). False positive results have been reported (Tatineni et al .2008, Teixeira et al. 2008a). It is probably due to the fact that all the sequences used are highly conserved. Tatineni et al. (2008) suggested use of a combination of different methods for final diagnosis.

Irey et al. (2006b) asserted that if the wrong method was used to sample from a less than optimal tissue type during a less than optimum time of year, it is possible or even probable, that the presence of '*Ca*. Liberibacter' would not be confirmed and the sample would be a false negative.

The sensitivity of the SYBR Green RTi-PCR technique for detection of '*Ca.* L. americanus' in field samples is such that a positive amplification is still obtained with only 10 liberibacters per PCR reaction mixture. This sensitivity is at least as high as that of similar techniques published for the detection of the African, Asian and/or American liberibacter. Nested PCR with universal primer pair FD1/RP1 followed by primer pair GB1/GB3 (n-PCR), was almost as sensitive as RTi-PCR (Teixeira et al. 2008).

Urasaki et al. (2008) reported '*Ca*. L. asiaticus' detection by Cycleave ICAN. The cycling probe enables the reaction to be done in one tube and to provide rapid results without electrophoresis.

[249] Turechek WW, Hartung JS, McCallister J. 2008. Development and ptimization of a real-time detection assay for *Xanthomonas fragariae* in strawberry crown tissue with receiver operating characteristic curve analysis. Phytopathology 98 (3): 359-368.

Kawano et al. (2008a, b) developed a detection system that comprises the amplification of 16S rDNA by isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN) and the detection of the amplified products with cycling probe technology. ICAN, which only requires two DNA–RNA chimeric primers, can be carried out using the 16S rDNA sequence. The cycling probe is a chimeric DNA–RNA probe that hybridises to an amplified target sequence, not to a nonspecific product, primer dimers. Once the probe hybridises, the RNA part of the probe is cleaved by Tli RNaseH. Thus the fluorescer ROX and quencher Eclipse on each side of the probe are separated, and red fluorescence is emitted. With this cycling probe technology, they (Kawano et al. 2008a, b) rapidly obtained red fluorescence from '*Ca*. L. asiaticus'-positive samples, and prevented the occurrence of false-positives. The performance of Cycleave ICAN and the conventional PCR system (Urasaki et al. 2008a) were compared using the 16S rDNA primers designed by Jagoueix et al. (1996). The sensitivity of the Cycleave ICAN was considered to be at least 25 times higher than that of the PCR–agarose gel system. The cycling probe enables the reaction to be done in one tube and to provide rapid results without electrophoresis. Compared with the PCR system, the Cycleave ICAN could shorten the time for the detection of '*Ca*. L. asiaticus' e.g., 4000 samples processed per person per year.

Palacio-Bielsa et al. (2009) reviewed protocols for PCR detection of liberibacters and other plant-pathogenic bacteria according to ISPP nomenclature: they cited name of primers and target DNA, sample treatment in the original article, variant of PCR protocol, reference and observations for protocols.

Kim & Wang (2009) compared the detection of '*Ca*. L. asiaticus' with different qPCR-based methods with primers/probe targeting either 16S rDNA, beta-operon DNA, 16S rRNA, or beta-operon RNA. The 16S rDNA copy number of '*Ca*. L. asiaticus' was estimated to be three times of that of the beta-operon region, thus allowing detection of lower titre of '*Ca*. L. asiaticus'. Quantitative reverse transcriptional PCR (QRT-PCR) indicated that the 16S rRNA averaged 7.83 times more than that of 16S rDNA for the same samples. Dilution analysis indicated that QRT-PCR targeting 16S rRNA is 10 times more sensitive than qPCR targeting 16S rDNA. Thus QRT-PCR was able to increase the sensitivity of detection by targeting 16S rRNA.

Lin et al. (2008, 2010) developed an ultra-sensitive dual-primer TaqMan PCR for HLB molecular diagnosis. This system uses two sets of primers, analogous to the standard nested PCR and two sequential amplification steps. However, unlike two-tube nested PCR, this dual-primer Taq-Man PCR is carried out in a single closed tube. Computational algorithms were used to carefully design set of primers and probes that had the least interactions and avoid any possible self and cross dimer formations. PCR conditions were optimised such that each pair of primers worked sequentially during the amplification process. Specificity of the designed primers was validated by *in silico* BLAST and PCR tests with other citrus disease pathogens, and citrus and insect vector DNAs. The specificity of this detection system is high because the target amplicon was amplified sequentially by specific dual primers and the fluorescent signal is detected only from the probe which specifically hybridizes with the target amplicon. The sensitivity of this dual primer detection system is significantly higher than that of a standard Taq-Man PCR and has comparable sensitivity to a two-tube nested PCR. However, the standard two-tube nested PCR procedure requires a second amplification from the first amplified product in a separate tube. This processing of the previously amplified products could cause the cross contamination and lead to false positives, making this approach risky for practical application unless extreme caution is taken. Also, the cost of consumable reagents is reduced by nearly half with this new single-tube dual primer Taq-Man PCR protocol compared to the conventional two-tube nested PCR. Like other real-time PCR, this new system is gel-free and results are available immediately after the PCR is completed. Therefore, it is particularly suitable for high through-put processing of large numbers of clinical samples. In a recent survey from HLB infected citrus groves, the comparative detection rate was ~12% higher using the single-tube dual primer

Taq-Man PCR protocol than that using the single primer pair real-time PCR method. Cloning and re-sequencing of the amplicons confirmed the detections were true positives.

Benyon et al. (2008) noted that nested PCR, coupled with improved sampling and bacterial DNA isolation methods (Zhou et al. 2008c), are essential in order to detect '*Ca.* L. asiaticus' in certain phenotypes of HLB, or from psyllids that carry a low titre of the bacterium.

Increasing knowledge of sequence variation within '*Ca*. Liberibacter' 16S rRNA indicates species-specific primers will miss some of the sequence variants. Shatters et al. (2009) presented a method with at least equal sensitivity to the Taqman assay and the ability to amplify more variant sequences and to discriminate the variations using a high resolution melt (HRM) protocol.

Trivedi et al. (2009) developed a method to quantify viable '*Ca*. L. asiaticus' with the aid of ethidium monoazide (EMA) to differentiate live from dead cells. Hu (2012) used propidium monoazide (PMA: a novel DNA-binding dye) with qPCR protocols to estimate live '*Ca*. L. asiaticus' populations in plant and psyllid materials. An optimized PMA-qPCR was developed by Hu & Brlansky 2014 to provide an accurate way to determine live bacterial genomes in plant hosts of Las.

Phloem metagenomic DNA has provided a PCR-independent means of verifying the presence of '*Ca*. L. asiaticus' in infected tissue and strongly suggests that no other disease agent was present in phloem. Analysis of these metagenomic data suggests that this approach has a detection limit of one '*Ca*. Liberibacter' cell for every 52 phloem cells (Tyler et al. 2009).

Lin *et al.* (2010) developed two novel surveillance systems for detection and identification of '*Ca.* Liberibacter' species. The first system is called 'single tube dual primer Taq-Man PCR' (STDP) and is analogous to a conventional nested PCR but the whole process is completed in a single closed tube. The second system is SYBR-based Real-time PCR. This detection system employs a pair of liberibacter universal primers designed in a common sequence region among the known '*Ca.* Liberibacter'species; '*Ca*. L. asiaticus', '*Ca*. L. africanus', '*Ca*. L. americanus' and '*Ca*. L. solanacearum'. The polymorphisms, due to deletions, insertions and nucleotide substitutions in amplicons representing unique sequencing characteristics among four '*Ca.* Liberibacter' species, can be reliably distinguished and identified based on high resolution melting curve analyses. This molecular diagnostic system, using only one pair of primers, greatly simplifies detection and eliminates possible competition between primer/primer and/or primers/probes that could occur in multiplex detection systems. Both systems are robust and cost-effective for reliable detection, quantitation and identification of '*Ca.* Liberibacter' species in plants and insects and provide high throughput capabilities suitable for large scale year around quarantine screening and epidemiological studies.

Gowda (2011) developed gene specific primer-pairs for the t-RNA methyltransferase, elongation factor (EF-TU) proteins, and used these primer pairs to prepare non-radioactive Digoxigenin (DIG) labeled DNA probes. Hybridization observed with probes for EFTU, were much greater than any other probe tested in detecting HLB even in non-symptomatic branches with in an infected tree.

The sequencing of the '*Candidatus* Liberibacter asiaticus' genome using a metagenomic approach (Duan et al. 2009) revealed 2 unique hypothetical genes within a prophage region which has allowed intragenic repeats of the prophage sequence to develop and validate new real-time PCR methods with increased sensitivity of CLas detection in psyllids and plants. The prophage sequence is also present in '*Candidatus* Liberibacter americanus' (Morgan et al 2012). In this work Morgan *et al* (2012) demonstrated a new utility of nearly identical tandem-repeats of two '*Ca.* L. asiaticus' prophage genes for real-time PCR by SYBR Green 1 (LJ900fr) and TaqMan(®) (LJ900fpr). When compared with conventional 16S rDNA-based real-time PCR, targeting the repeat sequence reduced the relative detectable threshold by approximately 9 and 3 real-time PCR cycles for LJ900fr and LJ900fpr, respectively. Additionally, both LJ900 methods detected '*Ca.* L. asiaticus' from otherwise non-detectable samples by other methods. Additionally, Morgan *et al* (2012) demonstrated the

presence of the hyv(I)/hyv(II) repeat sequence within the *'Ca.* Liberibacter americanus' strain. The method thereby provides sensitive HLB detection with broad application for scientific, regulatory, and citrus grower communities.

