Research Article

Received: 22 April 2024 Revised: 21 October 2024 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.8528

'*Candidatus* Liberibacter asiaticus' infection alters the reflectance profile in asymptomatic citrus plants

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Abstract

BACKGROUND: Huanglongbing (HLB) is the primary and most destructive disease affecting citrus, caused by a pathogen transmitted by an insect vector, *Diaphorina citri*. There are no curative methods for the disease, and rapid and accurate methods are needed for early detection in the field, even before symptoms appear. These will facilitate the faster removal of infected trees, preventing the spread of the bacteria through commercial citrus orchards.

RESULTS: It was possible to determine ranges of hyperspectral bands that demonstrated significant differences in relative reflectance between treatments consisting of healthy and infected plants from the first days of evaluation, when plants infected with '*Candidatus* Liberibacter asiaticus' (CLas) were still in the asymptomatic stage of the disease. From the Week 2 of evaluation [58 days after infection (DAI) of plants] until the last week, spectral differences were detected in the red edge region (660–750 nm). From the Week 6 onwards (86 DAI), spectral differences between healthy and symptomatic plants were observed in bands close to the visible region (520–680 nm).

CONCLUSION: Spectral differences were detected in the leaves of *C. sinensis* infected by CLas before the appearance of symptoms, making it feasible to use the hyperspectral sensor to monitor the disease. Our results indicate the need for future studies to validate the use of hyperspectral sensors for managing and detecting HLB in commercial citrus orchards, contributing to the integrated management of the disease.

Keywords: huanglongbing; greening; precision agriculture; remote sensing; hyperspectral; early detection

1 INTRODUCTION

Diseases that affect cultivated plants constitute one of the main problems that directly reduce the quantity and quality of agricultural production.[1] The detection and identification of plant injuries are necessary to quickly understand the need to implement management, contributing to the reduction of damage and production losses.[2] Monitoring citrus diseases and pests in the early stages is essential to ensure the efficiency of phytosanitary management, as several diseases caused by fungi, bacteria, viruses and arthropods limit citrus production.[3]

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), is one of the main pests of citrus as it is a vector of the bacteria associated with the most destructive disease of the crop, huanglongbing (HLB), also called greening.[4–6] The disease is caused by the bacterium '*Candidatus* Liberibacter spp.' which causes significant losses in citrus production.[4,7] In the Americas, '*Candidatus* Liberibacter asiaticus' (CLas) has greater prevalence and dissemination, and is the main bacteria affecting citrus farming.[8]

HLB has represented a significant challenge for citrus production as there are no curative management methods, only preventive ones.[9,10] Among these methods, one of the management practices used is the elimination of symptomatic plants as quickly as possible because this tree can be a source of inoculum, promoting the spread of the disease in the rest of the orchard.[11,12] The detection of symptomatic plants is done by visual inspection and is adopted by many producers. However, it is a very time-consuming and often ineffective method, as the manifestation of symptoms can take a few months to appear.[11,13]

Therefore, it is of great relevance to develop an effective method to prevent and control the spread of HLB in extensive citrus groves, thus improving the quality and yield of production.[14] Likewise, early detection of pest outbreaks and effective control methods are intrinsic to integrated pest management (IPM), allowing control decisions to occur before pests are well-established and cause significant production losses.[15] IPM

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depends directly on monitoring insect pests to support decision-making on a given level of infestation or injury, and select the appropriate control method.[16] The main challenge for producers is related to pest monitoring tools and techniques, which are time-consuming, expensive and, at times, unreliable.[15,16]

To date, real-time PCR testing is the most accurate method for detecting and diagnosing HLB in citrus plants. However, samples must be collected in the field and tested individually in the laboratory,[17] making the process laborious and time-consuming and not being used considering the extent of trees in commercial areas. Furthermore, despite being efficient, PCR is expensive[17] as it requires specialized equipment and professionals, making it unfeasible in large-scale orchards.[14]

Implementing highly efficient and affordable contactless methods to detect and monitor plant diseases and pests over large areas could facilitate plant protection.[18] Early detection of *D. citri* is necessary to impose adequate control measures in cultivation areas where the disease has not yet occurred.[6] In areas where the disease is already present, monitoring of *D. citri* is necessary for site-wide management based on insect density fluctuations.[6]

