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A review of techniques for detecting Huanglongbing (greening) in citrus

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Abstract

Huanglongbing (HLB) is the most destructive disease of citrus worldwide. Monitoring of health and detection of diseases in trees is critical for sustainable agriculture. HLB symptoms are virtually the same wherever the disease occurs. The disease is caused by *Candidatus Liberibacter* spp., vectored by the psyllids *Diaphorina citri* Kuwayama and *Trioza erytreae*. Electron microscopic technique was the first method for HLB detection. Nowadays, scientists are working on the development of new techniques for a rapid HLB detection as there is no sensor commercially accessible for real-time assessment of health conditions in trees. Currently, exploration is the most widely used mechanism for monitoring HLB, which is an expensive, labor-intensive and time-consuming process. Molecular techniques such as polymerase chain reaction are used for the identification of HLB disease, which requires detailed sampling and processing procedures. Furthermore, investigations are ongoing in spectroscopic and imaging techniques, profiling of plant volatile organic compounds and isothermal amplification. This study recognizes the need for developing a rapid, cost-effective, and reliable health monitoring sensor that would facilitate advancements in HLB disease detection. This paper compares the benefits and limitations of these potential methods for HLB detection.

Keywords: HLB, Detection, Trees, Monitoring, Health

Introduction

Citrus production is one of the most important economic agricultural activities in the world. According to the Food and Agriculture Organization (FAO 2014), approximately 122 millions of tons of citrus fruits are produced annually, corresponding to roughly US \$ 17 billion from the sale of juices and fresh fruit worldwide in 2008. Economic losses in citrus

production have occurred in recent years due to diseases such as citrus greening disease or Huanglongbing (HLB), which has become one of the greatest challenges for citrus growers across the world (Cevallos-Cevallos *et al.* 2012), because it reduces fruit yield and quality and severely debilitates citrus trees (Qureshi and Stansly 2009). The world's largest citrus producers, Brazil, China, India, Mexico and the United States are especially threatened by the HLB (Do Brasil Cardinali *et al.* 2012; FAOSTAT 2009). HLB has been associated with Gram negative bacteria, obligate parasite, phloem-limited α -proteobacterium, *Candidatus Liberibacter* spp. (Gottwald 2010). The genus *Candidatus Liberibacter* has three known species: *Candidatus Liberibacter asiaticus* (Las), *Candidatus Liberibacter africanus* (Laf) and *Candidatus Liberibacter americanus* (Lam). HLB can be transmitted by grafting from citrus to citrus and by dodder to periwinkle. *Trioza erytreae* and *Diaphorina citri* psyllids are natural vectors (Bové 2006). Early disease detection relies primarily on scouting for disease symptoms in the field, such as yellow shoots, blotchy mottle leaf and lopsided fruit with green color remaining on the stylar end, aborted seeds and high levels of starch in the leaves (Gonzalez *et al.* 2009). Nevertheless visual inspection is one of the most applied methods to diagnose citrus greening, this approach is highly influenced by subjective interpretation, and diagnostic errors can be higher than 30% (Pereira *et al.* 2011) and diagnosis may be worsened by other biotic and/or abiotic plant health related problems (Lin *et al.* 2010). HLB symptoms can be confused with diseases as *Citrus Tristeza Closterovirus* (CTV), *Phytophthora* infection, citrus blight and certain nutrient deficiencies (Shokrollah *et al.* 2011). Methods such as electron microscopy, serology, DNA probes, enzymatic assay, enzyme-linked immunosorbent assays (ELISA), conventional polymerase chain reaction (PCR) (Yamamoto *et al.* 2008) and quantitative PCR (qPCR) are used for the diagnosis and confirmation of HLB (Kogenaru *et al.* 2014). X-ray fluorescence (XRF) and laser-induced breakdown spectroscopy (LIBS) combined with chemometric strategies are used to