McCollum et al (2013) provided insights into the relationship between Ct values, CLas titers and HLB symptoms.

Wang et al (2013) examined extraction methods: DNA extracted by DNAquick Plant System kit was the best, and DNA extracted by Plant Genomic DNA kit and CTAB method were better than DNAsecure Plant kit, and that extracted by TIANcombi DNA Lyse&Amp PCR kit was the worst. '*Candidatus* Liberibacter asiaticus' pathogen was detected by the four methods except TIANcombi DNA Lyse&Amp PCR kit. The DNAquick Plant System kit was easy to operate, cost less time and had better extraction effects, therefore it could satisfy the need for detecting the HLB pathogen.

Fujikawa & Iwanami (2012) designed new primers from the Las 16S rDNA and used a very small DNA template for PCR. Fujikawa et al (2013) found that the Las bacterial cells in the midribs of infected leaves were extracted rapidly and easily by pulverization and centrifugation with mini homogenization tubes and the midrib extract was suitable for highly sensitive direct PCR.

Donnua et al. 2012 used two genes (*omp* and *phpol2*) in a duplex conventional PCR and found it to be more reliable and less costly than two separate single conventional PCR reactions.

Fujikawa et al (2013) and Fujikawa (2014) described a combination of techniques (70% ethanol fixation and RNA later fixation of samples, Las bacteria extraction with Biomasher III and direct PCR using the Las606/LSS primer set) for rapid detection of HLB in large scale diagnosis.

Nageswara-Rao et al (2013) developed 32 different gene-specific primer pairs across the '*Ca*. L. asiaticus' genome.

Govindarajulu et al (2013) and Ananthakrishnan et al (2013) reported the design of a single pair of degenerate primers and a hybridization probe corresponding to the rpoB region and their application for the detection of all three citrus '*Ca*. Liberibacter species', enabling detection of '*Ca.* Liberibacter' at the genus level. In addition, species-specific primers and probes based on the rplJ/rplK genes were designed and used for detection at the species level in a multiplexed format. Both the genus-and species-specific assays were validated in both SYBR Green I and TaqMan formats, and with both plant and insect extracts that contained the pathogen. These one-step qPCR diagnostic methods are useful for the detection of all species of Liberibacter infecting citrus. In addition, the degenerate genus-specific primers and probe successfully detected '*Ca*. Liberibacter solanacearum', a psyllid transmitted pathogen associated with disease in tomato, carrot, and potato.

Lu et al (2013) produced two polyclonal antibodies against the POTRA domain of the Omp protein of '*Candidatus* Liberibacter asiaticus'.

Orange juice processed from Huanglongbing (HLB) affected fruit is often associated with bitter taste and/or off flavor. It is extremely difficult to detect CLas in orange juice because of the low CLas population, high sugar and pectin concentration, low pH, and possible existence of an inhibitor to DNA amplification. The objective of this research was to improve extraction of DNA from orange juice and detection of CLas by qPCR. Homogenization using a sonicator increased DNA yield by 86% in comparison to mortar and pestle extraction. It is difficult to separate DNA from pectin; however, DNA was successfully extracted by treating the juice with pectinase. Application of an elution column successfully removed the unidentified inhibitor to DNA amplification. This work provided a protocol to extract DNA from whole orange juice and detect CLas in HLB-affected fruit (Bai et al 2013).

Kogenaru et al (2014) have designed qRT-PCR primers based on Las specific genes. Among them, 18 are suitable for the detection of Las from Las-infected plant and psyllid samples.

HLBaspr primers and probe produced unreliable qPCR results for detection of '*Ca*. L. asiaticus' in citrus root samples from Texas. cPCR usingLas606/LSS primers could detect '*Ca*. L. asiaticus' in trees where A2/J5 primers failed and the sensitivity of these primers was comparable to the LJ900fpr and CQULA qPCR assays. All the primers and probes used in this study could detect '*Ca.* L. asiaticus' from leaf samples without any problem. Nevertheless, fibrous root samples collected either close to the trunk or 1.8 m away from the trunk produced consistent detection of '*Ca*. L. asiaticus' (Kunta et al 2014).

Vazquez et al (2104) found that roots serve as a more reliable diagnostic sample compared to leaves. Las is often detected in the roots in asymptomatic trees (Johnson et al., 2014; Lopes et al., 2013; Louzada???.

However reports differ on the relative titers of Las in roots vs. leaves. In addition to the work already cited which shows higher levels of Las in roots compared to leaves (Johnson et al., 2014; Louzada, personal comm.), Li et al. (2009) also detected root Las titer at levels similar to or higher than they detected in above ground tissues. However, similar to this report, Trivedi et al. (2009) reported leaf titers one to two orders of magnitude greater than they observed in roots, and in Brazil, naturally field infected trees had higher Las titers in the leaves than in the roots and there was no correlation between canopy symptoms and Las root titer (Lopes et al., 2013). There is considerable evidence that the relationship between the citrus host and Las pathogen is dynamic and the relative titers and perhaps importance of root vs. foliar Las may change as the disease develops (cited from Stover et al 2014).

Louzada et al (2016) found that among randomly collected leaves, CLas was distributed in a patchy fashion. Detection of CLas varied with leaf symptomology with symptomatic leaves showing the highest frequency (74%) followed by their neighboring asymptomatic leaves (30%), while randomly distributed asymptomatic leaves had the lowest frequency (20%). Among symptomatic leaves, they found statistically significant differences in mean number of bacterial cells with respect to both increasing distance of the leaf from the trunk and cardinal direction. The titer of CLas cells was significantly greater on the north side of trees than on the south and west sides (this was in Texas). Moreover, these directions showed different spatial distributions of CLas with higher titers near the trunk on the south and west sides as opposed to further from the trunk on the north side. Similarly, we found spatial variation in CLas distribution among root samples. CLas was detected more frequently, and bacterial abundances were higher, among horizontally growing roots just under the soil surface (96%) than among deeper vertically growing roots (78%). Bacterial abundance declined slightly with distance from the trunk.

CLas is consistently detectable when at least one unit of DNA extract from a positive tree is pooled with five units of DNA from a healthy tree (3.713x106 CLas genomes/g HLB-infected fresh tissue). The use of composite DNA samples will enable a cost-effective quick screen of large number of survey samples in disease surveys (Alabi & Kunta 2014).

Chen et al. (2015) have identified a multi-copy gene in the HLB pathogen genome. By targeting this bacterial gene, a highly sensitive and accurate detection protocol has been developed.

Orce et al*. (2015)* have developed for the first time a set of qPCR primers based on the conserved 16S rDNA gene, which specifically and simultaneously detects in a singleplex reaction, all three bacterial species associated with HLB, and can differentiate *Ca.* Liberibacter asiaticus or africanus from americanus by their characteristic melting curves. The assay is claimed to be very sensitive, and it was possible to amplify expected DNA fragments with an efficiency of 98 % using the Syber Green system and a Ct value lower than tested methods for HLB diagnosis.

Kremer et al (2015) have developed a promising and novel diagnostic assay based on digital PCR (dPCR) for early and reliable detection of HLB. dPCR partitions samples into 20,000 picoliter wells in a single reaction, with each well carrying out independent PCR reactions simultaneously. The large number of positive and negative wells can be fitted to a Poisson distribution to allow absolute and precise quantification of the target molecules and statistical assessment of the measurement. Using probes targeting the Las 16s rDNA and the integrated prophage repeat sequences, we showed that as few as 1 to 2 copies of the targeted DNA molecules per microliter could be detected, with the prophage probe providing the best sensitivity. Early-stage HLB samples can be statistically differentiated from healthy individuals. Furthermore, this assay can quantitate the copy number of the 16S rDNA and the phage repeat DNA simultaneously, permitting the tracking of lytic activities of the Las prophage/phage accurately. The dPCR-based assay will not only provide a reliable and early diagnostic tool but also an enabling technology to advance research on HLB therapies.

Silica gel dehydrated midrib and bark citrus samples are amenable for DNA extraction and further processing by PCR and real-time PCR. FTA plant collecting card were useful for easily collect DNA samples in the field and processing by real-time PCR although at a lower concentration than extracted DNAs (Bella et al 2016).

Lou et al (2017) reported the development of a novel method, tandem repeat-based polymerase chain displacement reaction (TR-PCDR), for the detection of '*Candidatus* Liberibacter asiaticus' (Las). A uniquely designed primer set TR2-PCDR-F/TR2-PCDR-1R and a thermostable *Taq* DNA polymerase mutant with strand displacement activity were used for TR-PCDR amplification. Performed in a regular thermal cycler, TR-PCDR could produce more than two amplicons after each amplification cycle. Sensitivity of the developed TR-PCDR was 10 copies of target DNA fragment. The sensitive level was proven to be 100 times higher than conventional PCR and similar to real-time PCR. Data from the detection of Las with field samples using the above three methods also showed similar results. No false-positive TR-PCDR amplification was observed from healthy citrus samples and water controls. These results illustrated that the developed TR-PCDR method can be applied to the reliable, highly sensitive and cost-effective detection of Las.

A *C*Las secreted protein has been used as a biomarker for detecting HLB infected citrus by Pagliaccia et al (2017). Proteins secreted from *C*Las cells can presumably move along the phloem, beyond the site of ACP inoculation and *C*Las colonized plant cells, thereby increasing the chance of detecting infected trees. They generated a polyclonal antibody that effectively binds to the secreted protein and developed serological assays that can successfully detect *C*Las infection. This work demonstrates that antibody-based diagnosis using a *C*Las secreted protein as the detection marker for infected trees offers a high-throughput and economic approach that complements the approved quantitative polymerase chain reaction-based methods to enhance HLB management programs.

