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Article

Methods for detecting Huanglongbing in citrus

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Abstract

In Mexico, citrus farming represents one of the most important economic activities for national fruit growing, with a production of 8.8 million tonnes and an economic spillover of more than 47 billion pesos per year. Nonetheless, production is affected by pests and diseases, with Huanglongbing (HLB), 'yellow dragon' or citrus greening, standing out as the most devastating disease worldwide, caused by a proteobacterium of the genus *Candidatus* Liberibacter (Ca. L.). Because there is no effective method to control this disease, correct and timely detection can significantly reduce its spread. The purpose of this review is to compile methods used for detecting the presence of Ca. L. in citrus plants, covering general aspects of the symptomatology of the disease, molecular methods for accurate and rapid detection when sampling both in plants and in the vector. In addition, different protocols are mentioned that analyze some compounds produced during infection with Ca. L. and images in citrus with HLB.

Keywords:

diagnosis, images, PCR, sampling, spectrometry, symptoms.

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Introduction

In Mexico, citrus farming represents one of the most important economic activities for national fruit growing, with a production of 8.8 million tonnes and an economic spillover of 47.499 billion pesos per year (SIAP, 2022). Nevertheless, the production is affected by pests and diseases, with Huanglongbing (HLB), 'yellow dragon' or citrus greening, standing out.

Three species of Gram-negative alphaprotobacteria have been reported as causative agents: *Candidatus* Liberibacter *asiaticus* (CLas), *Ca*. L. *africanus* (CLaf) and *Ca*. L. *americanus* (CLam) (SENASICA, 2019). The pathogen invades the vascular system of the plant through the sieve tubes of the phloem, preventing the efficient transport of nutrients, causing a series of symptoms in leaves and fruits as well as the eventual death of the diseased tree (Bové, 2006).

The CLas and CLam species are vectored by the Asian citrus psyllid *Diaphorina citri*, while CLaf is transmitted by *Trioza erytreae*. This disease affects all cultivated citrus species and their hybrids. The CLas species is present in Mexico and is subject to official control. The bacterium was first detected in *D. citri* in the municipality of Tizimín, Yucatán, in 2009. Ten years after its initial detection, the spread of the disease comprises 342 municipalities distributed in 25 states of the Mexican Republic (SENASICA, 2019).

HLB is considered a destructive disease in citrus worldwide due to its negative effect on fruit production, and in Mexico it is no exception. Due to the impact of the disease, its management requires various actions such as the timely detection and elimination of diseased trees, the control of the population of the vector insect and the use of healthy plants produced in certified nurseries, in order to mitigate the harmful effects of the disease (SENASICA, 2012). In this sense, the timely diagnosis of CLas-infected trees in Mexico requires efficient sampling methods, timely sampling, and adequate sending to specialized sites in charge of detection (SENASICA, 2012).

The purpose of this review is to present the progress made in the research that has been carried out on HLB in Mexico and worldwide in recent years. Due to the devastating nature of HLB on citrus production in the country, we consider it essential to update the information that has been developed recently, mainly in relation to lines of research on the detection of this disease.

Detection by symptoms, sampling

Symptomatology

Symptoms vary depending on the species, variety, and age of the affected plant. Yellowing is observed on the leaves, followed by mottling with asymmetrical angular spots on both sides of the main vein. In some cases, thickening and lightening of the veins were observed, which have a corky appearance. The leaves of the terminal branches are small, upright, and display a variety of chlorotic patterns.

Occasionally, leaf symptoms may be mistaken for nutritional deficiencies of zinc, copper, or manganese; however, these mineral deficiencies exhibit a symmetrical pattern on both sides of the main vein in the affected leaves. As the discoloration is further away from the veins, the leaves turn pale yellow to light yellow with dark green areas unevenly distributed (EPPO, 2014).

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The symptoms in the tree begin with the appearance of yellow or chlorotic shoots, followed by a sectorial yellowing of the tree crown, which is related to the area where the vector is feeding (Bové, 2014). In advanced stages of the disease, intense defoliation of the affected branches occurs, which can show death in the apical region. During infection, it is common for the fruits that form to fall prematurely, but those that manage to remain on the tree develop asymmetrically.