successfully predict the condition of orchard plants infected with *Candidatus Liberibacter* spp. (Pereira and Milori 2010). Fourier transform infrared (FTIR) spectroscopy has also been used for the diagnosis of diseased citrus plants, and mid-infrared spectroscopy has been used to study citrus greening infection; however, except for LIBS method, these studies did not provide early diagnosis. Early detection and quarantine of Las-infected trees are important management strategies used to prevent HLB from invading HLB-free citrus producing regions (Kogenaru *et al.* 2014). In this paper, a review of current diagnostic techniques for HLB disease performed worldwide is described, because an early diagnosis and differentiation of *Candidatus Liberibacter* spp. is critical in reducing the local and via international trade spread and devastation of this disease, as well as minimizing the economic impact of potential false positive diagnoses.

Microscopic techniques for Huanglongbing disease detection

In 1970 Electron microscopy was the first laboratory technique used by Lafleche and Bove (1970) for the identification and confirmation of HLB (Bové 2006); however in citrus trees with HLB, the symptoms are difficult to identify. Yellow shoots, leaf blotchy mottle, and lopsided fruits with color inversion and aborted seeds are all characteristic, but they do not always occur together in the same tree (Cevallos-Cevallos *et al.* 2009; Folimonova *et al.* 2010); they can be distorted or masked by symptoms of other diseases or in some cases, induced by conditions unrelated to HLB (Cevallos-Cevallos *et al.* 2009). In 2009 Cevallos-Cevallos described techniques such as Light microscopy (LM) and transmission electron microscopy (TEM), which were used on leaves, petioles, stems, bark and roots that were sampled from HLB-affected and control Valencia trees. The samples were fixed in 3% glutaraldehyde, 0.1 M potassium phosphate buffer, pH 7.2, for 4 h at room temperature and then overnight in the refrigerator. They were then washed in the same buffer and post-fixed 4 h at room temperature in 2% osmium tetroxide in the above buffer. The

samples were then dehydrated in acetone series and embedded in Spurr's resin. For light microscopy, 1 mm sections were cut with glass knives and stained with methylene blue/azure A, post-stained in basic fuchsin. Light micrographs were taken on a Leitz Laborlux S compound microscope (Germany) with a Canon Powershot S31S digital camera (Tokyo, Japan). For TEM, the same blocks were thin sectioned (90–100 nm) with a diamond knife, collected on 200 mesh copper grids and stained with 2% uranyl acetate (aq.) and post-stained with lead citrate. Micrographs were made with an AMT (Advanced Microscopy Techniques Corp., Danvers, MA) digital camera on a Morgagni 268 (FEI Company, Hillsboro, OR) transmission electron microscope (Cevallos-Cevallos *et al.* 2009). In another way Svetlana and Diann in 2010, conducted microscopy studies in cooperation with FEI Company (The Netherlands), using a Morgani 268 TEM. Leaf and petiole samples were collected from trees induced with HLB disease; to prepare these samples for TEM, a routine fixation procedure was used in which they were fixed with the same technique used by Cevallos-Cevallos in 2009, but the difference is that Svetlana and Diann used resin for 3 days, then samples (1 μ m) were taken for light microscopy observations. Sections of 100 nm in size were mounted on 200-mesh formvar-coated copper grids and stained with 2% uranyl acetate (aq.) and lead citrate. The thin sections were further examined with a Morgagni 268 transmission electron microscope.

The microscopic techniques in different investigations are analogous; examinations of the disease-affected tissues using TEM revealed that the pathogenic bacterium possesses a cell wall of the Gram-negative type and exclusively resides within the sieve tubes of infected citrus trees (Svetlana and Diann 2010). These properties of the bacterium were used as a basis for the detection of HLB by electron microscopy, which had been the only reliable diagnostic technique for a number of years prior to the development of detection methods based on DNA hybridization and PCR.

