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REVIEW

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 microscopy was the first technique used 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, the most widely used mechanism for monitoring HLB is exploration, 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.

Key words: HLB, detection, trees, monitoring, health.

Résumé : La maladie du dragon jaune (MDJ) est la maladie des agrumes la plus destructrice au monde. Pour assurer la durabilité agricole, il est essentiel de contrôler la santé et la présence de maladies chez les arbres. Les symptômes de la MDJ sont pratiquement les mêmes quel que soit le point d'origine de la maladie. Celle-ci est causée par *Candidatus* Liberibacter spp. et transmise par les psylles *Diaphorina citri* Kuwayama et *Trioza erytreae*. La technique de microscopie électronique fut le premier mode de détection de la MDJ. De nos jours, des scientifiques travaillent à l'élaboration de nouvelles techniques de détection de la MDJ puisqu'il n'existe aucun détecteur commercial permettant l'évaluation de l'état de santé des arbres en temps réel. Actuellement, on doit la plupart du temps recourir à l'exploration pour surveiller la MDJ, un processus laborieux, coûteux et pénible. On a recours à des techniques moléculaires comme la réaction de la polymérase en chaîne pour identifier la MDJ, ce qui exige un prélèvement et un traitement minutieux. Par ailleurs, on évalue présentement des techniques de spectroscopie et d'imagerie, le profilage de composés végétaux organiques volatils et l'amplification isothermique. La présente étude met en relief le besoin de mettre au point un dispositif d'analyse de l'état de santé qui serait à la fois rapide, rentable et fiable et qui permettrait de faire progresser la détection de la MDJ. Cet article compare les avantages et les limites de ces méthodes aspirant à la détection de la MDJ. [Traduit par la Rédaction]

Mots-clés : MDJ, détection, arbres, surveillance, santé.

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 million 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

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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 parasites, phloem-limited alphaproteobacteria, and *Candidatus* (*Ca.*) Liberibacter spp. (Gottwald 2010). The genus *Ca.* Liberibacter has 3 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 (Etxeberria 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 like *Citrus Tristeza Closterovirus*, *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. 2006), and quantitative PCR (qPCR) are used for the diagnosis and confirmation of HLB (Kogenaru et al. 2014). X-ray fluorescence and laser-induced breakdown spectroscopy (LIBS) combined with chemometric strategies are used to successfully predict the condition of orchard plants infected with *Ca.* Liberibacter spp. (Pereira and Milori 2010). Fourier transform infrared 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 the 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 review, we describe the current diagnostic techniques for HLB disease performed worldwide, because an early diagnosis and differentiation of *Ca.* Liberibacter spp. is critical in reducing the local and international trade spread and devastation of this disease, as well as minimizing the economic impact of potential false-positive diagnoses.

Microscopic techniques for HLB disease detection

In 1970, electron microscopy was the first laboratory technique used by Lafleche and Bové (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 and Achor 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). Cevallos-Cevallos et al. (2009) 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' orange trees. The samples were fixed in 3% glutaraldehyde, 0.1 mol/L 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 for 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 LM, 1 mm sections were cut with glass knives and stained with methylene blue or 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 (aqueous) and post-stained with lead citrate. Micrographs were made with an AMT (Advanced Microscopy Techniques Corp., Danvers, Massachusetts) digital camera on a Morgagni 268 (FEI Company, Hillsboro, Oregon) TEM (Cevallos-Cevallos et al. 2009). In another way, Folimonova and Achor (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 et al. (2009), but the difference is that Folimonova and Achor (2010) used resin for 3 days, then samples (1 μm) were taken for LM observations. Sections of 100 nm in size were mounted on 200-mesh Formvar-coated copper grids and stained with 2% uranyl acetate (aqueous) and lead citrate. The thin sections were further examined with a Morgagni 268 TEM.

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 (Folimonova and Achor 2010). These properties

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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 HLB 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).