Advances in sensor technology have demonstrated the potential for automated monitoring of pests and their damage, optimizing the use of insecticides and reducing yield losses in agriculture.[15,19] Remote sensing is a science that has great affinity with the concept of precision agriculture (AP), encouraging the development of new methodologies and other approaches that produce spatial information that assists farmers in planning and making control and management decisions of the crop.[20] The AP concept prioritizes better management of agricultural inputs, such as water, fertilizers, herbicides and insecticides, among others, allowing appropriate management to occur precisely where and when necessary.[21]

Technological advances have contributed to the development of hyperspectral sensors and, consequently, a greater volume of data processing. Sensors that capture high spectral resolution can classify components into elements of a similar spectral pattern.[21] Spectral data obtained in the visible and near-infrared (NIR) regions can provide information about plant health, which can contribute to the rapid detection of abnormal and harmful conditions caused by diseases.[22]

For the early detection of HLB, some preliminary studies demonstrated the potential of using spectral images, and pointed out a difference in reflectance between infected and healthy citrus plants in the field.[11,17] A portable device based on multispectral reflectance and multicolor fluorescence imaging was developed for detecting diseases at different infected stages in citrus cultivars.[12] Furthermore, the polarized imaging technique developed by Pourreza *et al.*[23] could detect CLas infection in citrus leaves in a pre-symptomatic state and early symptomatic stages. The method also detected four infection levels whereas the leaves remained symptom-free.[23] Remote sensing technology can differentiate the accumulation of starch in leaves infected with HLB and symptoms caused by zinc deficiency, which can be confused by the similarity of the leaf spots.[24] However, detection using this technique was effective at a distance of 80 cm from the plants,[24] which would make detection difficult in large areas of citrus production.

Hyperspectral reflectance imaging has been successfully used to identify diseases, nutritional deficiencies, and defects in different species of fruits and vegetables.[25] Hyperspectral images provide spatial and spectral information on plant tissue, which allows the precise detection of changes in reflectance generated by damage caused by insects.[19] Furthermore, the use of hyperspectral images in citrus plants has been studied, demonstrating great potential for the early detection of HLB.[18,26,27] Hyperspectral imaging can provide unique spectral signatures and can, therefore, detect potentially infected trees quickly and efficiently over a large area, significantly reducing the monitoring cost.[25] In the study by Deng *et al.*[28] information from hyperspectral bands was used to detect HLB in citrus plants, with bands being selected based on the maximum entropy distance, finding 20 useful bands for detection located mainly in three regions: the visible, red edge region and NIR region.

Several researchers have been carrying out studies with spectral images; however, at the time of publication, this technology has not yet been widely commercialized for detecting HLB in citrus groves because, despite the progress made in research, there are still challenges that need to be addressed to validate its applicability in the field considering that studies with different degrees of infection remain scarce, especially in asymptomatic stages.[27] Therefore, more studies are needed to adopt this tool quickly and effectively, detecting the disease early in asymptomatic stages, thus preventing the spread of the disease in commercial areas. Hence, the research aimed to verify the potential of hyperspectral imaging in detecting differences in the spectral pattern between healthy and asymptomatic infected plants of *Citrus sinensis* (L.) Osbeck, variety 'Valência'.

2 MATERIALS AND METHODS

2.1 Maintaining healthy and infected citrus plants

The citrus plants (*C. sinensis*) used in the analyses were maintained throughout the bioassay in glasshouses at the Integrated Pest Management Laboratory, at the Department of Entomology and Acarology, at the Luiz de Queiroz College of Agriculture, in the University of São Paulo (ESALQ/USP), Piracicaba, Brazil. The control treatment (healthy plants) and those infected with CLas were kept in different locations to avoid contamination. The plants were irrigated using a sprinkler and dripper system installed in the glasshouses.

The treatments consisted of five citrus plants infected with CLas through bud grafting and five healthy plants, representing the control treatment of the experiment. Each citrus plant represented a repetition of its treatment (control or infected). Thus, after ≈50 days of plant infection, spectral evaluations began.