Molecular techniques for Huanglongbing disease detection

In recent years, molecular techniques of plant disease detection have been well established. The sensitivity of molecular techniques refers to the minimum amount of microorganism that can be identified in the sample. It is reported that the sensitivity of molecular techniques for detecting bacteria ranged from 10 to 10^6 colony forming units/mL (Sankaran *et al.* 2010). The commonly used molecular techniques for disease detection are ELISA, PCR and qPCR, other molecular techniques include immunofluorescence (IF), flow cytometry, fluorescence *in situ* hybridization (FISH), and DNA microarrays (Sankaran *et al.* 2010).

Huanglongbing is one of bacterium which has yet to be cultivated *in vitro*, consequently, the pathogen was given a provisional *Candidatus* status in nomenclature; currently three species of the pathogen are recognized from trees with HLB disease based on the amplification of a 1160 bp fragment of the 16S rDNA sequence of a *Candidatus* Liberibacter spp: Las, Laf, and Lam (Kim and Wang 2009; Bové 2006; Li and Levy 2006; Pietersen *et al.* 2014). Two PCR systems have been used in HLB disease. The first is based on 16S rDNA sequence, using many sequences of primers and probes (Fujikawa and Iwanami 2012). OI1/OI2c is able to amplify the rDNA Las and Laf species (Teixeira *et al.* 2005a). When the two *Ca.* Liberibacter spp are known or supposed to be present in any country, it is desirable to use the two forward primers, OI1 + OA1, and the common reverse OI2c primer in the same PCR mixture (Bové 2006). Sequence analysis shows that the rDNA amplified from Las has one *Xba*I restriction site, and yields, upon *Xba*I treatment, two fragments of sizes 520 bp and 640 bp., respectively. The rDNA amplified from Laf has an additional restriction site, and yields three fragments of 520 bp, 506 bp, and 130 bp. (Jagoueix *et al.*, 1996; Teixeira *et*

al., 2005a). The second PCR is centered on the *nusG-rplK* operon region (A2/ J5 and MHO353/MHO354) (Fujikawa and Iwanami 2012). The intergenic region between genes *rplA* and *rplJ* is 34 bp larger in Las compared to Laf. With forward primer f-*rplA*2, selected in the *rplA* gene, and reverse primer r-*rplJ*5 from the *rplJ* gene, a 703 bp DNA is amplified from Las, while a 669 bp DNA is obtained with Laf, if both *Ca. Liberibacter* spp. are present in the same sample. Amplification of the two DNAs is obtained, and upon agarose gel electrophoresis, two DNA bands are seen, the upper (703 bp) corresponding to Las, and the lower (669 bp) to Laf (Bové 2006). A third *Ca. Liberibacter* specie was identified in Sao Paulo, Brazil by the *nusG-rplKAL-rpoBC* gene clusters of Las and Laf, which were obtained and sequenced (Teixeira *et al.* 2005a,b). The same gene cluster has recently been obtained from Lam. additional *Ca. Liberibacter* genes, including the *omp* gene, have been isolated by using Random Amplified Polymorphic DNA (RAPD). The *omp* gene was used to study the genetic variability of Las (Teixeira *et al.* 2008). Currently, quantitative PCR has become the preferred detection method of *Ca. Liberibacter* spp. (Li *et al.* 2009). Compared to conventional PCR, qPCR offers both sensitive and rapid detection of these bacteria. qPCR is reported to increase the sensitivity for *Ca. Liberibacter* spp. detection by 10 times relative to nested PCR, and 100 to 1000 times relative to conventional PCR for these bacteria (Morgan *et al.* 2012). qPCR methods target genes with low copy number: three copy 16S rDNA, single copy b-operon or single copy elongation factor Ts (EF-Ts). The reported low sill limits of qPCR are around ten gene copies for 16S rDNA and b-operon procedures (Teixeira *et al.* 2008), and single gene copy for the elongation factor Ts (single closed tube with dual sets of primers; (Morgan *et al.* 2012). In another way, Kim and Wang (2009) compared the detection of HLB with different qPCR based methods to primer probes targeting either 16S rDNA or b-operon DNA. The 16S rDNA copy number of Las was estimated to be three times of that of the b-operon