To better improve the detection sensitivity, a droplet digital PCR (ddPCR) assay was developed by Zhong et al (2018) for the rapid detection of '*Candidatus* Liberibacter asiaticus' (Las), the putative causal agent of HLB. The detection sensitivity comparison using positive plasmid indicated that ddPCR was superior to quantitative PCR (qPCR) for detecting and quantifying Las at low concentrations. The Las detection of 40 field samples also showed that six of 13 asymptomatic samples (46.15%) with high Ct value (>35) were positive by ddPCR. This methodology showed great potential for early HLB infection diagnosis.

In field detection techniques:

Bertolini et al. (2010, 2012, 2014) developed a fast and simple procedure for accurate detection of *Candidatus* (*Ca.*) Liberibacter (L.) spp. ('*Ca.* L. africanus', '*Ca.* L. asiaticus' and '*Ca.* L. americanus') by Rt-PCR in plant tissues and in individual psyllids. Universal detection of *'Ca*. Liberibacter' species was achieved by a direct tissue-printing and spotting of plant leaf petiole extract or squashing of individual psyllids onto paper or nylon membranes. New primers were designed and used with TaqMan chemistry for accurate detection of the bacterium in immobilized targets (prints of 10 overlapping leaf pedicels/tree or squashed single vectors), by extraction with water and direct use for real-time PCR. This simplified method was validated and found to be efficient for detection of HLB-liberibacters in 100% of symptomatic and 59% of symptomless leaves collected from HLB-infected trees. The use of direct assays as template showed good agreement with use of purified DNA (Kappa 0,76±0.052). The squash assay allowed detection of the bacterium in 40% of mature *Diaphorina citri* that fed on symptomatic or on symptomless leaves of HLB-infected trees. A commercially ready-made kit based on this technology showed 96% accuracy in intra-laboratory performance studies and can be effectively adopted for use in rapid screening of HLB agents in extensive surveys, certification schemes or for epidemiological and research studies.

Siverio et al 2013 reported on annual surveys of 180,000ha of citrus (100 sampling points in selected orchards and 20 packinghouses) in Valencia Spain. In all these localized points arthropods caught on yellow sticky traps were identified monthly and citrus trees were visually inspected for HLB symptoms (62,500 trees inspected, 385 analyzed). Suspicious HLB-symptomatic leaves were directly printed on paper membranes during the field inspection and in the Canary Islands *T. erytreae* specimens were directly squashed. Samples were analyzed by real-time PCR using a commercial kit (HLB/100, Plant Print Diagnostics: http://plantprint.net/kits-and-products/bacteria-2/?lang=en).

Russel et al (2012, 2015) reported that Agdia has developed a new rapid molecular detection technique, AmplifyRP, allowing PCR – level sensitivity detection within minutes in the field. AmplifyRP uses a Recombinase-polymerase methodology for DNA amplification at a single temperature . A portable fluorescence reader or a lateral flow device (similar to Agdia's Immunostrip) can be used to visualise results in as little as 30 mins. See https://www.agdia.com/testing-services/Citrus-Greening.cfm

EnviroLogix Inc. has introduced a test that can be performed in the field in less than 30 minutes to determine whether a citrus tree is infected with HLB http://www.envirologix.com/solutions/catalog/410-11783-dnable-molecular-detection-kit-for-hlb-liberibacter-asiaticus-las-petiole-48-tests-per-kit/

Cary et al (2014) have developed a sensitive nucleic acid-based HLB diagnostic test based on Mesa Tech International, Inc.'s (MTI's) new point-of-use diagnostic platform, MTIDx, and targeting hyvI/hyvII genes of *Candidatus* Liberibacter asiaticus (Las) (Zhou et al., 2011). The MTIDx platform integrates sample preparation, rapid nucleic acid amplification and sequence-specific hybridization-based detection. The authors claim the simplicity of the test may offer end users with little or no specialized training the opportunity to obtain molecular test results comparable to laboratory-based PCR methods without costly instrumentation.

Keremane et al (2015) reported on the development of a field detection kit for testing psyllids for Las using loop-mediated amplification technology (LAMP) conducted in a Smart-DART^{TM} detection unit operated from an Android device. The LAMP detection method is claimed to be 100 times more sensitive than traditional real time PCR. The method was also validated as effective for identifying Las in plant DNA extractions.

Recently an anti-outer membrane protein A (OmpA) polyclonal antibody (Ding et al 2015) was highly effective for the detection of CaLas from citrus tissues in a simple tissue printing format. The antibody was also used to capture bacteria from periwinkle extracts. About 80% of all field samples analyzed tested positive with both immune tissue printing and qPCR; whereas 95% were positive with at least one of these two methods. When asymptomatic citrus tissues were tested, the tissue printing method gave a higher rate of detection (83%) than the qPCR method (64%). This is consistent with a lower concentration of CaLas DNA, but a higher proportion of viable cells, in the asymptomatic tissues. The immune tissue printing method also highlights the detail of the spatial distribution of '*Ca*. Liberibacter asiaticus' in diseased citrus tissues. Both the immune capture PCR and immune tissue printing methods offer the advantages of low cost, high throughput, ease of scaling for multiple samples and simplicity over current PCR-based methods for the detection of '*Ca*. Liberibacter asiaticus' (Ding et al. 2017).

Detection of 'Ca. Liberibacter' in Psyllids

Identifying ACP carrying CLas is more a problem of sampling than of detection since qPCR and other conventional assays can easily detect small copy numbers of bacterial DNA if it can be recovered from the insects. Because only a relatively small proportion of the ACP individuals are actually competent vectors (Coy and Stelinski, 2015), the insect sample size must be large enough to determine infection pressure accurately. Furthermore, the trapping method used to collect ACP must allow quality DNA to be recovered from the insects. Sticky cards are effective for trapping insects but not for the recovery of quality DNA.

Psyllids positive for '*Ca.* L. asiaticus' were found prior to finding HLB positive plants in surveys. Thus a positive result from a psyllid analysis may be informative and useful for undertaking appropriate management decisions, but negative results may not indicate absence of HLB in an area. 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 (Manjunath et al. 2008a).

The following are references additional to those reported above on detection of citrus liberibacters in psyllids.

Bové et al. (1993) cloned a DNA fragment (In 2.6) from a Poona (India) strain of the HLB organism and used it as a probe for the detection of '*Ca.* L. asiaticus' in single Asiatic citrus psyllids collected in India and Malaysia.

Li & Ke (2002) used nested PCR to detect '*Ca.* L. asiaticus' in single Asian citrus psyllids.

Hung et al. (2004) developed a PCR-based assay for monitoring '*Ca.* L. asiaticus' in vector psyllids using a rapid DNA extraction from psyllid bodies and PCR amplification. The entire procedure for '*Ca.* L. asiaticus' detection in psyllids can be completed within 5 h. Using this method, '*Ca.* L. asiaticus' can be accurately detected in psyllid adults, as well as nymphs, in different instar stages. The assay is sensitive enough for '*Ca*. L. asiaticus' detection in single-psyllid extracts from adult, fifth, fourth and third instars.

Manjunath et al. (2008a) adapted the TaqMan based real-time quantitative polymerase chain reaction methodology of Li et al. (2006) for detection of '*Ca.* L. asiaticus' in *D. citri* in Florida.

In related studies in Brazil, Manjunath et al. (2008b) found that liberibacters can be identified in *D. citri* 'long before symptoms become visible in plants' and that this demonstrated 'the usefulness of psyllid analysis in monitoring different management practices'.

Postnikova et al. (2009) described a high throughput DNA extraction for use with individual psyllids. The method utilizes single steel bead, 2–3 mm in diameter (Biospec Products) in each well of a ninety-six well racked collection microtube plate (QIAGEN) along with a single psyllid and 200 µl of DNazol direct (MRC). Plates are shaken at 1800 rpm on a TissueLyser (QIAGEN) two times for 90 s, followed by a short centrifugation at 3000 rpm. The supernatant can be used immediately as a template for PCR. DNA can also be extracted from batched psyllids by varying the number of beads and volume of DNAzol Direct. Time to extract DNA from 200 psyllids is less than two hours.

See the above methodology of Bertolini et al. (2010, 2014) for direct plant tissue-print and insect squash-blot methods for large-scale detection of *'Ca.* Liberibacter' spp. by real-time PCR with new primers.

When bulk tested for HLB-associated liberibacters, no more than 5 psyllids should be combined for sample processing. Testing composite samples larger than 5 insects increases the risk of obtaining false positives of HLB-associated liberibacter due to DNA dilution (TWG Report 2010).

Sala et al (2013) evaluated the detection of '*C*Las' in ACP adults submitted to different storage methods and time of storage by qPCR.

LJ900 primers detected Las from otherwise nondetectable levels in plant and insect samples (Ammar et al 2013).

The '*Ca.* L. asiaticus' incidence among *D. citri* populations collected on HLB-affected citrus plants in the field in Florida varied from 10% to 100% (Li et al. 2008). The bacterial populations complied with a normal distribution with some skewness among the psyllid populations. One quarter of the *D. citri* populations in Florida carried '*Ca.* L. asiaticus' populations of less than 104 '*Ca.* L. asiaticus' cells/psyllid, which was on the borderline of the low detection limit of real-time PCR, but beyond the detection range of conventional PCR.

Coy et al (2014) found that the false-negative rate of the presence/absence qPCR assay for '*C*Las' insect samples was greater than 50%, demonstrating that psyllids can harbor titers of *C*Las below the detection limit of the assay. Their results suggest that suboptimal qPCR efficiency is not uncommon for the *16S* presence/absence qPCR assay, which combined with low-abundant *C*Las template in some samples, likely contributes significantly to the under-reporting of *C*Las infection in psyllid and plant samples.