2.2 Spectral data collection

The spectral evaluation was performed using the hyperspectral sensor Pika-L (Resonon Inc., Bozeman, MT, USA), with a 23-mm objective lens. This sensor collects spectral data in the range 400–1000 nm, from 281 bands (spectral resolution) by 900 pixels (spatial resolution), with a spectral bandwidth of 2.1 nm. Hyperspectral data were collected in a dark room with artificial lighting mounted on a tower, from 15 W, 12 V LED light bulbs mounted in two angled rows, one on either side of the lens, with two bulbs in each row. A voltage stabilizer (Tripp-Lite, PR-7b, www.radioreference.com) was used to stabilize the power supply to the lighting system. A polyethylene plastic board (Type 822, Spectronon Pro; Resonon Inc.) was used for white calibration, and the lens had its cover for dark calibration. Then, the relative reflectance of the samples could be calculated based on white and dark calibration.

Weekly spectral readings were taken from three to four leaves of each plant (repetition). The leaves were chosen from different plant branches, from the most apical to the basal branches. Leaves that had some damage to the plant tissue were not used for evaluation.

Spectral evaluations began 50 days after infection (DAI) of plants with CLas. To this end, the samples (leaves) were positioned on a linear platform below the sensor, which moves and reads automatically, controlled by the SPECTRONON software (Resonon Inc.). Each leaf was positioned on the platform with its adaxial face facing the sensor, performing a sample reading in a pre-defined region of interest. For data extraction, the region of interest selected in the software was defined as the entire leaf.

The numerical data of the generated spectra were transferred to the computer for recording, processing and analysis. Spectral data collection was carried out until the appearance of the characteristic visual symptom of HLB (Fig. 1), totalling 15 weekly readings. Spectral evaluations were carried out weekly at 50, 58, 64, 72, 79, 86, 94, 99, 107, 114, 122, 129, 134, 143 and 150 DAI of plants with CLas through bubbles.

2.3 Extraction of reflectance values

The hyperspectral data corresponding to the spectrum of each leaf were manually extracted from the dataset. This process involved contrast adjustment using specific tools from SPECTRONON-PRO software, selection of the region of interest, and generation of average reflectance values for each spectral band of the leaves. Additionally, based on the average spectral reflectance, a single spectrum was obtained for each treatment to evaluate their spectral response.

2.4 Data preprocessing

Originally, the electromagnetic spectrum of the sensor used ranged from 384.73 to 1021.76 nm, encompassing 300 spectral channels. For the analysis, a spectrum range of 400–1000 nm with 281 channels was adopted for all analyzed dates. This range was chosen because noise in the dataset was concentrated in the wavelengths 384.73–398.77 nm and 1001.47–1021.76 nm, which were eliminated. To express the reflectance factor on a scale from 0 to 1, all pixel values were divided by 10 000. This normalization not only simplifies the interpretation of the resulting reflectance values, but also facilitates comparison between different spectral bands.

2.5 Multivariate analysis

Principal component analysis (PCA) was used to reduce the size of the dataset. It selected variables based on their contributions to explaining the existing variability. In the end, ≈10% of the initial dataset was used for mean comparison through ANOVA.

2.6 Analysis of variance

For each selected variable, a one-way ANOVA with Tukey's honestly significant difference (HSD) *post hoc* test was performed, comparing the means of the tested treatments during the evaluation period.

Figure 1. Comparison of leaves taken from plants (a) infected with '*Candidatus* Liberibacter asiaticus' and (b) uninfected after ≈200 days of infection.

The aforementioned analyses were conducted in R[29] using basic functions, loops, and the following packages: FactoMineR, factoextra, stats and agricolae.

3 RESULTS

It was possible to observe that, from the spectral data obtained from infected and healthy plants, some spectral band ranges showed differences in reflectance between the treatments tested from the first day of evaluation, that is, after 50 DAI by plant buds of citrus (Table 1). After data extraction, spectral bands that showed significance between treatments were determined to reduce redundancy and increase detection efficiency (Table 1).

From the first day of leaf evaluation, there were differences between healthy and HLB-infected plants in the NIR range between 730 and 1200 nm (Table 1). From the second to the last evaluation, the spectral bands more frequently repeated in the different evaluations are close to the red edge region (660–750 nm) (Table 1).

Furthermore, after the Week 6 reading, ≈86 DAI, significant differences were observed between infected and healthy plants in the visible region (520–680 nm) in all weeks evaluated until the last week, after 150 DAI (Table 1).