region, consequently allowing their detection. Quantitative Reverse Transcriptase PCR (qRT-PCR) showed that 16S rRNA averaged 7.83 times more than 16S rDNA for equal samples. Dilution analysis also indicates 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. On June of 2013, Nageswara-Rao *et al.* made an effort to develop a variety of candidate gene markers specific to Las for early detection of HLB disease; the effectiveness of the primer pairs developed were also tested for cross-species amplification, if any, against the other two HLB-causing species Laf and Lam. Teixeira *et al.* (2005a) reported three primer pairs successfully developed from thirty-two different gene-specific primer pairs, across Las genome. The possibility of these primer pairs for cross-genome amplification across Laf and Lam were tested and their conclusions of the applicability for detection and differentiation of *Candidatus Liberibacter* spp. was discussed. A list of specific primers used in conventional PCR and qPCR to identify *Ca. Liberibacter* spp., is reported in Table 1.

In another way DNA dot and Southern hybridization was employed by Hung *et al.* (1999). In this study, DNA cloning methods were developed and used to detect HLB in infected citrus hosts. One of the clones containing a 0.24 kb HLB-specific DNA fragments was labeled with biotinylated nucleotides by a PCR-labeling technique. A dot hybridization assay with a biotin-labeled DNA probe has been successfully used for detecting HLB in various citrus hosts including mandarins, tangors, sweet oranges and pummels. This probe could specifically react with all HLB strains from several Asian countries, but not with those from South Africa. The developed probe is specified with sensitivity enough to detect minute levels of HLB infection and, therefore, can be used in quarantine of the Asian Greening disease (Hung *et al.* 1999).

Meanwhile, the first study of transcriptional profiling in response to *Candidatus Liberibacter* spp. infection using microarray technology was developed by Albrecht and Bowman in 2008. Microarray technology has been used in several studies of bacterial plant diseases such as bacterial blight (Li *et al.* 2006) and bacterial spot (Gibly *et al.* 2004), as well as viral, fungal, and other diseases (Espinoza *et al.* 2007; Panthee *et al.* 2007). Albrecht and Bowman (2008) investigated the gene expression of sweet orange plants (*Citrus sinensis* L. Osbeck) in response to infection with Las in comparison with non-infected healthy plants using the Affymetrix GeneChip® citrus genome microarray. Due to the compatibility of the interaction, significant transcriptional changes were expected to happen between genes associated with cellular modifications caused by infection processes or involved in general defense and stress-responses. Citrus genome array provides new insights into the molecular basis of citrus response to this pathogen. Of the more than 33,000 probe sets on the microarray, 21,067 were expressed in the leaves, of which 279 and 515 were differentially expressed (false-discovery rate (FDR) ≤ 0.05) 5–9 and 13–17 weeks after inoculation, respectively. Outstanding was the pathogen-induced accumulation of transcripts for a phloem-specific lectin PP2-like protein. Transcriptional changes and their relation to disease symptom development are discussed (Albrecht and Bowman 2008).

Although there are laboratory-based PCR methods that can accurately detect HLB, there is a need for a sensing or screening system that would provide reliable detection of citrus greening symptoms in real-time and under field conditions. Since PCR technique is expensive and time-consuming (Hawkins *et al.* 2010), having a prescreening technique that can detect the infected trees will reduce the demand for exploration. Also it will reduce the quantity of samples for PCR testing which could result in lower disease management costs and more effective disease recognition.