HLB is one bacterium that has yet to be cultivated in vitro, consequently, the pathogen was given a provisional *Candidatus* status in nomenclature; currently 3 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 *Ca.* Liberibacter spp.: Las, Laf, and Lam (Kim and Wang 2009; Bové 2006; W. Li et al. 2006; Pietersen et al. 2010). 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). Primer pair OI1 and OI2c is able to amplify the rDNA Las and Laf species (Teixeira et al. 2005*a*). When the 2 *Ca.* Liberibacter spp. are known or supposed to be present in any country, it is desirable to use the 2 forward primers, OI1 + OA1, and the common reverse OI2c primer in the same PCR mixture (Bové 2006). Sequence analysis shows that the 16S rDNA amplified from Las has one *Xba*I restriction site and yields, upon *Xba*I treatment, 2 fragments of sizes 520 and 640 bp. The 16S rDNA amplified from Laf has an additional restriction site and yields 3 fragments of 520, 506, and 130 bp (Jagoueix et al. 1996; Teixeira et al. 2005*a*). The second PCR is centered on the *nusG–rplK* operon region (primer pair A2 and J5, and primer pair MHO353 and MHO354) (Fujikawa and Iwanami 2012). The intergenic region between genes *rplA* and *rplJ* is 34 bp larger in Las than in Laf. With forward primer f-*rplA2*, selected in the *rplA* gene, and reverse primer r-*rplJ5* 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 2 DNAs is obtained, and upon agarose gel electrophoresis, 2 DNA bands are seen: the upper (703 bp) corresponding to Las, and the lower (669 bp) to Laf (Bové 2006). A third *Ca.* Liberibacter species was identified in São Paulo, Brazil, by the *nusG–rplKAJL–rpoBC* gene clusters of Las and Laf, which were obtained and sequenced (Teixeira et al. 2005*a*, 2005*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, qPCR has become the preferred detection method of *Ca.* Liberibacter spp. (Li et al. 2009). Compared with 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 by 100–1000 times relative to conventional PCR for these bacteria (Morgan et al. 2012). qPCR methods target genes with a low copy number: 3 copies of 16S rDNA, 1 copy of beta-operon, or 1 copy of elongation factor Ts (EF-Ts). The reported low sill limits of qPCR are around 10 gene copies for 16S rDNA and beta-operon procedures (Teixeira et al. 2008), and 1 gene copy for the EF-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 with primer probes targeting either 16S rDNA or beta-operon DNA. The 16S rDNA copy number of Las was estimated to be 3 times that of the beta-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. In June of 2013, Nageswara-Rao et al. (2013) 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 2 HLB-causing species Laf and Lam. Teixeira et al. (2005*a*) reported 3 primers pairs successfully developed from 32 different gene-specific primer pairs, across the 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 *Ca.* Liberibacter spp. was discussed. The specific primers used in conventional PCR and qPCR to identify *Ca.* Liberibacter spp. are provided in Table 1.

In an alternative method, 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 fragment was labeled with biotinylated nucleotides by a PCR-labeling technique. The dot hybridization assay with a biotin-labeled DNA probe was 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

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

HLB bacterium	Primer sequence (5′–3′)*	DNA region amplified	Type of PCR	Reference
Ca. Liberibacter americanus	F: AGTCGAGCGAGTACGCAAGTACT R: CAACTTAATGATGGCAAATATAG	16S rDNA	Conventional PCR	Nageswara-Rao et al. 2013; Teixeira et al. 2005*a*
	F: GAGCAGTACGCAAGTACTAG Tp: AGACGGGTGAGTAACGCG R: GCGTTATCCCGTAGAAAAAGGTAG	16S rDNA	qPCR	Kim and Wang 2009; W. Li et al. 2006; Li et al. 2009
Ca. Liberibacter asiaticus	F: CGCGTATGCAATACGAGCGGCA R: GCCTCGCGACTTCGCAACCCAT	16S rDNA	Conventional PCR	Nageswara-Rao et al. 2013; Teixeira et al. 2005*a*
	F: TCGAGCGCGTATGCAATACG Tp: AGACGGGTGAGTAACGCG R: GCGTTATCCCGTAGAAAAAGGTAG	16S rDNA	qPCR	Kim and Wang 2009; W. Li et al. 2006; Li et al. 2009
	F: GCCGTTTTAACACAAAAGATGAATATC Tp: ATAAATCAATTTGTTCTAGTTTACGAC R: ACATCTTTCGTTTGAGTAGCTAGATCATTGA	hyv_{I} and hyv_{II}†	qPCR	Morgan et al. 2012
Ca. Liberibacter africanus	F: GCGCGTATTTTATACGAGCGGCA R: GCCTCGCGACTTCGCAACCCAT	16S rDNA	Conventional PCR	Nageswara-Rao et al. 2013; Teixeira et al. 2005*a*
	F: CGAGCGCGTATTTTATACGAGCG Tp: AGACGGGTGAGTAACGCG R: GCGTTATCCCGTAGAAAAAGGTAG	16S rDNA	qPCR	Kim and Wang 2009; W. Li et al. 2006; Li et al. 2009

Note: PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; HLB, Huanglongbing.
*F, Forward primer; R, Reverse primer; Tp, Taqman probe.
†Intragenic tandem-repeats sequence.

countries but not with those from South Africa. The developed probe proved to be specific and had sensitivity enough to detect minute levels of HLB infection; therefore, it can be used in quarantine of the Asian Greening disease (Hung et al. 1999).