APPENDIX 5: FACTORS AFFECTING SAMPLING FOR DETECTION OF CITRUS LIBERIBACTERS

Irey et al. *(2006b) asserted that "if the wrong method was used to sample from a less than optimal tissue type during a less than optimum time of year, it is possible or even probable, that the presence of 'Ca. Liberibacter' would not be confirmed and the sample would be a false negative".*

During symptom development, CLas is not evenly distributed within the vascular system (Gottwald, 2010) and its titer levels fluctuate throughout the year (Irey, 2008).

In Florida, visual symptoms are most evident from mid summer to early spring; using Ct values as a proxy, the highest titre of HLB bacteria is from mid summer to late winter; and the highest incidence of infection occurs in trees 6–9 years old or 2-3 m tall and most often in oranges and grapefruit, least in tangerines and tangelos; infected trees are most likely to be found at the perimeter of blocks (Irey et al. 2009). HLB symptoms appear throughout the year in Brazil, but with greater intensity in

autumn and winter (April to August), because in addition to mottled leaves, affected shoots also show leaves with Zn/Mn like deficiency patterns (Lopes et al. 2006a).

Higher incidences of HLB infected trees were observed associated with grove and block 'edges' i.e., where there was a break in the trees that created an interface between continuous trees and an open space, there tended to be an increase in infected trees. Examples of edges include grove and block boundaries, roads, irrigation ditches, ponds, interfaces with natural areas, and interfaces between young and mature trees. Some of these observations can be used in making scouting decisions e.g., to determine if HLB is present in an orchard for the first time, scouting efforts should be initially directed towards young trees and concentrating on edges may be warranted (Irey et al 2008).

Scouting only detects 50-60% of symptomatic trees and there is one asymptomatic tree for every symptomatic tree (Irey 2006a,b), and the latency period between infection and symptom development ranges from 6 months to 2 years depending on tree size and age. During the latency period, psyllids can acquire the pathogen from asymptomatic trees. This has become more important with the finding by Lee et al (2015) that young flush becomes infectious within 15 d after receiving CLas inoculum. As Halbert et al (2015) predicted, transmission can bypass the latent period in the plant making it possible to have positive ACP throughout an orchard before ever seeing a symptomatic tree.

Sampling protocol

Sample collection procedures used in Florida are outlined in Gomez (2008). Each sample collected should contain green twigs of 15 cm to 20 cm long with approximately 20 leaves, preferably with the petiole still attached and with good, recognisable symptoms. Measures should be taken to avoid the presence of the insect vector in the sample bag. Samples should contain leaves showing blotchy mottling (Carlos et al. 2006).

The total number of HLB symptomatic plants must be recorded. Samples should, if possible, be photographed before they are removed from plants. The entire plant should also be photographed and include the region of the plant exhibiting symptoms. Other photographs should include a perspective of where the symptomatic plant is located with respect to other plants. A minimum of 3 photographs per symptomatic plant is recommended, unless several plants in a block are symptomatic, in which case fewer photographs may be need to be taken.

Collection of samples from symptomatic field trees

The procedures used in Brazil are: trees with HLB-like symptoms are labelled along with the first and last trees of the row to indicate presence of a suspect tree within that row, for sample collection and ultimately tree eradication. After sample collection, a second label is attached to the suspect tree. If the laboratory result is negative, the seal is removed. In the case of a positive laboratory result, a third marking is made in paint on the trunk, indicating the tree is to be eradicated (Massari & Libanori 2006).

The sampling protocol for the Florida Southern Gardens HLB Diagnostic Laboratory (http://www.flcitrusmutual.com/content/docs/issues/canker/sg_samplingform.pdf) specifies:

1. Samples should be collected from the symptomatic areas/branches of the trees.
2. Samples should consist of short sections (4-6 inches 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, the peduncle should be left on the sample (i.e., trim the fruit off as close to the button as possible leaving the peduncle 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's ear' appearance (small, upright leaves);
    f. branches with leaves that show chlorosis and 'vein-corking';
    g. branches with Zn and/or Fe deficiencies that are not related to blight or other known causes.
4. Place the leaves/twigs into a sealable (e.g., zip-lock) plastic bag and keep the sample cool and out of the sunlight.

Sample handling and shipping[250]

Surveyors should forward specimens, complying with the recommended sampling and handling procedures and provide a completed Survey Form including details on the address, owner, GPS co-ordinates, varieties affected, disease symptoms and site features. For guidance, see QDPI&F Work Instruction ST-W-007[251]; QDPI&F Work Instruction ST-W-008[252]; and QDPI&F Work Instruction ST-W-011[253].

Criteria for the determination of a positive or negative result

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

Test results fall in one of three categories:

- HLB positive – test results indicate that '*Ca*. Liberibacter sp.' was detected in the sample;
- no HLB found – test results did not indicate that '*Ca*. Liberibacter' was present in the sample; or
- an inconclusive test result (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.

In field studies, when trees become infected, PCR assay results are generally negative or inconclusive for 9 to 12 months or longer before confirmation of the infection. This is known as the *cryptic period*, when the plant is infected but we are unable to detect infection (Gottwald and McCollum 2017). Recently, early detection methodologies such as metabolomics, proteomics, volatile organic compound detection, canine detection, etc., have been used to directly or indirectly explore this cryptic period to a greater extent. Gottwald and McCollum (2017) believe that it is critical to detect

[250] PLANTPLAN "Sampling procedures and protocols for transport, diagnosis and confirmation of EPPs"
[251] Telford G. 2007. Collecting a Sample from a Plant that is Suspected of being Infected with Citrus Canker. QDPI&F Work Instruction ST-W-007.
[252] Telford G. 2007. Packing and Dispatch of Samples for Diagnostic Analysis. QDPI&F Work Instruction ST-W-008.
[253] Telford G, Benham M, Jarrett K. 2007. Completion of Form ST-F-001 (Citrus Canker Survey Form – Orchard). QDPI&F Work Instruction ST-W-011.

the disease further back in time into the cryptic period in order to adequately control HLB. Epidemiological models demonstrate that early detection followed by hasty removal of infected trees in the cryptic (asymptomatic) stage is highly advantageous and requires tree replacement of only 2-3% per year capable to sustain viable production at very low disease incidence (Cunniffe et al. 2015[254]).

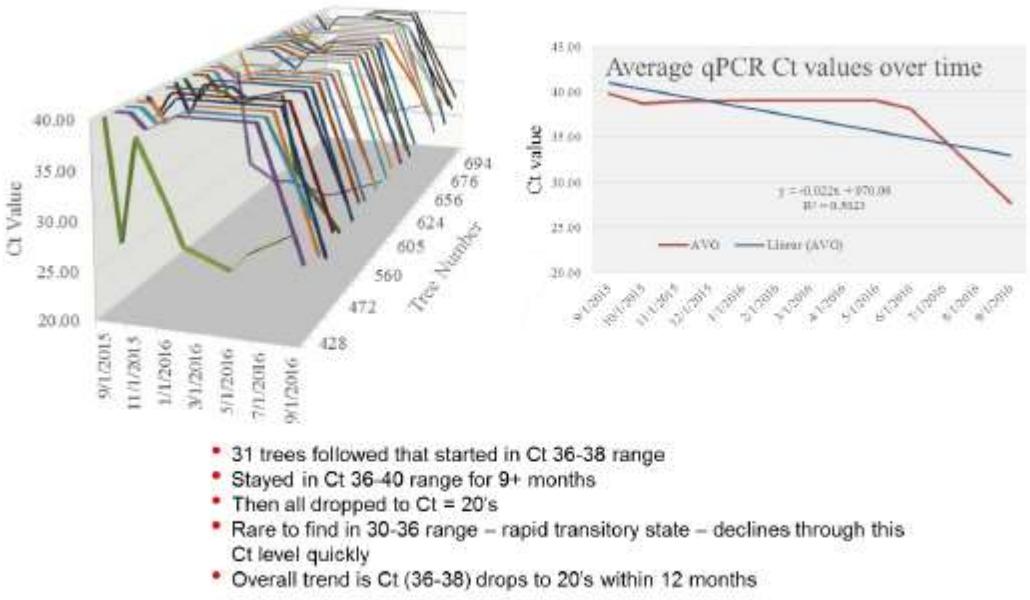


Fig. 3. qPCR Ct progression over time from a Florida commercial citrus block. Left panel indicates Ct values over time for 31 individual trees. Right panel indicates average Ct value for the 31 trees and regression analysis for the population.

The concept of a minimum infectious dose for *CLas* proliferation/HLB disease development has significant implications. First, it may confound efforts to develop detection technologies other than qPCR. If there are systemic signals (transcriptome, proteome, metabolome, microbiome, volatile organic compounds) induced by even nascent infections, if the infection has to be confirmed by qPCR, if HLB symptoms are not visible, we are back to the sampling problem, and if *CLas* cannot be found any alternative detection technology is dismissed.

However, absence of confirmation (by qPCR) is not confirmation of absence.

From Gottwald & McCollum (2017).

In Australia, similar symptoms to those of HLB are caused by Australian citrus dieback (Broadbent et al. 1976, Broadbent 2000) and sometimes by severe stem pitting strains of CTV in grapefruit. A phytoplasma has been associated with Australian citrus dieback (Davis et al. 1997, Monique Garnier, pers. comm., 1998). **It is recommended that a test for phytoplasmas should be be used on all negative suspect samples with HLB-like symptoms.** Overseas phytoplasmas have also been associated with HLB-like symptoms (see Appendix 8).

Tissue type

Asymmetric blotchy leaves should be used for sampling for PCR.