Spectroscopic and imaging techniques for Huanglongbing disease detection

The most accurate HLB diagnosis involves PCR (Hansen *et al.* 2008), but the identification of infected trees and sampling of leaves is time consuming. Moreover, the average accuracy achieved in visually inspecting and identifying infected trees by scouts is reported to be between 47% and 59% (Futch *et al.* 2009). Specific regions in the electromagnetic spectra have been found to provide information about the physiological stress in plants, and consequently, diseased plants usually exhibit different spectral signature than non-stressed healthy plants in those specific ranges. The spectral reflectance from the tree canopy in the visible and infrared regions of the electromagnetic spectra can be used as an indication of plant stress (Sankaran *et al.*, 2010). Differences in the spectral reflectance of healthy and diseased plants can be seen in the visible-infrared region (Purcell *et al.* 2009). Spectroscopy in the range of visible and near infrared has been investigated for disease detection in a great variety of crops since it is a rapid and non-destructive tool that can be used in real time crop assessment under field conditions (Sankaran *et al.* 2010). For example, Naidu *et al.* (2009) recognized viral infection (leafroll) in grapevines (*Vitis vinifera* L.) under field conditions using leaf spectral response from field portable spectrometer equipped with a leaf-probe. Hyperspectral reflectance in the range of 350–2500 nm was employed by Delalieux *et al.* (2007) to detect apple scab (*Venturia inaequalis*). The study concluded that the features along two spectral ranges in near infrared (1350–1750 nm and 2200–2500 nm) showed higher performance in the ordering of infected and healthy leaves at early stages. Both, the spectral regions in the range 580–660 nm and 688–715 nm showed better classification power for developed stages of infection.

Sankaran and Ehsani (2011) showed the evaluation of spectral features extracted from visible-near infrared Spectra spectroradiometer for their potential to detect citrus greening disease and did an effort to lower the cost of the optical sensor while maintaining their performance. Sankaran *et al.* defined spectral features: (i) spectral reflectance bands and (ii)

vegetation indices (VIs), which were derived from 350-2,500 nm spectral reflectance data using two feature extraction methods: stepwise discriminant analysis and stepwise regression analysis. Following the selection of spectral features, the features were assessed using two classifiers: quadratic discriminant analysis (QDA) and soft independent modeling of classification analogies (SIMCA) to determine the overall and individual classification accuracies. The classification results indicated that both spectral features (spectral bands and VIs) yielded good overall (higher than 80%) and healthy class (higher than 85%) classification accuracies using the QDA-based algorithm. The SIMCA-based algorithm yielded good average citrus greening classification accuracy (higher than 83%) using selected spectral features. Sankaran and Ehsani (2011) demonstrated the applicability of utilizing spectral features for detection of greening in citrus, thus laying the foundation for the development of new projects, protocols and tools for rapid and early HLB disease. For example, Mishra *et al.* (2011) focused on a low-cost, multi-band active optical sensor study to evaluate the identification of HLB-infected trees from the healthy trees. The sensor measured reflectance of tree canopy in four bands: two visible bands at 570 nm and 670 nm, and two near infrared bands at 870 nm and 970 nm. Extensive field measurements were conducted using this sensor. Analysis of the data showed that due to the large variability in the data, it was not possible to discriminate healthy from infected trees based on a single measurement from a tree; however, using multiple measurements from a tree it was possible to achieve high classification accuracies. With five measurements from each tree, classification methods such as k-nearest neighbors, support vector machines, and decision trees resulted in classification errors of less than 5%. Mishra *et al.* results demonstrated and confirmed the potential of a multi-band active optical sensor for detecting HLB-infected citrus trees under field conditions. In another way, Garcia-Ruiz *et al.* (2013) presented a new methodology of high-resolution aerial imaging for HLB detection using a low-cost, low-

altitude remote sensing multi-rotor unmanned aerial vehicle (UAV). A multi-band imaging sensor was attached to a UAV that is capable of acquiring aerial images at desired resolution by adjusting the flying altitude. Moreover, Garcia-Ruiz *et al.*, obtained results using UAV-based sensors that were compared with a similar imaging system (aircraft-based sensors) with lower spatial resolution. Data was comprised from six spectral bands (from 530 to 900 nm) and seven vegetation indices derived from the selected bands. Garcia-Ruiz *et al.* used stepwise regression analysis to extract relevant features from UAV-based and aircraft-based spectral images. At spatial resolutions, 710 nm reflectance and NIR-R index values were found to be significantly different between healthy and HLB-infected trees. During classification studies, accuracies in the range of 67–85% and false negatives from 7% to 32% were acquired from UAV-based data, while corresponding values were 61–74% and 28–45% with aircraft based data. Among the tested classification algorithms, support vector machine (SVM) with kernel resulted in better performance than other methods such as SVM (linear), linear discriminant analysis and quadratic discriminant analysis. Thus, high-resolution aerial sensing has good prospects for the detection of HLB-infected trees.