The color inversion from yellow/orange to green in the fruits is a characteristic symptom of HLB, which is why the disease is also known as citrus greening. Other damage to the fruit is the thickening and deformation of the central core. The peduncles of the fruits also show color inversion as the end turns yellow to orange while the stylar end remains green. The vascular bundles within the fruit axis at the peduncular end have a brownish spot (Bové, 2006).

Sometimes the albedo thickens at the peduncular end and not at the stylar end. The seeds of the affected fruits are small, stunted, dark brown to black, and aborted (EPPO, 2014). It is important to consider that the symptoms associated with HLB (asymmetrical mottling, yellowing, and thickening of the vein) are the product of the interference caused by the pathogen on the movement of nutrients through the phloem tissues, thus promoting the development of the disease in the parts of the plant that present nutritional deficiencies (Paredes-Tomás *et al*., 2015).

This interference is not unique to *Ca*. Liberibacter since there are reports of the presence of two phytoplasmas associated with symptoms similar to those caused by HLB: Pigeon pea witches'-broom phytoplasma reported in Brazil and Mexico (Teixeira *et al*., 2008; Alanís-Martínez *et al*., 2013) and *Candidatus* Phytoplasma asteri reported in China, where about 50% of the samples analyzed were positive for both phytoplasma and *Ca*. Liberibacter (Chen *et al*., 2009). Periodic inspections in citrus plantations aimed at searching for plants with characteristic symptoms of HLB, as well as taking samples of plant material for subsequent analysis, are activities that allow the early detection of infected trees and thus mitigate the spread of the disease (SENASICA, 2012).

Sampling

Sampling methods are critical for the detection, identification, and quantification of *Ca*. Liberibacter species since their distribution in host plants can be irregular. Trees infected with CLas may have symptomatic leaves only on some branches of the tree and others may remain free of infection or have low bacterial titration, due to an irregular distribution in the tree (Teixeira *et al*., 2008). In addition to differences in the distribution of CLas in trees, there are differences in the expression of characteristic symptoms associated with the disease.

In Mexico, it has been observed that the intensity of asymmetric mottling manifests itself differently depending on the species. In acidic citrus such as Persian lime or Mexican lime, the spots are more angular and more contrasting in color compared to the mottling of sweet citrus (Figure 1). Additionally, symptoms may be present or absent at the onset of infection, so asymptomatic plants should not be ruled out. In suspected asymptomatic trees, 1 to 4 one-year-old branches with 5 to 10 leaves are collected, which are taken from four points on the top of the tree.

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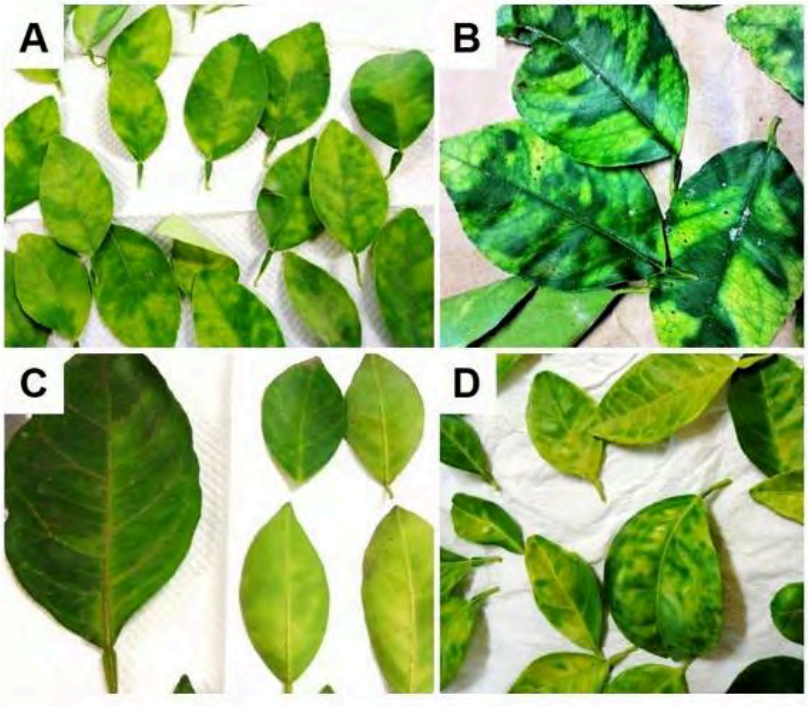
Figure 1. Symptoms of HLB in citrus leaves. A) Mexican lime: yellowing followed by mottling with asymmetrical angular spots on both sides of the main vein; B) Persian lime: thickening of the central vein causing a corky appearance, presence of green islands and leaf distortion; C) mandarin: diffuse mottling, lightening and thickening of the veins; and D) sweet orange: corking and lightening of the veins, leaf distortion, presence of green islands. Photographs: Alanis-Martínez.