In the first evaluation carried out at 50 DAI, it was possible to observe that healthy plants presented a higher reflectance factor when compared to plants previously infected with CLas, close to the NIR region (730–1000 nm) (Fig. 2). In the 400–700 nm range, it was impossible to observe differences in the curves regarding the reflectance factor between both treatments (Fig. 2).

After 86 DAI, or Week 6, it was possible to observe slight differences in the spectral curves in the regions of 500–600 nm and 900–1000 nm, regions close to visible and NIR, respectively (Fig. 3). However, the differences between the spectral curves in the NIR region of healthy and infected plants were smoother than the differences observed in Week 1 of evaluation (Fig. 2).

In the last evaluation, after 150 DAI, the spectral curves demonstrated relevant differences in the region close to the red edge (680–730 nm) (Fig. 4). However, in the visible region (500–600 nm), it was possible to observe a lower reflectance factor in healthy plants than in infected plants (Fig. 4).

Table 1. Evaluation (days after infection, DAI) and spectral band ranges (nm) with significance between treatments (infected and healthy plants) of *Citrus sinensis* leaves, determined by principal component analysis ($P < 0.05$)

Evaluation (DAI)	Spectral band ranges (nm)	*P*
50	730–1020	< 0.05
58	700–730	< 0.05
64	700–740	< 0.05
72	660–720	< 0.05
79	700–740	< 0.05
86	380–520; 600–720; 940–1020	< 0.05
94	520–680; 680–740	< 0.05
99	520–580; 700–740	< 0.05
107	520–610; 690–750	< 0.05
114	530–580; 700–740	< 0.05
122	520–610; 700–750	< 0.05
129	520–610; 700–740	< 0.05
134	520–590; 700–740	< 0.05
143	530–570; 700–740	< 0.05
150	530–580; 700–740	< 0.05

4 DISCUSSION

Studies related to monitoring *D. citri* and early detection of HLB have been in-depth, mainly through hyperspectral sensors.[18,26,27] The precision obtained with the use of remote sensors can contribute to greater assertiveness in decision-making regarding the management of diseases such as HLB, in addition to providing accurate detection of the location of attack by pests or diseases, identifying infected areas in large orchards in a faster and more effective way than current monitoring methods.

Our study observed spectral differences between healthy and diseased citrus plants, mainly in the red edge, NIR and visible regions. The spectral reflectance of leaves can detect differences between plants based on physiology, biochemistry and structure. This is to the consequence of plant pigments that absorb electromagnetic radiation in the visible wavelengths (400–700 nm).[30] Spectra in the visible and NIR range are often used for analysis because almost all major structures and functional groups of organic compounds can be detected.[22]

The visible range and NIR range are the most useful spectral regions in plant analysis because they can verify changes in leaf pigmentation and mesophyll cell structure.[22] In addition, the visible region (400–750 nm) provides information about the spectral characteristics of pigments, such as chlorophylls, anthocyanins and carotenes. The NIR region (780–2500 nm) is associated with the measurement of overtones and combinations of molecular vibration.[31] Vegetation interacts with solar radiation, absorbing at red and blue wavelengths and reflecting at green wavelengths, both located in the visible region and reflecting at wavelengths in the NIR region.[22]

Therefore, several research studies have been conducted using visible–NIR spectroscopy, especially because it is a nondestructive and rapid method that provides chemical and biological data of a plant.[22,31] The visible–NIR region generally refers to the wavelength range where the visible spectrum (400–700 nm) and the NIR region (700–2500 nm) overlap.[32] Leaf reflectance changes significantly as a result of stress generated by pathogens at specific wavelengths in the visible range (380–750 nm), and generates variation in the far-red range (690–720 nm), which provides a faster and more consistent indication of infections than other regions of the electromagnetic spectrum.[33] However, the extent to which different pathogens can produce different spectral signatures in plants and the degree to which the spectral response to a specific stressor can vary between species.[34]