Meanwhile, Pourreza *et al.* (2014) studied a less expensive HLB detection based on particular symptoms, such as starch accumulation in the citrus leaf. The ability of narrow-band imaging and polarizing filters in detecting starch accumulation in symptomatic citrus leaf was evaluated in this study. A custom-made image acquisition system was developed for this purpose in which leaf samples were illuminated with polarized light using narrow-band high-power LEDs at 400 nm and 591 nm, and the reflectance was measured by two monochrome cameras. Two polarizing filters were mounted in perpendicular directions in front of the cameras so that each camera acquired an image with reflected light in only one direction (parallel or perpendicular to the illumination polarization). The results of this study showed that the starch accumulation in HLB-symptomatic leaves rotated the polarization planar of

light at 591 nm, and this property can be effectively used in a fast and inexpensive HLB detection system. Based on the same property of starch accumulation in citrus leaf, developed a sensor that includes a highly sensitive monochrome camera, narrow band high power LEDs, and polarizing filters. The sensor was first tested and calibrated in a simulated field condition in a laboratory. Then, it was tested in a citrus grove. Two simple image descriptors; mean and standard deviation of gray values, were used for the purpose of classification. The results showed that the sensor clearly highlighted the starch accumulation in the HLB-infected leaf and differentiated it from visually analogous symptoms of zinc deficiency (Pourreza *et al.* 2015).

Aksenov *et al* (2014) developed a method of disease detection based on chemical analysis of released volatile organic compounds (VOCs) that emanate from infected trees. They found that the biomarkers “fingerprint” is specific to the causal pathogen and could be interpreted using analytical methods such as gas chromatography/mass spectrometry (GC/MS) and gas chromatography/differential mobility spectrometry (GC/DMS). This VOC-based disease detection method has a high accuracy of ~90% throughout the year, approaching 100% under optimal testing conditions, even at very early stages of infection where other methods are not adequate. Detecting early infection based on VOCs precedes visual symptoms and DNA-based detection techniques (real-time polymerase chain reaction, RT-PCR) and can be performed at a substantially lower cost and with rapid field deployment.

The development of techniques for fast detection spectroscopic should continue to develop, mainly focused on lower costs of equipment and rapid detection to reduce false positive results

Profiling of plant volatile organic compounds for disease detection

Disease symptom development in HLB-affected plants is associated with starch accumulation in the leaf tissue, a phenomenon which is sometimes used as a diagnostic tool, though it is also observed in response to nutritional deficiencies and viral infection. Changes in transcript levels were therefore expected to occur within the group of genes associated with carbohydrate metabolism, specifically starch synthesis (Albrecht and Bowman 2008). Metabolomics is a developing field of analytical chemistry focused on the identification of metabolites. Usually employed in pharmaceutical applications, metabolomics has become a powerful tool in agriculture and food science (Gibney *et al.* 2005; Cevallos-Cevallos *et al.* 2009) and has been used to characterize metabolic changes in plants after biotic and abiotic stresses, as well as biotic contamination of foods (Peluffo *et al.* 2010). Metabolomics techniques have been able to identify changes in the metabolite profile of different citrus varieties (Cevallos-Cevallos *et al.* 2012), including those affected by HLB (Cevallos-Cevallos *et al.* 2011). In 2012 Cevallos-Cevallos *et al.* conducted a study to determine GC-MS-based metabolomics differences between two citrus varieties that are sensitive to HLB ('Madam Vinous' sweet orange (MV) and 'Duncan' grapefruit (DG)) and two tolerant citrus varieties ('Carrizo citrange' (CAR) and 'Poncirus trifoliata' (TR)). Cevallos-Cevallos *et al.* also monitored metabolomics changes occurring during HLB infection of sensitive varieties as a first step towards understanding the HLB tolerance mechanism of citrus. The gas chromatograph (GC) used in this experiment was model HP5890 coupled to an HP5971 series mass spectrometer (MS) from Hewlett Packard, (Santa Clara, CA). Chromatogram analysis was completed using HP ChemStation software (Cevallos-Cevallos *et al.* 2012); Cevallos-Cevallos *et al.* found 61 compounds with signal-to-noise ratios of at least 3 that were detected by GC-MS. Higher levels of the amino acids L-proline, L-serine, and L-aspartic acid, as well as the organic acids butanedioic and tetradecanoic acid, and accumulation of galactose in healthy plants