254 Cunniffe NJ, Stutt ROJH, DeSimone RE, Gottwald TR, Gilligan CA. 2015. Optimising and communicating options for the control of invasive plant disease when there is epidemiological uncertainty. PLoS Comput Biol. 11(4): e1004211.

Asymmetric blotchy mottle, the diagnostic symptom for sampling for PCR confirmation in Brazil (Pat Barkley).

Li et al. (2009) found that the populations of '*Ca.* L. asiaticus' inferred from the distribution of 16S rDNA sequences specific for '*Ca.* L. asiaticus' in leaf midribs, leaf blades, and bark samples varied by a factor of 1000 among samples prepared from the six citrus species tested and by a factor of 100 between two sweet orange trees tested. In naturally infected trees, above-ground portions of the tree averaged 10^{10} '*Ca.* L. asiaticus' genomes/g of tissue. Similar levels of '*Ca.* L. asiaticus' genomes were observed in some, but not all, root samples from the same plants.

A study was conducted by Kunta et al (2014) in Texas to compare the population of *Candidatus* Liberibacter asiaticus (CLas) cells in different plant parts including peduncle, columella, leaves, seeds, young shoots, flower buds, flowers, and bark of 6-year-old known infected grapefruit and sweet orange trees. Except for bark tissue, there was no significant difference in the concentration of CLas cells in other plant parts between the two cultivars. Within the cultivar, the bacterial concentration also varied with the plant part, with peduncle, columella, midrib having significantly higher titer of CLas compared with other plant parts. Consistently lowest bacterial titer were recorded in young shoots, leaf blade, and especially leaf margins relative to the midrib.

Comparisons of Las concentrations in different leaf and tissue parts of three Las-infected citrus cultivars by Lin et al (2017) showed high average Las concentrations were detected in mature and old leaves, whereas young leaves and new flush contained low Las concentrations. According to the PCR analysis, the Las concentrations, from high to low, were fruit, mature leaf, old leaf (completely yellowing), bark of young twig, root, new flush and bark of trunk. For the seasonal dynamics analysis, higher Las concentrations were detected in the cooler temperatures of autumn and spring, whilst lower concentrations of Las were detected in the high and low temperatures of summer and winter.

Caution is needed to ensure that a positive IST is not caused by factors other than HLB (Miles et al., 2009), particularly Australian citrus dieback, CTV, winter yellows, girdling and borer damage.

Taba et al. (2006) claimed the IST test is a rapid, simple, portable and inexpensive test that can be used in the field to complement visual diagnosis and differentiate HLB symptoms from mineral deficiencies.

The iodine starch test or 'scratch method' used by Takushi et al. (2007), is based on the observation by Schneider (1968) that significant accumulation of starch occurs in HLB affected leaves and the finding by Kawano (2006) that the average quantity of starch in HLB affected leaves was 6 times higher than in healthy leaves.

Eng (2007) found that the accuracy of the scratch test ranged from 74.5% to 89.5% for mandarins and for pomelo, from 12.5% to 51.7%, dependent on the correct selection of infected leaves.

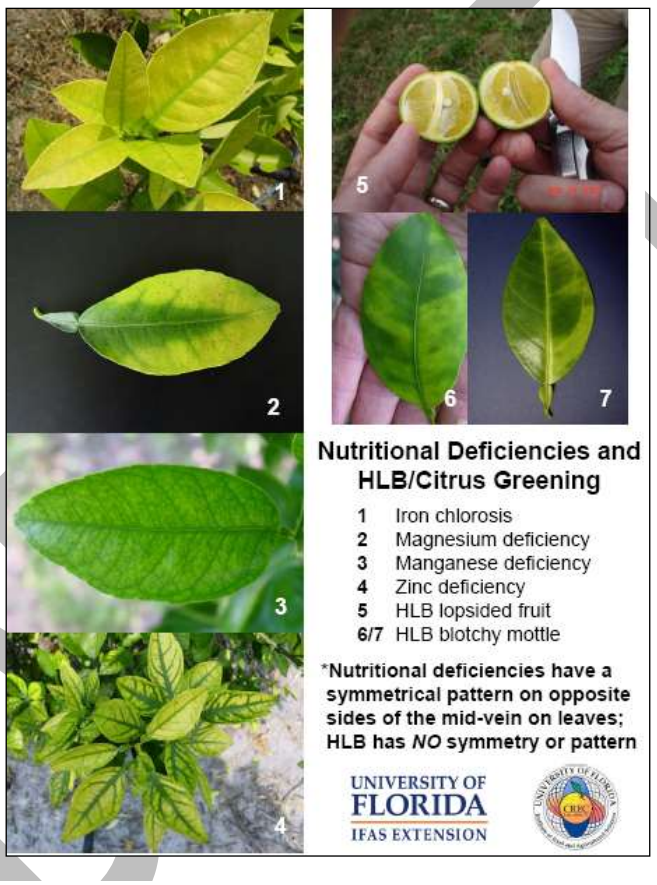
Accumulation of starch granules in HLB-infected leaves evidenced by an iodine reaction (Onuki et al. 2002) was shown to give 8.9% false negatives and 3.0% false positives when compared to PCR tests (Le & Nguyen 2003). Takushi et al. (2007) demonstrated that the scratch method (using 50 mM of iodine solution), and PCR were more than 90% in agreement. They also reported that this method did not give HLB-positive reactions for healthy, nutrient-deficient or other diseased leaves.

Of leaf samples collected from 1759 HLB suspect trees in Florida, 85% of the samples were positive by RT-PCR versus 78% positive for the starch test. The starch and the RT-PCR tests agreed for 76% of the samples. The best correlation between the tests was obtained for leaves exhibiting the classic blotchy mottle leaf symptom and the worst correlation was obtained with leaves showing the green island symptom type. Overall, the RT-PCR testing was more consistent in detecting HLB infected trees than the starch test. Therefore, the starch test should be considered a useful tool for HLB diagnosis in the field, but not as a substitute for PCR-based testing (Chamberlain & Irey 2008).

Whitaker et al (2014) detailed a comprehensive statistical analysis of starch levels in citrus leaves and compared them with real-time polymerase chain reaction (PCR) detection of the presumptive causal agent *Ca*. Liberibacter asiaticus'. Starch content was found to reliably predict the PCR results (the proxy for HLB presence) during the ''warm season'' but not in the ''cool season" when 43% of samples were incorrectly classified compared to 8% in summer.

APPENDIX 7: CONFUSION OF HLB SYMPTOMS WITH OTHER DISEASES AND NUTRIENT DISORDERS

The distinction of HLB symptoms from nutrient deficiency symptoms can be difficult (Catling 1970). Halbert & Manjunath (2004) stated that leaf mottling resulting from HLB infection differs from symptoms of nutrient deficiencies, as it usually crosses leaf veins, rather than occurring between or along the veins.



Distinction between some symptoms of nutritional deficiencies and HLB.

In Australia, similar symptoms to those of HLB are caused by Australian citrus dieback (ACD) (Broadbent et al. 1976, Broadbent 2000). ACD has been attributed to phytoplasma (Broadbent et al 1976, Davis et al 1997, Constable, pers.comm). At Nangiloc, Ca. Phytoplasma asteris wasd associated with ACD in grapefruit (Constable pers. comm.). Recently various phyoplasmas have been associated with HLB-like symptoms overseas[255].

[255]Recently, phytoplasmas were reported in Brazil (pigeon pea witch's broom phytoplasma (16Sr IX) (Teixeira et al. 2008, Silva et al. 2014) and Guangdong Province, China ('Ca. phytoplasma asteri' 16Sr I) (Chen et al. 2009), India '*Ca.* Phytoplasma trifolii' (16SrVI group) associated with blotchy-mottle leaf symptoms. These phytoplasmas can infect citrus plants alone or in combination with 'Ca. L. asiaticus' (Teixeira et al. 2008; Chen et al. 2009). Lou et al (2013) found that a few grapefruit trees with blotchy-mottle leaf symptoms in a HLB-infected orchard in China were positive for a variant (16SrII-A*) of phytoplasma subgroup 16SrII-A. Wulff et al (2015) found that sunn hemp is a major source of inoculum of the HLB-phytoplasma in Brazil caused by 16Sr group IX phytoplasmas, and transmitted by S. *marginelineatus* to sweet orange. Transmission from sweet orange to sweet orange occurs only rarely, if at all. In 2018, Wulff et al reported HLB-samples testing negative for Las, Lam and 16SrIX phytoplasma, were infected with 16SrIII phytoplasmas. Co-infection with Las and 16SrIII was also found. 16SrIII phytoplasmas highly related are commonly found in *Melia azedarach*, a widespread tree in Brazil and Argentina. In Mexico Arratia-Castro et al reported 'Ca. Phytoplasma asteris' associated with HLB-like symptoms. A new emerging citrus decline (CDD) widely spread in Southern Kerman region of Iran, klling around 10% of cultivated citrus trees is associated with the presence of liberibacters and phytoplasmas (Alizadeh et al 2017).

HLB-like symptoms may also be caused by blight (Broadbent et al. 1996), starch accumulation associated with B deficiency (see Haas & Klotz 1931a, b)[256], winter yellows[257] (Broadbent & Fraser 1979), severe stem pitting strains of *Citrus* tristeza virus in grapefruit and Phytophthora root rot. Fruit re-greening symptoms of HLB can mimic symptoms of the rind of naturally re-greened Valencia orange fruit in late summer and autumn. Diseases and disorders that can be confused with HLB are described at https://www.daf.qld.gov.au/__data/assets/pdf_file/0003/54183/Citrus-HLB-similar-symptoms.pdf and presented in Figs below:

Australian citrus dieback-affected grapefruit leaves with asymmetric, sometimes dull, blotchy-mottling that crosses leaf veins (Pat Barkley).