There were significant differences between the reflectance of HLB-infected and healthy plants in the red edge region (660–750 nm) from the second evaluation (Week 2) carried out 58 DAI and, from 86 DAI (Week 6) onwards, significant differences were observed in spectral bands in the visible region (520–680 nm). These results corroborate those obtained by Mei *et al.*,[26] who observed differences in the average reflectance of HLB-positive plants in the band range 400–700 nm. The spectral reflectance of leaves can be influenced by different factors, such as growth conditions, the structure of leaves and their constituents, and the environment.[26] Thus, these factors can influence internal leaf and morphological characteristics, such as color, texture, structure, pigment, moisture and composition of plant tissues.[26] Although hyperspectral data can provide rich spectral and spatial information related to the pathological properties of citrus leaves,

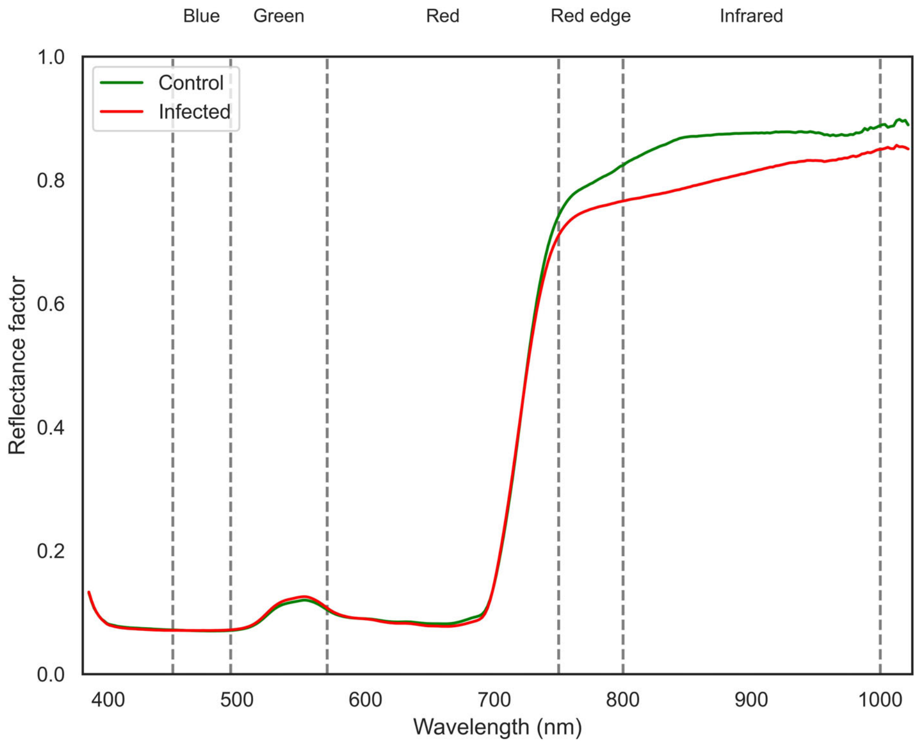

Figure 2. Spectral reflectance curve of plants infected by '*Candidatus* Liberibacter asiaticus' and healthy plants (control) in Week 1 of evaluation (~50 days after infection).