were characteristic of the most sensitive variety MV. Only galactose was significantly higher in DG when compared to the tolerant varieties TR and CAR. The tolerant varieties showed higher levels of L-glycine and mannose when compared to sensitive varieties MV and DG.

Profiling of the sensitive varieties MV and DG over a 20-week period after inoculation of those with the HLB-containing material revealed strong responses of metabolites to HLB infection, which differed from the response of the tolerant varieties. Significant changes of the L-threonine level in leaves from old mature flushes and L-serine, L-threonine, scyllo-inositol, hexadecanoic acid, and mannose in leaves from young developing tints were observed in MV. Significant variations in myo-inositol in old flushes and L-proline, indole, and xylose in new flushes were detected in DG (Cevallos-Cevallos *et al.* 2012). Results were confirmed and compared with PCR, showing that it is necessary to continue further studies to better understand the metabolic profile of the HLB disease.

Isothermal amplification combined with a lateral flow dipstick for rapid and sensitive detection of *Candidatus Liberibacter*.

A modern DNA amplification method known as Loop Mediated Isothermal Amplification (LAMP) was adjusted for the detection of Las by Rigano *et al.* (2014). This methodology was combined with a Lateral Flow Dipstick (LFD) device for visual recognition of the resulting amplicons, eliminating the need for gel electrophoresis. Rigano *et al.* 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. By serially diluting purified DNA from an infected plant, the sensitivity of the assay was found to be 10 picograms. This sensitivity level was verified to be similar to the values obtained running a real time PCR in parallel. This methodology was able to detect Las from different kinds of samples including infected citrus plants and psyllids. Rigano *et al.* Results indicated that the methodology

constituted a step forward in the development of new tools for the management, control and eradication of this destructive citrus disease. LAMP is based on the principle of autocycling strand displacement DNA synthesis performed by Bst DNA polymerase, for the detection of a specific DNA sequence (Notomi *et al.* 2000). The technique uses four to six primers that recognize six to eight regions of the target DNA and provides very high specificity (Nagamine *et al.* 2002). Amplification can be carried out in a simple and inexpensive device like a water bath at temperatures between 60 to 65°C. LAMP produces large amounts of DNA (Notomi *et al.* 2000) and shows high tolerance to biological contaminants (Kaneko *et al.* 2007), thereby simplifying sample preparation. Although LAMP products can be detected by gel electrophoresis, this procedure reduces the suitability for field applications. Rigano *et al.* (2014) assay focused on the detection of the DNA sequence of the *tufB-secE-nusG-rplKAL-rpoB* gene cluster present in the microorganism.

The analysis of the amplification products was done by gel electrophoresis or dot-blotting of the amplification products on a nylon membrane followed by staining with Mupid Blue, methods that are not compatible with field applications. The study targets a hypothetical protein-coding sequence present in the Las genome for the detection of this pathogen. To overcome the limitations associated with gel electrophoresis in the investigation, Rignano *et al.* (2014) coupled the LAMP amplification with a Lateral Flow Dipstick (LFD), which permits an accurate and straightforward detection of LAMP amplicons, eliminating the need of complex equipment and data analysis. Using LAMP and LFD technologies defines the development of an original molecular diagnostic tool for the detection of Las.