Australian citrus dieback induced dieback and mottling of whole tree (left) and chlorotic leaf symptoms initially restricted to one branch (right) (Pat Barkley).

[256] Boron deficiency can cause vein corking (Haas & Klotz 1931a, b) as portions of cambium of the phloem disintegrate, and the xylem tissue to lesser extent, if at all. A copious amount of gum is formed, which finds its way to the exterior through a split in the cortex (Haas & Klotz 1931b) distinguishing this form of vein corking from the effects of HLB. Boron deficiency also leads to abnormal accumulation of carbohydrates. This, coupled with destruction of phloem, interferes with translocation (Haas & Klotz 1931b).
[257] Winter yellows results from starch accumulation in fully expanded late autumn flush (see Broadbent & Fraser 1979).

Small, rounded grapefruit and leaves with yellow veins (left), and off-season fruit of variable size (right) caused by Australian citrus dieback (Pat Barkley).

Off-season flowering (left and right) and yellow veins, and severe chlorosis (right) caused by Australian citrus dieback (Pat Barkley).

Severe Australian citrus dieback (left) and symptomatic grapefruit trees adjacent to native vegetation (right) (Pat Barkley).

Cold injury to sweet orange leaves at Kulnura, NSW, 2008 (left: GAC Beattie), and winter yellows on sweet orange at Griffith, 2008 (right: Pat Barkley).

Winter yellows in the Riverina (Pat Barkley).

Symptoms of girdling injury (GAC Beattie: Guangdong, late autumn 2008).

Chlorosis of leaves on a single branch of *M. paniculata* (left) caused by borer injury (right): Mundubbera – 17 November 2008 (GAC Beattie).

Left, vein corking caused by boron deficiency (GAC Beattie: Guangdong late autumn 2008). Right, vein corking of West Indian lime leaves caused by citrus tristeza virus (Pat Barkley: photograph held by NSW Department of Primary Industries).

Root rot at Stanbridge, Riverina (Pat Barkley 2008).

In Australia, the disease most closely resembling HLB in terms of symptom expression is Australian citrus dieback (a phytoplasma-associated disease: Broadbent et al. 1976, Davis et al. 1997, Broadbent 2000; Garnier, pers. comm; Constable, pers.comm.). In China, Pakistan, Mexico, and Brazil, phytoplasmas have been found associated with HLB-like symptoms (Chen et al. 2008, Lou et al 2014; Arratia-Castro et al 2014; Teixeira et al. 2008d; Mannan et al 2009; Marques et al. 2012; Wulff et al 2015).

In 2007, a phytoplasma of group 16SrIX, closely related to the pigeon pea witches' broom phytoplasma (99% 16SrDNA sequence identity) was found associated with HLB-like symptoms in São Paulo State (SPS). The phytoplasma associated with HLB-like symptoms is probably transmitted to citrus by an insect vector[258] becoming infected on an external non-citrus source. *Crotalaria junceae* L. [Fabales: Leguminosae] plants, grown in between citrus rows for soil improvement, have been found to be infected with the phytoplasma. Wulff et al (2015) found that i) sunn hemp is a major source of inoculum of the HLB-phytoplasma, (ii) *S.marginelineatus* becomes infected on sunn hemp and transmits the phytoplasma to sweet orange,and (iii) transmission from sweet orange to sweet orange occurs only rarely, if at all. 16Sr group IX phytoplasmas, very closely related to the SPS HLB-phytoplasma, have also been detected in citrus in Minas Gerais and Bahia states (Brazil) and Mexico. 16SrII-C subgroup phytoplasma were found associated with *C. aurantiifolia* in Brazil in asymptomatic plants (Silva et al 2014). In 2018 Wulff et al reported HLB-samples testing negative for Las, Lam and 16SrIX phytoplasma, were infected with 16SrIII phytoplasmas. Co-infection with Las and 16SrIII was also found. The 16S rRNA gene sequences from 22 samples were obtained and sequenced, confirming that 16Sr group III phytoplasma is associated with HLB symptoms in SP and MG States. 16SrIII phytoplasmas highly related are commonly found in *Melia azedarach*, a widespread tree in Brazil and Argentina.

In Guangdong, China in 2006 and 2007, 110 out of 141 citrus samples showing typical symptoms of HLB from 11 different cities, were PCR-positive for a strain of '*Candidatus* Phytoplasma asteri'. Many of the samples (48.9%) were mixed infections with '*Ca*. L. asiaticus' (Chen et al. 2008, 2009). In 2010, in a survey in Guangxi Province, China, to detect and characterize phytoplasmas in a huanglongbing (HLB)-infected grapefruit (*Citrus paradisi*) orchard, 87 leaf samples with symptoms of blotchy mottle were collected from symptomatic grapefruit trees, 77 (88.5%) were positive for '*Candidatus* Liberibacter asiaticus' and 5 for both phytoplasma and '*Ca.* L. asiaticus'. Phylogenetic analysis and virtual restriction fragment length polymorphism analysis confirmed the phytoplasma was a variant (16SrII-A*) of phytoplasma subgroup 16SrII-A. As phytoplasmas were only detected in blotchy-mottle leaves, the 16SrII-A* phytoplasma identified was related to HLB-like symptoms (Lou et al 2014).

An association of '*Candidatus* Phytoplasma asteris' with HLB-like symptoms has been found in citrus groves in Mexico (Arratia-Castro et al 2014).

In Pakistan, '*Candidatus* Phytoplasma asteris' (Group 16SrI) was found infecting mango, citrus (Mannan et al. 2009), loquat, geranium, periwinkle, radish, blackberry and potato (Fahmeed et al. 2009).

[258] It is very unlikely that *D. citri* would vector phytoplasmas. The superfamily containing the largest number of vector species is the Membracoidea, within which all known vectors to date are confined to Cicadellidae. The second largest group is the Fulgoromorpha, in which four families of vector species are found. The smallest suborder is Sternorrhyncha, in which only two genera in the Psyllidae, *Cacopsylla* and *Bactericera*, are confirmed vectors (Weintraub & Beanland 2006).

In Taiwan, the citrus phytoplasma detected from a healthy-looking tree was identical in sequence alignment with the purple coneflower (*Echinacea purpurea*) witches' broom phytoplasma (Tseng et al. 2012). The Taiwan citrus phytoplasma from a healthy Wentan Pommelo tree was identified to be a Peanut witches' broom (PnWB) phytoplasma (16SrII-A), the same as that found in grapefruit trees with HLB symptoms in Guangxi, China. However, inoculation trials with the Taiwan citrus symptomless phytoplasma and one isolate of PnWB phytoplasma into healthy citrus plants showed infection but did not induce visible symptoms. It is difficult to conclude that the infection of the PnWB phytoplasma was associated with HLB symptom expression (Feng et al 2014). Furthermore, artificial inoculation of periwinkle leaf yellowing (PLY) phytoplasma (16SrI-B) into the healthy citrus plants demonstrated no infection.

In Puerto Rico a group 16SrIX phytoplasma has been found infecting citrus (*Citrus sinensis* and *C. limon*), as well as coffee (*Coffea arabica*), periwinkle (*Catharanthus roseus*), and tabebuia (*Tabebuia heterophylla*) (Caicedo et al 2015).

In Central India '*Candidatus* Phytoplasma trifolii' (16SrVI group) has been found in Nagpur mandarin (*Citrus reticulata*) showing HLB-like symptoms (Das et al 2016).

A new emerging citrus decline disease (CDD) widely spread in Southern Kerman region of Iran, is killing around 10% of cultivated citrus trees. Early CDD symptoms include leaf pale green color, no production of fresh sprouts, and general retardation of the growth. Late symptoms include the evident tree decline along with reduction and decay of the root system. Single and double infection by '*Ca*. L. asiaticus' and '*Ca*. P. aurantifolia'have been found in CDD-affected citrus trees (Alizadeh et al 2017).

Symptoms of Australian citrus dieback

Symptoms of Australian citrus dieback (ACD) are most frequently observed in grapefruit and are especially prominent in autumn. ACD affects most varieties although symptoms have not been seen in rough lemon, Rangpur lime or lemons.

For photos of symptoms of ACD see Apendix 7. Symptoms appear first in one branch and the chlorotic leaf patterns (suggestive of zinc or iron deficiency) develop in young growth. Irregular blotches or small green round spots of green in yellow tissue and yellowing of leaf veins and entire leaves are common symptoms. Leaf fall is heavy on badly affected branches. Trees remain in a chronic unproductive state and don't die. Fruit of affected grapefruit are small but not distorted or bitter as with HLB or lopsided as with CTV stem pitting.

Occurrence of ACD

ACD has been observed in all citrus growing regions in Australia. Incidence of new infections is coincident with above average rainfall during the spring and autumn, which encourages growth of native shrubs and weeds and a build-up in populations of native insects. The very wide but sporadic distribution of ACD and its appearance in isolated orchards (sometimes in remote areas surrounded by native vegetation) and in mature trees from known healthy budlines suggest that infections result from movement of a pathogen from another host by a native vector (possibly flattids) that visits citrus only rarely (Broadbent in Compendium of Citrus Diseases 2000). Mr. N Grylls of CSIRO had circumstantial evidence that *Siphanta atomaria* could transmit ACD (Broadbent et al. 1977). There was also a corresponding decline in health of adjacent *Acacia* shrubs with which a bacterium-like organism was associated.

ACD has been attributed to phytoplasma (Broadbent et al. 1976, Davis et al. 1997, Garnier, pers. comm., Constable, pers.comm). Constable considered the phytoplasma associated with ACD in grapefruit at Nangiloc to be tomato big bud.