Meanwhile, the first study of transcriptional profiling in response to *Ca.* Liberibacter spp. infection using microarray technology was developed by Albrecht and Bowman in 2008 (Albrecht and Bowman 2008). Microarray technology has been used in several studies of bacterial plant diseases, such as bacterial blight (Q. 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 noninfected 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 ≤ 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 the 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 HLB 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 nonstressed 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

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variety of crops, since it is a rapid and nondestructive 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 a 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 2 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 spectral ranges of 580–660 and 688–715 nm showed better classification power for developed stages of infection.

Sankaran and Ehsani (2011) evaluated spectral features extracted from visible to near-infrared spectra via a spectroradiometer for their potential to detect citrus greening disease, and tried to lower the cost of the optical sensor while maintaining their performance. Sankaran and Ehsani (2011) defined the following as spectral features: (*i*) spectral reflectance bands and (*ii*) vegetation indices (VIs), which were derived from the 350–2500 nm spectral reflectance data using 2 feature extraction methods: stepwise discriminant analysis and stepwise regression analysis. Following the selection of spectral features, the features were assessed using 2 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, multiband active optical sensor study to evaluate the identification of HLB-infected trees from the healthy trees. The sensor measured reflectance of tree canopy in 4 bands: 2 visible bands at 570 and 670 nm, and 2 near-infrared bands at 870 and 970 nm. Extensive field measurements were conducted using this sensor. Analysis of the data showed that owing 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, by using multiple measurements from a tree, it was possible to achieve high classification accuracies. With 5 measurements from each tree, classification methods such as *k*-nearest neighbors, support vector machines, and decision trees resulted in classification errors of <5%. The results of Mishra et al. (2011) demonstrated and confirmed the potential of a multiband 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 multirotor unmanned aerial vehicle (UAV). A multiband 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. (2013) obtained results using UAV-based sensors that were compared with a similar imaging system (aircraft-based sensors) with lower spatial resolution. Data were comprised from 6 spectral bands (from 530 to 900 nm) and 7 vegetation indices derived from the selected bands. Garcia-Ruiz et al. (2013) 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 and 591 nm, and the reflectance was measured by 2 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 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 this property of starch accumulation in citrus leaf, a sensor was developed 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

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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 spectroscopic detection should continue to develop, focusing mainly on lower costs of equipment and rapid detection to reduce false-positive results.

Profiling of plant VOCs for disease detection

Disease symptom development in HLB-affected plants is associated with starch accumulation in the leaf tissue, a phenomenon that 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). Cevallos-Cevallos et al. (2012) conducted a study to determine GC–MS-based metabolomics differences between 2 citrus varieties sensitive to HLB ('Madam Vinous' sweet orange (MV) and 'Duncan' grapefruit (DG)) and 2 tolerant citrus varieties ('Carrizo citrange' (CAR) and 'Poncirus trifoliata' (TR)). They 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 used in this experiment was model HP5890 coupled to an HP5971 series mass spectrometer from Hewlett Packard (Santa Clara, California). Chromatogram analysis was completed using HP ChemStation software (Cevallos-Cevallos et al. 2012). Cevallos-Cevallos et al. (2012) 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 the accumulation of galactose in healthy plants were characteristic of the most sensitive variety MV. Only galactose was significantly higher in DG when compared with the tolerant varieties TR and CAR. The tolerant varieties showed higher levels of L-glycine and mannose when compared with 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 *Ca.* 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. The Rigano et al. (2014) 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 pg. 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. The results of Rigano et al. (2014) 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 4–6 primers that recog-

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nize 6–8 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 and 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. The Rigano et al. (2014) assay focused on the detection of the DNA sequence of the *tufB–secE–nusG–rplKAJL–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, Rigano et al. (2014) coupled the LAMP amplification with a LFD, which permits an accurate and straightforward detection of LAMP amplicons, eliminating the need for 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 5 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 include light microscopy (LM) and transmission electron microscopy (TEM). Molecular techniques include conventional 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 spectroscopy in the design of new tools and optical sensors for the treetops. Profiling of plant volatile organic compounds currently employs gas chromatography – mass spectrometry (GC–MS). The most recent technique in 2014 for detection HLB disease could be isothermal amplification, which combines loop-mediated isothermal amplification (LAMP) with a 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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