In the case of small nursery trees, 4 to 6 mature leaves are collected from each tree or plant. In trees with symptoms, samples of 1 to 4 branches with symptomatic leaves are collected. For the detection of the bacterium, the optimal tissue is the midrib and petiole of the leaves (NAPPO, 2012).

When leaves are not available, or it is sought to analyze fruits, it is advisable to take tissue from the extremity of the peduncle or from the bark of the peduncle (Li *et al*., 2009; Ding *et al*., 2017). On the other hand, the analysis of the vector insect is an additional strategy to detect CLas when symptomatic plants are not yet observed, or to delimit the incidence of the disease in a given region.

The positive result of an analysis of psyllid samples is useful for carrying out management actions. Because psyllid nymphs acquire the bacterium through feeding, a positive nymph sample would indicate that the plant from which they were collected is infected, while a CLas-positive adult may indicate the presence of infected trees in the area (Manjunath *et al*., 2008).

Although this strategy is expensive because it involves analyzing a large number of psyllid samples, derived from the presence of high populations of *D. citri* in some citrus-growing regions, the collection and analysis of the vector is a useful strategy because it works as a guide to visually healthy diseased plants that are the source of inoculum.

In Mexico, periodic sampling is carried out in commercial groves, nurseries, urban and rural areas to determine if psyllids are carriers of the bacteria. In groves, psyllids are collected from different trees in order to cover the entire area. These should be stored in 70% ethyl alcohol in properly sealed vials until analysis (SENASICA, 2012).

Isolation in culture media and problems

In the last two decades, great efforts have been made to isolate (culture) the CLas bacterium in pure culture; however, it has only been possible to culture it temporarily. Davis *et al*. (2008) tried to

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isolate CLas using babaco basal medium (BBM) formulations, in response to the growth of another bacterium that appears to be related to *Ca*. Liberibacter, based on the identities of the 16S rRNA gene.

The other bacterium found in the cultures was an actinobacterium related to *Propionibacterium acnes* identified with the 16S rRNA gene, suggesting that the bacteria might be benefiting each other in the culture and not surviving independently.

Sechler *et al*. (2009) developed a culture medium called 'Liber A', composed of citrus vein extract, a growth factor (NADP) and an inhibitor of eukaryotic protein synthesis (cycloheximide). *Ca*. Liberibacter asiaticus and *Ca*. L. americanus initially grew in this medium; nevertheless, after four to five transfers, culture viability declined. Subsequently, Parker *et al*. (2014) showed that media supplemented with commercial citrus juice prolonged the viability of CLas for up to 18 days, suggesting that conditions such as pH, sugars, abundance of specific mineral elements, and amino acids could contribute to the viability of the bacterium.

On the other hand, Fujikawa *et al*. (2018) showed that the survival of CLas depends on the interaction of this pathogen with a specific subset of microbiota. Recently, the establishment and maintenance of CLas cultures for prolonged periods in biofilms derived from citrus tissues infected with the pathogen has been reported (Ha *et al*., 2019). These results suggest that CLas is able to survive independently of insects or host plants. Nonetheless, reproducibility of these findings by other research groups is needed.

Molecular detection

A wide variety of qualitative methods have been developed for the detection and identification of the three species of *Ca*. Liberibacter. These methods include visual symptom identification, biological indexing, microscopy, chemifluorescence and adsorption enzyme immunoassay, (ELISA) assay. However, such tests require expensive equipment, personnel trained in sample preparation, and long periods to perform them (Lafleche and Bové, 1970; Roistacher, 1991; Garnier and Bové, 1993).