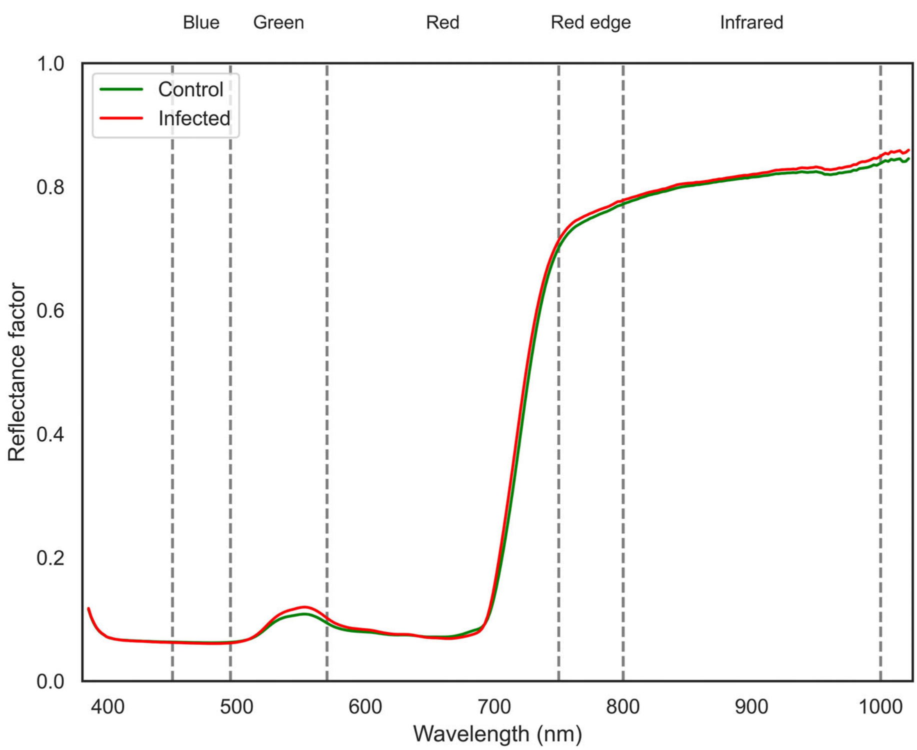

Figure 3. Spectral reflectance curve of plants infected by '*Candidatus* Liberibacter asiaticus' and healthy plants (control) in Week 5 of evaluation (~80 days after infection).

it also contains redundant information. Therefore, selecting representative and analytically influential wavelengths is necessary to reduce irrelevant information.[27]

In the first evaluation (Week 1), after 50 DAI, the reflectance of healthy plants was higher in the NIR region (730–1000 nm). However, after 79 and 150 DAI (weeks 5 and 15), it was observed that

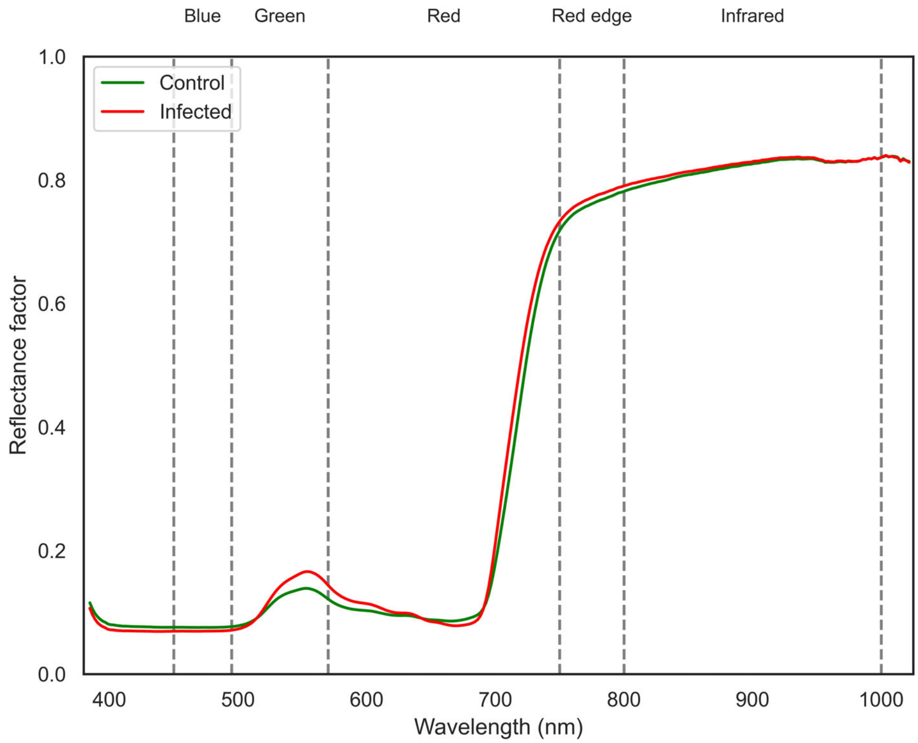

Figure 4. Spectral reflectance curve of plants infected by '*Candidatus* Liberibacter asiaticus' and healthy plants (control) on the Week 15 evaluation (∼150 days after infection).

the reflectance in the visible region (500–600 nm) was lower for healthy plants when compared to infected plants. Similar results were obtained by other authors, such as that observed by Sankaran *et al.*,[30] where the reflectance of healthy trees in the red edge and NIR region was higher than those of trees with HLB. Similar results also were observed by Weng *et al.*,[27] using the combination of hyperspectral images and carbohydrate metabolic analysis for the detection of HLB.