Seed Transmission

Examination of the evidence that seeds of *Poncirus* genus and hybrids may transmit *Candidatus* Liberibacter species, the causal agent of citrus greening *Supplement to the global pest list of Citrus spp. pathogens and an examination of evidence for seed transmission* https://www.aphis.usda.gov/plant_health/plant_pest_info/citrus/downloads/supplement-global-pest-list.pdf

For descriptions of seed development in *Citrus* Schneider 1968b; Frost and Soost, 1968; Koltunow, 1993; Albrecht & Bowman 2009.

Reduced fruit production is a common symptom of HLB, and many seeds are aborted or are small and dark. Capoor et al. (1974) reported no evidence of seed transmission of '*Ca*. L. asiaticus' when seeds from fruits of infected grapefruit and sweet orange were grown. A large number of seeds from symptomatic fruit and both normal looking and brown and abortive seeds were used in the study. However, Tirtawidjaja et al. (1981) suggested that some stunted, chlorotic plants were produced by seeds from small fruits produced by HLB infected trees, but noted that further work was required to clarify the results. No symptoms were seen in seedlings grown from seed from normal sized fruit produced by diseased trees. Ke et al. (1988) found no symptoms in seedlings grown from 888 Fuju mandarin seeds from HLB trees observed over 5 years.

In the October 2007 Environmental Assessment Final Rule for Movement of Commercially Packed Citrus Fruit from the Citrus Canker Quarantine Areas under IV Environmental Impacts it states 'we have received reports of preliminary scientific evidence indicating that when seedlings are generated from seed that is taken from plants infected with citrus greening, a small percentage of those seedlings are themselves infected with citrus greening. In response to this and to be abundantly cautious, APHIS has amended the Federal Order for citrus greening to prohibit movement of seed for planting from areas quarantined for citrus greening'.

Sagaram et al. (2008), using PCR, found that '*Ca.* L. asiaticus' was distributed in bark tissue, leaf midrib, roots, and different floral and fruit parts, but not in the endosperm and embryo, of infected citrus trees.

Zhou et al. (2008a) detected '*Ca.* L. asiaticus' in up to 53% of all seeds tested both from HLB-infected periwinkle (*Cantharanthus roseus*) and dodder (*Cuscuta pentagona* Engelm.) without resorting to nested PCR. The PCR amplicons were confirmed by sequence analysis. Germination rates of these '*Ca*. L. asiaticus'-positive seeds from both plant species were normal. Over 80% of the periwinkle plants germinated from the infected seeds showed initial HLB symptoms of vein yellowing and leaf yellowing only when they were stressed by nutrient deficiency. Surprisingly, the disease progressed slowly, and did not cause plant death, and all symptomatic plants became asymptomatic after the stress was removed. The '*Ca*. L. asiaticus' titre remained very low; in most cases, detected only by nested PCR or regular PCR by increasing the concentration of the bacterial DNA. The periwinkles infected with '*Ca.* L. asiaticus' via seed transmission were maintained for over six months. Zhou et al. (2008a) suggested that although '*Ca.* L. asiaticus' was transmitted in periwinkle seeds a second, undescribed component of an HLB disease complex was not. Seed transmission of Las was tested in grapefruit, sweet orange, sour orange, lemon, and trifoliate orange. A very low titer of Las was detected from the embryos and seedlings using nested PCR and real-time PCR. Most, if not all the seedlings did not show typical HLB symptoms and contained a relatively low Las bacterial titer for HLB, even in the four to five year old seedlings. The results indicated that the seed-transmitted Las

could not cause typical HLB disease by themselves. Psyllid transmission studies on the Las-positive seedlings were performed. A high percentage of psyllids acquired Las bacterium but did not have the same bacterial levels as those from HLB-affected citrus plants. However, it is the first time that a seed transmitted plant was confirmed by PCR using several Las-specific primer sets. Further study with graft transmission and electron microscopy confirmed the unique nature of seed transmission of HLB (Duan 2013 http://research.citrusrdf.org/reports/2013/07/16/Duan-162-Final_report-2013CMA.pdf).

Hartung et al. (2008) germinated seeds from '*Ca*. L.asiaticus' symptomatic fruit of '*Murraya paniculata'*, rough lemon and Meyer lemon, sour orange, grapefruit and Valencia sweet orange and kept 319 seedlings in a greenhouse for nearly 3 years. The seedlings were observed regularly for symptoms of HLB and tested for '*Ca.* L. asiaticus' by Q-PCR three times using a 16SRNA based procedure. The large majority of seedlings did not show symptoms, and none of the seedlings tested positive for '*Ca*. L. asiaticus' by a real-time PCR. However, 9 of 89 sour orange seedlings showed abnormal growth patterns, which included stunting, defoliation and chlorosis. One of these sour orange seedlings in particular was severely stunted and showed symptoms similar to HLB. In the abstract of this paper, the authors stated that 'This seedling was positive for the presence of 'Ca. L. asiaticus' when tested with a different set of primers that targeted the 16S region 'Ca L. asiaticus'. The amplicon was sequenced from duplicate reactions and was found to have a 100% match to the 16S gene sequence from several strains of 'Ca. L. asiaticus' deposited in Genbank.' Yet in his talk when the paper was presented, Hartung claimed there were no positive PCRs, no symptoms and therefore no seed transmission. Certainly the photograph of the sour orange seedling that was shown, suggested a tetraploid or a zygotic seedling, rather than a sour orange with HLB symptoms. This was confirmed in Hartung et al. (2010). Hartung et al (2010) used two groups of 360 or more seedlings each of various citrus species were grown from seed removed from fruit on trees that were symptomatic for HLB and confirmed to be infected with '*Ca*. L. asiaticus' by PCR tests. These seedlings were tested multiple times over periods of up to 3 years. No symptoms typical of HLB, such as blotchy leaf mottle, chlorotic shoots, or dieback of branches, were observed in these seedlings, and none of these 723 seedlings tested positive for the presence of '*Ca*. L. asiaticus' even after repeated testing by sensitive quantitative PCR assays.

Shatters (2008) observed HLB-like symptoms in seedlings of Duncan grapefruit grown in insect-free controlled environment greenhouses. Using a newly developed qPCR-based method for the detection of '*Ca*. L. asiaticus' 16S rDNA sequence, he was able to detect the '*Ca*. L. asiaticus' sequence in less than 10% of these seedlings; however, the detection of '*Ca*. L. asiaticus' did not necessarily correlate with symptoms. In subsequent studies with Ruby Red grapefruit and Hamlin orange, the 16S rDNA sequence was detected in seedlings that were surface sterilised and germinated in sterile Magenta jars (Shatters 2008). Sequence analysis of the 16S rDNA sequence indicated that the amplified DNA was 100% identical to previously reported '*Ca*. L. asiaticus' sequence and only 98% of the bases were identical to '*Ca.* L. americanus'. Dissection of the sterile-grown seedlings showed that the highest detectable level of '*Ca*. L. asiaticus' was in the seedling roots. As plants grew, HLB-symptomatic plants developed more slowly than asymptomatic plants, however, most lost HLB symptoms over time.

Graham et al. (2008) reported that seed coats of Pineapple sweet orange seeds from infected fruit in late autumn 2006 in Florida contained high titres of '*Ca.* L. asiaticus', based on RT-PCR assay. Seeds minus their seed coats were germinated. All surviving seedlings from seed with RT-PCR positive seed coats, as well as 45 plants with negative seed coats, were sampled and tested by RT-PCR. From the 59 plants sampled, 7 plants were either positive or questionably so. Ct values for one plant were

28.22 and 31.38 for a second plant. Ct values for the remaining suspect plants were between 32.3 and 33.4 (a Ct value between 30 and 32 was considered questionable for HLB). Upon re-assay, 3 of the 7 plants were positive and yielded ~700 bp 16s rDNA sequences for '*Ca*. L. asiaticus' after nested PCR (Benyon et al. 2008). The three plants testing positive were transferred to the UDSA-ARS facility in Fort Pierce to conduct psyllid transmission studies, so as to partially fulfil Koch's postulates. Of these three plants, only one tested positive in subsequent RT-PCR testing. In addition to the 59 plants tested, 356 plants not tested in the original group of 56 were tested and none were positive or questionable. In late autumn 2007, fruit were collected from 8 symptomatic trees. Extracted seed was classified as healthy (28%), off coloured-gummy (29%) or aborted (43%). Healthy (359) and off-coloured (344) seed were planted and the resultant 723 seedlings were assayed by RT-PCR in February 2008. Six had Ct values less than or equal to 32 after two assays. In 2008, HLB was confirmed for two Carrizo citrange seed source trees in a Florida citrus nursery. From each positive tree, fruit were collected for extraction and germination of at least 200 seedlings. In seedlings assayed by RT-PCR for transmission, 142 seedlings from the first source yielded two seedlings with Ct values of 32 or less, and of 148 seedlings from a second source, 5 seedlings had Ct values of 32 or less. The conclusion was that transient infection of seedlings with '*Ca*. L. asiaticus' had occurred (Graham et al. 2008)**.** Confirmation of seed transmission clearly requires:

- multiple primers for various loci; and
- psyllid and graft transmission of the pathogen initially found in seed (Graham et al. 2008)

In response, Florida citrus nurseries began treating rootstock seed trees located outdoors with insecticide applications to reduce risk of psyllid transmission of 'Candidatus Liberibacter asiaticus' (Las), the putative causal agent. In 2008, a survey identified two 'Carrizo' citrange trees with symptoms of HLB. To assess the potential for seed transmission from HLB-affected seed source trees, assays of seedlings derived from seed extracted from symptomatic fruit were begun in 2006 (Graham http://research.citrusrdf.org/reports/2011/12/22/Graham_HLBSeedTrans_final_report_12-11.pdf). From 2006 to 2008, 1557 seedlings germinated from 'Pineapple' sweet orange seeds from trees were assayed by quantitative polymerase chain reaction (qPCR) using 16S rRNA gene primers. Of these seedlings, a single plant was positive for (Las+), although additional tests were negative. In 2009, no Las+ plants were detected among 332 'Murcott' tangor seedlings from trees in Hendry Co. From nurseries in 2008, one Las+ seedling was detected in 290 seedlings from fruit located on symptomatic branches of two 'Carrizo' citrange trees, but it's Las+ status was not confirmed after repeated testing. In 2009, a single Las+ result was obtained for one of 100 Cleopatra mandarin seedlings, whereas no Las+ seedlings were detected for 125 seedlings from seeds from two trees of 'Swingle' citrumelo, 649 seedlings from four trees of 'Kuharske' citrange, or 100 seedlings from one tree of 'Shekwasha' mandarin. Despite the occasional Las+ qPCR tests, no plants developed HLB symptoms. The most probable explanation for these results is transient transmission of Las from seed obtained from HLB-infected trees with no subsequent disease establishment.