Starting in the second half of the 1990s and with the advancement of molecular biology techniques, particularly polymerase chain reaction (PCR), this technique began to be used to detect *Ca*. Liberibacter using sequences of 16S ribosomal DNA (16S rDNA), ribosomal protein genes of the # operon, and other regions of the bacterial genome such as the group of genes *tuf*B, *sec*E, *nus*G, *rpl*KAJL, and *rpo*B (Jagoueix *et al*., 1996; Teixeira *et al*., 2005; Li *et al*., 2009; Lin *et al*., 2010; Arredondo-Valdés *et al*., 2016).

PCR-based molecular diagnostic methods are fast, sensitive, specific, and reliable and have been widely used for the clinical diagnosis of *Ca*. Liberibacter associated with HLB in plants and vector insects (Lin *et al*., 2010). Currently, there are many PCR-based molecular detection assays, including conventional PCR, SSR nested PCR, LAMP, immune-capture PCR, droplet digital PCR, and qPCR, which have been used to detect HLB-associated bacteria (Jagoueix *et al*., 1996; Li *et al*., 2009; Lin *et al*., 2010; EPPO, 2014; Arredondo-Valdés *et al*., 2016; Ding *et al*., 2017; Zhong *et al*., 2018).

Conventional PCR for detecting HLB in citrus is a fast technique and is efficient for the accurate diagnosis of HLB. The primer used amplify 226 bp. Although the technique detects almost all strains of CLas bacteria, it has limitations in its use. Other conventional or endpoint PCR assays use specific primers that amplify 16S rDNA sequences.

OI1/O2c primers amplify 1160bp, while 606/LSS amplify 500bp and detect all three *Ca*. Liberibacter species. GB1/GB3 primers are specific for CLam and amplify a 1 027 bp fragment (Jagoueix *et al*., 1996; Teixeira *et al*., 2005; Fujikawa and Iwanami, 2012). Some conventional PCR assays generate large amplicons (> 1 000 bp), resulting in lower sensitivity and reproducibility in detections (Lin *et al*., 2010).

Sometimes it is not possible to detect the bacterium (or its detection is inconsistent) due to the irregular distribution in the plants or the low bacterial concentration. For such reasons, PCR

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protocols have improved sensitivity in detecting *Ca*. Liberibacter. Nested PCR reactions significantly increase sensitivity by using a second pair of primers in a second reaction (Hong *et al*., 2019).

A nested PCR assay for *Ca*. Liberibacter detection uses the fDI and rD1 primers in the first amplification reaction, whose amplified product serves as template DNA in the second amplification using the OI1 and OI2c primers (Ding *et al*., 2004). Both pairs of primers were reported for the identification of CLas and CLaf (Jagoueix *et al*., 1996). This technique allows the detection of the bacterium even at low concentrations and has been used to improve the detection of *Ca*. Liberibacter species associated with HLB in clinically asymptomatic trees, especially in nursery plants as well as in assays to analyze possible seed transmission (Deng *et al*., 2007).

Another PCR variant currently used and accepted for the diagnosis of HLB by regional and national plant protection organizations in various parts of the world [the North American Plant Protection Organization (NAPPO), the International Regional Organization for Agricultural Health (OIRSA, for its acronym in Spanish), the Ministry of Agriculture, Fisheries and Food (MAPA, for its acronym in Spanish), the National Service for Agri-Food Health, Safety and Quality (SENASICA, for its acronym in Spanish)], is the quantitative PCR (qPCR) technique, which simultaneously amplifies and quantifies products as they are generated in real time.

The qPCR technique is accepted by regulatory entities as a diagnostic method to determine the presence of the bacterium in both citrus samples and the vector because the sensitivity of detection of *Ca*. Liberibacter is up to 10 times more relative to nested PCR and 100 to 1 000 times more relative to conventional PCR (Morgan *et al*., 2012). One of the qPCR assays commonly used for detecting HLB employs the primers and probe developed by Li *et al*. (2009), which are targeted at the sequence of the 16S region of rDNA.