Thus, the reflectance of HLB-infected asymptomatic leaves in the visible region was slightly higher than that of healthy leaves but lower than that of CLas-infected and iron-deficient symptomatic leaves.[26] Sankaran *et al.*,[35] using a vehicle-mounted visible–NIR system to detect HLB-infected citrus plants, also observed that the average reflectance of HLB-infected trees was higher than healthy trees in the visible region of the magnetic spectrum. Kumar *et al.*[25] and Li *et al.*[36] also presented similar conclusions using aerial multi- and hyperspectral images. In the visible range (400–700 nm), the average reflectance of healthy plants was lower than that of HLB-infected samples, whereas the reflectance of healthy plants in the 700–1000 nm range was much higher than HLB-infected plants.[25,36]

Differences in the reflectance of infected and healthy plants in the visible and NIR regions may occur because healthy vegetation absorbs much energy in the visible region. By contrast, it has high reflectance in the NIR region.[35] This is to the consequence of leaf pigments such as xanthophylls, chlorophylls and carotenoids, which strongly absorb light energy in the visible region yet have little or no absorption in the NIR region.[37,38] Our study observed no differences in the spectral curve close to the visible region in Week 1 of evaluation owing to the citrus plants being in the initial stage of CLas infection. Significant differences were detected only in the NIR region (730–1000 nm); however, as the evaluation time progressed, it was possible to observe an increasing in the difference in the reflectance of healthy and diseased plants in the visible region, for example, when comparing the spectral curves after 79 and 150 DAI.

Considering the initial time of infection and the beginning of data collection, we can verify that significant differences in the relative reflectance between infected and healthy plants were detected in spectral band ranges of 520–680 nm in the visible region after ≈5 months of infection of plants with CLas. In a glasshouse, the period for the appearance of visual symptoms is 3–6 months, while in the field, in large trees, it can take more than a year for visual detection.[39] As the plants were maintained in a glasshouse, the significant differences between the reflectance of healthy and infected plants in the visible region are probably related to changes in pigmentation resulting from the appearance of visual symptoms of HLB.

IPM has made significant advances using modern and technological techniques, such as artificial intelligence and remote sensing, to promote sustainable agricultural development. These new tools have been essential in complementing traditional methods, promoting a balance between increased productivity and sustainability.[40] Currently, remote sensing is an effective tool for detecting, predicting, and managing insects and plant diseases in various economically important crops. This technology provides fast and accurate predictions, minimizing pest damage and production costs.[41] Commercially, digital tools available to producers include drones, automated traps and satellites. These devices offers remote and real-time access to plant health, insect populations, climate, soil moisture and other relevant parameters.[19] In addition to being important for disease monitoring and control, these devices allow the detection of even small changes in vegetation health and the presence of plant pathogens.[42]

The use of hyperspectral sensors for monitoring and early detection of HLB in citrus plants represents a significant advancement for IPM. By providing more accurate and timely detection of infections and stress caused by pathogens, this technology enables more effective and targeted interventions, reducing the need for indiscriminate applications of agricultural pesticides.

In this context, practices such as the use of remote sensing have been widely developed for use in citrus crops,[20,27,28,35] owing to the complexity in controlling the insect vector *Diaphorina citri* and in managing the disease Huanglongbing. The data obtained in this study demonstrate that significant differences in reflectance are found between healthy and infected plants in the red edge region (660–750 nm) from 58 DAI onwards. Thus, the use of spectral images in the early detection of HLB has excellent potential for monitoring infected plants because visual symptoms of HLB begin to appear ≈6 months after transmission of the bacteria, which makes it challenging to eliminate diseased plants and, consequently, increases the spread of the disease in citrus crops.

The differences in reflectance can serve as a basis for the diagnosis and early detection of HLB in asymptomatic plants. To this end, the importance of more in-depth studies in the laboratory and the field is highlighted in order to improve the use of hyperspectral sensors that can be used by citrus growers, becoming an applicable management tool for HLB. The results presented here will contribute to future studies in improving techniques using sensors to detect areas infected with bacteria associated with HLB and, consequently, to manage the insect vector and the disease in commercial citrus orchards.

ACKNOWLEDGEMENTS

This study was funded by the Brazilian Federal Agency for the Support and Evaluation of Graduate Education (CAPES)-001, with financial support and the awarding of a scholarship.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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