HLB-like symptoms, such as yellow shoot, blotchy mottle and vein corking on the leaves were observed in a low percentage of the seedlings, (primarily on trifoliate orange plants) grown from the seeds of typical HLB-affected and atypical HLB-affected citrus trees of sweet orange, pomelo and trifoliate orange during 2007–2009. When various primer sets that target different genetic loci of the bacterial genome of '*Ca*. L. asiaticus' were used, '*Ca*. L. asiaticus'was detected from all three citrus hosts, ranging from 2.0% to 41.7% using PCR, nested PCR and quantitative PCR. It is important to note that the '*Ca*. L. asiaticus' detected from the seedlings remained at a very low titre, unlike that in graft- or psyllid-transmitted HLB-affected citrus plants, and most, if not all, of the '*Ca*. L. asiaticus'-

positive seedlings did not develope typical HLB symptoms over three years. The molecular mechanism of the low-titre, non-lethal, but seed-transmissible '*Ca*. L. asiaticus' (Benyon et al. 2009) was studied further by Benyon et al. (2010) for seedlings grown from the seeds of typical and atypical HLB-affected citrus trees of sweet orange, grapefruit, pomelo and trifoliate orange from 2007-2010. HLB-like symptoms, such as yellow shoot and vein corking on leaves were observed on the seedlings in a greenhouse. Using various primer sets that target different genetic loci of the '*Ca*. L. asiaticus' genome, '*Ca*. L. asiaticus' was detected from all seedlings of the four 'species' of citrus, ranging from 1.9 % to 78.5% depending on which method was used: conventional PCR, nested PCR or quantitative PCR. In addition, '*Ca*. L. asiaticus' was also detected by a newly developed sensitive qPCR method from 61.3% of the psyllids reared on these seedlings. The sequencing of PCR products confirmed the amplicons belong to '*Ca*. L. asiaticus'. However, the '*Ca*. L. asiaticus' titres in the seedlings remained at very low levels, and most, if not all, of the '*Ca*. L. asiaticus'-positive seedlings did not develop typical HLB symptoms over a three year period. The results indicated that the seed-transmitted '*Ca*. L. asiaticus' did not cause HLB by themselves, suggesting they differed from the HLB-causing strains.

Shokrollah et al. (2009) found no evidence of seed transmission of HLB when seeds of *C. reticulata* cv. Limau Madu were collected from HLB-infected orchard trees and germinated in a screenhouse. However, only 20 seedlings were tested.

Albrecht & Bowman (2009) reported that none of 13,000 seedlings from '*Ca*. L. asiaticus'-positive seed-source trees displayed leaf symptoms characteristic of HLB such as, blotchy mottle or yellowing of the veins. Of 3557 rootstock seedlings from '*Ca*. L. asiaticus'-positive fruit, 338 and 348 were sampled and tested at 4 to 5 months and 6 to 7 months, respectively, after sowing. The seeds were selected from fruit with the strongest PCR signals obtained after peduncle analysis. PCR analysis of petiole and midrib samples of the seedlings detected two of 338 seedlings (0.3%) positive for the bacterium 5 months after sowing. However, signal strength of the PCR products was weak. Both seedlings were of the mandarin genotype Sun Chu Sha and originated from seeds of the same fruit. Repeated PCR analysis of both seedlings 7, 9, and 15 months after sowing resulted in negative results and neither plant displayed abnormal growth characteristics or leaf symptoms typical of HLB. To confirm these findings, more than 900 'Valencia' sweet orange seedlings produced from infected fruit were analysed in the second experiment. PCR analyses were conducted soon after germination to ensure detection of a possibly transient presence of the bacterium in the plant tissue. Three of the 431 seedlings tested (0.7%) yielded weak PCR products specific for '*Ca*. L. asiaticus'. Repeated PCR analysis at a later time point yielded negative results, corresponding to the results from rootstock seedlings. Similar to rootstock seedlings, none of the 918 seedlings from infected sweet orange fruit developed mottled leaves or other growth abnormalities associated with HLB.

Albtrecht & Bowman (2009) concluded that 'provided that PCR products detected during the early development of citrus seedlings are not the result of cross-contamination, detection suggests that bacterial cells or bacterial DNA must somehow be translocated into the tissue of the developing seedling.' 'With regard to the nucellar form of embryony in citrus, it seems probable that a few bacterial cells may be translocated from the vascular plexus of the chalaza into the nucellus during the initial stages of seed development where they may become enveloped by the developing nucellar embryo. This would explain why *Ca*. L. asiaticus can be detected at a very low frequency in seedlings within the first few months after germination. Alternatively, bacterial transmission may occur externally through contact of the cotyledons or the embryo with the adhering infected seedcoat. The fact that *Ca*. L. asiaticus was not detected at later time points during seedling development and that no disease symptoms typical for HLB were observed suggests that the

pathogen did not exist in a viable form or in susceptible tissues or that environmental conditions were not conducive to the multiplication necessary for virulence' (Albrecht & Bowman 2009).

On 6 April 2010 the USDA-APHIS issued its federal import quarantine order[259] preventing the entry into the USA of seed from countries with huanglongbing and citrus variegated chlorosis**.** In a May 19 Federal Order, the U.S. Department of Agriculture's Animal and Plant Health Inspection Service announced it had removed the citrus and poncirus seed species from the citrus greening list[260].

No seed transmission of '*Ca*. L. africanus' could be demonstrated by van Vuuren *et al.* (2011). Fruit from 26 Laf-infected branches of six citrus varieties showing greening symptoms were collected and the seed harvested. Varieties included Minneola tangelo; sweet oranges, Premier midseason, Clanor midseason and Olinda Valencia; Eureka lemon and Troyer citrange. Branches from which fruit was collected were confirmed to contain '*Ca*. L. africanus' by real-time PCR testing using Taqman probe HLBp and HLBaf & HLBr primers as described by Li *et al*. (2006). The seed of each sample was sorted into five categories ranging from healthy looking to totally aborted based on their appearance before planting. Germination was done in seed trays under vector-free conditions at 24-28°C. No symptoms developed and all these indicators tested negative for '*Ca*. L. africanus', indicating the absence of a transmissible agent.

Using real-time quantitative PCR (qPCR) Hilf (2011) detected pathogen DNA in nucleic acid extracts of 36 and 100% of peduncles from 'Sanguenelli' and from 'Conners' fruits, respectively. He also detected pathogen DNA in extracts of 37 and 98% of seed coats and in 1.6 and 4% of extracts from the corresponding seeds of 'Sanguenelli' and 'Conners', respectively. Small amounts of pathogen DNA were detected in 10% of 'Sanguenelli' seedlings grown in the greenhouse, but in none of 204 extracts from 'Conners' seedlings. Pathogen DNA was detected in 4.9% and in 89% of seed coats peeled from seeds of 'Sanguenelli' and 'Conners' which were germinated on agar, and in 5% of 'Sanguenelli' but in none of 164 'Conners' seedlings which grew from these seeds on agar. No pathogen DNA was detected in 'Ridge Pineapple' tissue at 3 months post-grafting onto 'Sanguenelli' seedlings, even when pathogen DNA had been detected initially in the 'Sanguenelli' seedling. Though the apparent colonization of 'Conners' seeds was more extensive and nearly uniform compared with 'Sanguenelli' seeds, no pathogen DNA was detected in 'Conners' seedlings grown from these seeds.

Hilf et al (2013), using TEM and fluorescence in situ hybridisation (FISH) analyses with probes complementary to CLas 16S rRNA gene revealed bacterial cells in the vascular tissue of intact seed coats of grapefruit and pommelo.

Pollen transmission

Stover and McCollum (2011) found that numbers of '*Ca.* L. asiaticus' genomes in anthers were 0.15% to 1% of levels detected in mature symptomatic leaves and suggest that there may be a risk of spreading HLB through pollinations.

259 http://edocket.access.gpo.gov/2010/2010-7736.htm **Federal Register** /Vol. 75, No. 65 /Tuesday, April 6, 2010 /Rules and Regulations **17289**

260 http://www.thepacker.com/news/usda-amends-citrus-greening-rule-allow-seeds

[i] https://www.dallasnews.com/business/business/2017/07/28/pest-responsible-destructive-citrus-disease-found-luggage-dfw-airport

[ii] https://www.ippc.int/en/countries/trinidad-and-tobago/pestreports/2017/06/detection-of-huanglongbing-or-citrus-greening-candidatus-liberibacter-asiaticus-in-trinidad/