This procedure allows the detection and identification of the different species of *Ca*. Liberibacter by using the mitochondrial cytochrome oxidase (COX) gene as an endogenous reaction control. Another technique that has been used for detecting of *Ca*. Liberibacter is droplet digital PCR (ddPCR), which is based on the use of oil and water emulsion droplets, where each sample is fractionated into 20 000 droplets and PCR amplification of the template DNA molecules is performed on each droplet individually, allowing absolute quantification of nucleic acids in a sample, without the need for a standard curve (Hindson *et al*., 2011).

In comparative studies of ddPCR and qPCR techniques for the detection of *Ca*. Liberibacter, ddPCR has been reported to be superior in detecting and quantifying CLas at low concentrations. As an example, the detection of CLas in 40 field samples showed that six out of 13 asymptomatic samples with a high C_T value (>35) were detected as positive when using ddPCR, so this methodology has great potential for the early diagnosis of infection caused by CLas (Zhong *et al*., 2018).

In Mexico, the official protocol for the detection of *Ca*. Liberibacter uses the qPCR technique with the primers and probe proposed by Li *et al*. (2009). It is important to highlight that the protocol proved to be the best method for detecting CLas and CLaf in an international evaluation of three real-time PCR diagnostic methods recommended by EPPO and FAO, in which eight laboratories participated.

This evaluation considered parameters of specificity, analytical sensitivity, repeatability, and reproducibility. The recommended methods for detecting HLB are the method of (Li *et al*., 2009) and duplex conventional PCR (Teixeira *et al*., 2005). The combination of these methods can minimize the risk of releasing infected material, which is of paramount importance for citrus propagative material certification programs, as these methods produce very few false-positive results.

Although PCR and qPCR are important methods for diagnosing plant diseases, other factors such as the sampling method, sample size and type, as well as the quality and quantity of DNA used influence the efficiency of HLB detection. DNA extraction procedures should be chosen according to quality and integrity criteria to avoid partial or total inhibition of PCR (Yang *et al*., 2021).

Plant tissues contain substances such as polysaccharides, polyphenols, pectin, and xylan, which can be extracted in conjunction with DNA. These substances have an impact on the efficiency of

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PCR reactions, affecting the sensitivity of the technique (Demeke and Jenkins, 2010). The purity and quantity of DNA are factors that influence the detection of CLas.

Selecting the extraction method depends to some extent on the costs and time available for this activity. The CTAB method (Doyle and Doyle, 1987), commonly used for the extraction of DNA from plant samples for the detection of HLB, has been modified with the aim of determining parameters such as concentration and purity of the DNA obtained and their effect on the detection of HLB-associated species (Nauman *et al*., 2021).

Several commercial kits are now available for the rapid and effective isolation of genomic DNA. These kits use detergent to break down the cell wall as an initial step in extracting DNA from plant material and some use matrices based on silica or magnetic beads that allow DNA to bind. The success of DNA extraction depends on the type of sample. A kit that may be suitable for extracting DNA from one type of matrix may not be suitable for another (Demeke and Jenkins, 2010).

In this sense, modifications are also made to these methodologies to improve the yield of the DNA, or to reduce the execution time (Oppert *et al*., 2019). In general, commercial DNA extraction kits simplify the procedure and reduce the risk of cross-contamination.

Other detection methods

There are other methods for detecting HLB, some of these are close to 100% accuracy in detecting the disease, close to the accuracy of the PCR test. Spectrometry detection is a viable and rapid option for detecting HLB today. There are several methods of detection, analysis, and recognition of disease patterns in images by means of cameras, and others that acquire spectroscopic information through different devices (Ranulfi *et al*., 2016).

Spectroscopic imaging makes it possible to identify unknown molecules using computer vision to detect HLB. This can be done using a combination of images from the electromagnetic spectrum with special cameras. Another option is by acquiring images in the visible color space; i.e., color images (Ranulfi *et al*., 2016).

Spectrometry

Volatiles released by plants are closely related to plant metabolism and can serve as an indicator of plant health. Chemical analysis of the released compounds emanating from infected trees has been studied for detecting HLB. Aksenov *et al*. (2014) employed analytical methods such as gas chromatography/mass spectrometry and gas chromatography/differential mobility spectrometry to detect volatile organic compounds released by HLB-infected citrus plants.