Conclusions

This paper reviewed and summarized some of the techniques that have been used for HLB disease detection. The five major categories for HLB detection disease are: microscopic,

molecular, spectroscopic, profiling of plant volatile organic compounds and isothermal amplification combined with lateral flow dipstick techniques. The microscopic techniques includes light microscopic (LM) and transmission electron microscopic (TEM). Molecular techniques include conventional PCR (PCR), quantitative-PCR (qPCR), quantitative Reverse Transcriptase-PCR (qRT-PCR), random amplified polymorphic-DNA (RAPD) and microarrays. Spectroscopic and imaging techniques utilize visible and near infrared spectroscopic in the design of new tools and optical sensors for the treetops. Profiling of plant volatile organic compounds currently employs gas chromatography – mass spectrometer (GC-MS). The most recent technique in 2014 for detection HLB disease could be the isothermal amplification, which combines loop mediated isothermal amplification (LAMP) with lateral flow dipstick (LFD). This review suggests that these methods of HLB disease detection show a good potential to detect plant diseases accurately. Nevertheless it is necessary for the scientific community to continue to expand the effectiveness of the techniques and generate new research in the management and control of the disease, and thus achieve lower economic losses worldwide.

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Table 1 Nucleotide sequence of primers used for the amplification of *Candidatus* Liberibacter species by end point PCR and qPCR.

HLB bacterium	Primer sequence (5'—3') *	DNA region amplified	Type PCR	References
<i>Ca. Liberibacter americanus</i>	F: AGTCGAGCGAGTACGCAAGTACT	16S rDNA	Conventional PCR	Nageswara-Rao <i>et al.</i> , 2013; Teixeira <i>et al.</i> , 2005 ^a
	R: CAACTTAATGATGGCAAATATAG			
	F: GAGCAGTACGCAAGTACTAG	16S rDNA	qPCR	Kim, 2009; Li and Levy, 2006; Li <i>et al.</i> 2009
	Tp: AGACGGGTGAGTAACGCG			
<i>Ca. Liberibacter asiaticus</i>	R: GCGTTATCCCGTAGAAAAAGGTAG	16S rDNA	Conventional PCR	Nageswara-Rao <i>et al.</i> , 2013; Teixeira <i>et al.</i> , 2005 ^a
	F: CGCGTATGCAATACGAGCGGCA			
	R: GCCTCGCGACTTCGCAACCCAT	16S rDNA	qPCR	Kim, 2009; Li and Levy, 2006; Li <i>et al.</i> 2009
	F: TCGAGCGCGTATGCAATACG			
	Tp: AGACGGGTGAGTAACGCG	hyv _I and hyv _{II} **	qPCR	Morgan <i>et al.</i> 2012
	R: GCGTTATCCCGTAGAAAAAGGTAG			
<i>Ca. Liberibacter africanus</i>	F: GCCGTTTAAACACAAAAGATGAATATC	16S rDNA	Conventional PCR	Nageswara-Rao <i>et al.</i> , 2013; Teixeira <i>et al.</i> , 2005 ^a
	Tp: ATAAATCAATTGTCTAGTTTACGAC			
	R: ACATCTTTCGTTTGAGTAGCTAGATCATTGA	16S rDNA	qPCR	Kim, 2009; Li and Levy, 2006; Li <i>et al.</i> 2009
	F: GCGCGTATTTTATACGAGCGGCA			
<i>Ca. Liberibacter africanus</i>	R: GCCTCGCGACTTCGCAACCCAT	16S rDNA	Conventional PCR	Nageswara-Rao <i>et al.</i> , 2013; Teixeira <i>et al.</i> , 2005 ^a
	F: CGAGCGCGTATTTTATACGAGCG			
	Tp: AGACGGGTGAGTAACGCG	16S rDNA	qPCR	Kim, 2009; Li and Levy, 2006; Li <i>et al.</i> 2009
	R: GCGTTATCCCGTAGAAAAAGGTAG			

* F: Forward primer; R: Reverse primer. Tp: Taqman probe

**Intragenic tandem-repeats sequence.