The study of volatile organic compounds produced by citrus plants was carried out *in situ* and *in vitro*. This detection method has a high accuracy, of 90%, throughout the year, close to 100% under optimal testing conditions, even in very early stages of infection. Xue *et al*. (2022) employed an extractive electrospray ionization mass spectrometry (EESIMS) method to analyze metabolites in uninfected and HLB-infected leaves of Newhall navel orange.

The results of this study showed that uninfected leaves could be easily distinguished from HLB-infected leaves with EESIMS by combining it with a multivariate analysis. They found nine phenolic compounds involved in the phenylpropanoid pathway, mainly p-coumaric acid, naringin, and apigenin, these being the main components to distinguish uninfected leaves from infected Newhall navel oranges. They verified the results of the EESIMS analysis by using qPCR to determine the bacterial content of orange leaves.

The study provides a new strategy for the early detection of HLB. With this study, the authors suggest the mechanism of regulation of the phenylpropanoid pathway in the response of citrus to *Candidatus* Liberibacter asiaticus. Wetterich *et al*. (2017) detected HLB by combining a fluorescence imaging spectrometry technique and two machine learning methods, discriminating HLB from zinc deficiency stress in samples from Florida, USA. The classification methods were: 1) support vector machine (SVM); and 2) the artificial neural network (ANN). According to the

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classification results, they had an accuracy of 92.8% with the SVM method and 92.2% with ANN, which together indicates that the detection of HLB can be differentiated from zinc deficiencies.

The diagnosis of HLB-asymptomatic citrus using migration and transformation of elements by laser-induced breakdown spectroscopy (LIBS) is another method used to detect HLB. LIBS is an atomic spectrometry technique for the analysis of material components. Through absorption analysis, the infective progress on navel orange can be effectively monitored in real time as it detects the inhibitory effect of the pathogen on navel orange absorption.

A study conducted with this technique used healthy and HLB-asymptomatic navel oranges collected in the field to improve detection efficiency, using LIBS coupled with SVM algorithms to distinguish between healthy navel oranges and HLB-asymptomatic navel oranges. According to the results obtained, the technique has a classification accuracy of 100%.

LIBS combined with chemometric methods appears to be a promising tool for rapidly distinguishing healthy and HLB-asymptomatic samples despite having similar elemental compositions. The advantage and novelty of this technique is that it is significantly better than PCR in diagnosing HLB-asymptomatic citrus (Yang *et al*., 2022).

On the other hand, in a study conducted by Ponce *et al*. (2018), rapid and efficient discrimination between healthy and *Ca*. L-infected citrus was demonstrated by laser-induced breakdown spectroscopy combined with chemometric analysis.

The method involves fingerprinting healthy and diseased plants based on their organic and inorganic constituents and using a multi-pulse laser coupled to a microscope to take spectra of the plant's phloem. Healthy and HLB-diseased trees were differentiated with a high degree of accuracy. The method takes only a few minutes, is inexpensive, requires no chemicals or sample preparation, and generates no waste.

Satellite imagery

Yzquierdo-Alvarez *et al*. (2022) developed a practical, fast, and low-cost methodology for detecting plants with HLB symptoms using Sentinel-2 satellite imagery. Sentinel-2 imagery was used to perform supervised classification to discriminate healthy Persian lime trees and trees with HLB, both of which were verified by qPCR. Based on the spectral signatures obtained from the satellite image through supervised classification, 11 classes were generated for the study.

The results obtained showed that, in the green (560 nm), red (665 nm) and near-infrared (705 nm) wave regions, the spectral response of trees with HLB was higher than that of healthy trees with an accuracy of 0.84%, so the use of Sentinel-2 satellite imagery may be useful to timely detect plants with HLB symptoms.

Neural networks

He *et al*. (2022) used a combination of multicolor fluorescence imaging with multispectral reflectance imaging synchronously for rapid HLB detection based on the MobileNetV3 lightweight convolutional neural network (LCNN) using a mobile device. The LCNN was applied for HLB symptom detection trained on the navel orange dataset. The discriminant performance of the models (MobileNetV3) established in multicolor fluorescence imaging, multispectral reflectance imaging and their combinations respectively, was evaluated.

As a result, they obtained that the LCNN used can achieve an accuracy of 92.1% with a false negative rate of 12.1% (number of times= 33) when combining multicolor fluorescence with multispectral reflectance images as input to the MobileNetV3 model using navel orange data. On the other hand, the fine-tuning model's learning transfer method obtained a higher transfer capacity than the reuse model with 96.5% accuracy for Ponkan.

In a study conducted by Syed-Ab-Rahman *et al*. (2022), they detected and classified citrus diseases using an end-to-end anchor-based deep learning model. The model based on convoluted neural networks, a class of deep neural networks, employs two main stages: 1) it proposes the possible

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target disease areas by using a region proposal network; and 2) it classifies the most likely target area to the corresponding disease class using a classifier. The proposed model can detect and classify HLB with 94.6% accuracy.

Gómez-Flores *et al*. (2019) designed a method to detect HLB based on the intensity-invariant texture analysis of images in the visible spectrum. They employed the ranklet transformation to convert the input image into a representation of invariant intensity from which they extracted the common texture features.

They used a random forest classifier to distinguish between different classes of citrus leaves, including healthy, nutrient-deficient, and with HLB. They achieved about 95% accuracy in distinguishing between negative and positive HLB classes, and about 81% accuracy in identifying between six classes of citrus leaves. One advantage of this method is its possible use within a mobile app that can be used in the field to detect HLB-symptomatic citrus plants.

Starch concentration image analysis

In a study using a homemade computer vision system with two imaging modes, they evaluated the contribution of two symptoms, based on HLB-induced foliar starch accumulation, to the detection of this disease (Xu *et al*., 2022). A 660 nm light source was used and the absorption of different wavelengths by chlorophyll and lutein was measured. The transmission imaging was designed to detect an abnormal accumulation of starch within the leaf.

An image was taken with 590 nm polarized light penetrating the leaf, then the linear polarization angle was calculated using the Stokes vector. The multilayer perceptron, random forest, and logistic regression classifiers were then evaluated. The random forest classifier in the reflection experiment showed a classification accuracy of 96.67%. On the other hand, the transmission experiment with the logistic regression classifier had a recognition rate of 83.33% (Xu *et al*., 2022).

Drones

Recently, great advances have been made in precision agriculture, using technologies that use unmanned aerial vehicles, such as drones, to autonomously identify and monitor plant diseases. The importance of precision agriculture lies in the fact that the diagnosis of diseases in crops of agronomic interest is one of the main tasks to increase food production, thereby reducing time and working hours (Neupane and Baysal-Gurel, 2021).

When it comes to HLB detection, several studies have been conducted using drones. Garcia-Ruiz *et al*. (2013) captured high-resolution aerial images, and together with a multiband imaging sensor, found accuracy values in the range of 67 to 85% and false negatives between 7% and 32%, and although aerial detection showed encouraging results for the detection of infected trees, the values were low with respect to other detection techniques.

Currently, the company VetorGEO has developed a system based on the use of drones that use high-resolution multispectral sensors from Agrowing Ltd., which capture a large number of images that allow learning through the artificial intelligence of algorithms, which is used to calibrate the spectral signature of HLB symptomatology in different varieties of orange regardless of the phenological development of the trees, time since the infection occurred, use of irrigation systems, type of management of groves, etc. [Ira Dvir (Agrowing) and Neto Salvador (VetorGeo), pers. comm.].

The technology developed has made it possible to evaluate large areas cultivated with citrus in Brazil. The results obtained by this diagnostic tool are comparable to those obtained by endpoint PCR [Ira Dvir (Agrowing) and Neto Salvador (VetorGeo), pers. comm.] so it is expected that in the near future it can be used on a large scale.

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Conclusions

Protocols for detecting HLB have been refined over time. Artificial media culture of *Candidatus* Liberibacter spp. has made progress; nevertheless, it remains a challenge, which is why innovative studies are required on the interaction of the bacterium with the microbiota present in the phloem of infected plants. Molecular techniques have been perfected, various methods have been developed that involve the determination of different compounds produced during infection and the processing of images that allow a timely detection of HLB; however, more research is still needed so that they can be incorporated into precision agriculture.

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