Spectral reflectance in the detection of Persian lime (*Citrus latifolia* Tanaka) leaves infected with Huanglongbing under fertilization treatments

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ABSTRACT

Objective: Evaluate the spectral reflectance method, to determine whether Persian lime leaves exposed to fertilizer treatments exhibit wavelengths associated with symptoms of Huanglongbing (HLB).

Design/Methodology/Approach: An experiment with a randomized complete block design with fertilizer treatments was implemented to observe changes in reflectance, chlorophyll content, severity of HLB symptoms, and the number of bacteria in Persian lime leaves. Grafted plants inoculated with buds from a diseased tree were placed under greenhouse conditions for a 12-month period.

Results: Leaves of diseased plants exhibited significantly higher reflectance in the visible spectrum (VIS) and lower chlorophyll content than leaves of healthy plants. A reverse, but less marked trend was observed in the near-infrared spectrum (NIR). Fertilization treatments did not have a significant impact on reflectance, chlorophyll content, severity of the symptoms, and bacterial count.

Study Limitations/Implications: A spectral reflectance assessment should be extended to susceptible varieties that exhibit visible symptoms with greater rapidity. Spectrometry can be complemented with multispectral and machine learning techniques to facilitate the early detection of asymptomatic diseased plants.

Findings/Conclusions: Spectral reflectance in the visible range has effectively differentiated between leaves of healthy plants and leaves with mild symptoms of HLB. Higher reflectance and lower chlorophyll content in diseased leaves are associated with this phenomenon, even when management practices that could mask symptom expression in diseased plants, such as fertilization, are employed.

Keywords: *Candidatus* Liberibacter asiaticus, visible spectrum, spectrometer, fertilization.

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INTRODUCTION

Huanglongbing (HLB) is regarded as the most destructive citrus disease worldwide (Bové, 2006; da Graça *et al.*, 2016). In Mexico, *Candidatus* Liberibacter asiaticus (*C*Las) is the causative agent of this disease. This bacterium is spread to plants by the *Diaphorina citri* Kuwayama insect vector (Bové, 2006). Significant economic losses have been caused by HLB among citrus all over the world, due to reduced yields and increased production costs

associated with its management (Li *et al.*, 2020). In Mexico, the lemon industry has been particularly impacted: over 105,000 t and ≈10,000 ha of sown area were lost between 2008 and 2020. This loss led to a significant decrease in yields (23-57 kg per tree), particularly in cases the severity reached over 75 % (Villar-Luna *et al.*, 2024).

Persian lime has significant economic importance in Mexico. As an HLB-tolerant crop, it has slow symptom development and sustains growth and productivity even in the presence of the disease (Folimonova *et al.*, 2009). However, recent studies have estimated a reduction in fruit yield of approximately 2.4 t ha^{-1}, with losses of 17.3% in fruit weight and 18.6% in juice volume (Flores-Sánchez *et al.*, 2015; Ortiz-Saavedra, 2022).

Leaves of infected plants show various symptoms, such as asymmetric spots with yellow areas, as well as the discoloration and thickening of the midribs. Additionally, underdeveloped and distorted fruits drop prematurely. Plants may also show signs of secondary root dieback. These symptoms are more severe in susceptible than in tolerant varieties (da Graça *et al.*, 2016). Identifying diseased plants under field conditions is generally based on visible symptoms. In tolerant varieties, such as Persian lime, symptoms may appear gradually, even when their bacterial titers can be compared with those of susceptible varieties already showing symptoms (Folimonova *et al.*, 2009). This situation indicates the presence of affected plants without visible symptoms, which poses a significant challenge for designing effective management strategies that limit the spread of the inoculum source.

To date, the disease is diagnosed in the lab, using techniques such as Polymerase Chain Reaction (PCR). This diagnosis involves a significant expense and longer processing times, underscoring the importance of establishing an alternative, fast, and non-destructive method to identify HLB-infected trees under field conditions (Weng *et al.*, 2018). Spectral reflectance has been successfully implemented in agriculture to assess the response of plants to biotic and abiotic stress, with encouraging results for the early detection of HLB-infected plants (Sankaran *et al.*, 2013; Weng *et al.*, 2018).

Yzquierdo-Alvarez *et al.* (2021) analyzed satellite images of Persian lime plants infected with HLB in Mexico and reported that these plants showed higher reflectance than healthy plants in the green (560 nm), red (665 nm), and near-infrared (705 nm) spectral bands. However, further exploration is necessary to determine if this approach can be applied to the individual leaves of tolerant varieties, particularly in asymptomatic conditions or with mild HLB symptoms. Additionally, such a study should assess the sensitivity of this methodology to different agronomic management practices, including various fertilization conditions, which could further delay the onset of symptoms. Consequently, the objective of this study was to assess whether or not spectral reflectance is a method that can be used to determine if Persian lime leaves, subjected to fertilization treatments, show wavelengths associated with HLB symptoms.

MATERIALS AND METHODS

Plant material

Persian lime plants (*Citrus latifolia* Tanaka) used in this experiment were grafted on two-year-old *Citrus macrophylla* Macf rootstocks, obtained from a certified nursery in the town of Cañadas, municipality of Martínez de la Torre, Veracruz. A group of approximately 50 plants was re-grafted with buds obtained from a tree previously confirmed as HLB-positive by PCR. The 12-month experiment, which took place from summer to autumn of the following year, was conducted in a greenhouse at the Colegio de Postgraduados, Campus Veracruz (19.194167 N, −96.343611 W).

Confirmation of infection by qPCR

DNA was extracted from the leaf midribs at the beginning and end of the experiment, following the CTAB protocol (Doyle and Doyle, 1987). Before the start of the experiment, plant infection was confirmed by end-point PCR with primers OI/OI2C (Jagoueix *et al.*, 1994). Mixtures of 50 ng/μL^{-1} were prepared for each treatment, representing the four replicates. Primers and probes reported by Li *et al.* (2006) and Bao *et al.* (2020) were used for the 16S rDNA genes of *C*Las and COX as an endogenous gene. The $2^{-\Delta\Delta Ct}$ method was used to quantify the relative changes in *C*Las levels from the beginning to the end of the experiment. Additionally, the error propagation of the ΔCT of the 16S and COX genes was calculated (Livak and Schmittgen, 2001).

Fertilization treatments and experimental design

A randomized complete block design was conducted with three fertilization treatments and four replications per treatment. The treatments consisted of three levels of micronutrients: 1) B0, without micronutrients; 2) B1, with micronutrients applied to the soil (12 S, 6 Zn, 3 B, 6 Mn, 0.47 Fe g plant^{-1} yr^{-1}); and 3) B2: with micronutrients applied to the soil and the leaves (12 S, 6 Zn, 3 B, 6 Mn, 0.47 Fe to the soil+4.8% N, 4.9% Mg, 4.9% B, and 9.9% Zn to the leaves). In addition, a standard macronutrient fertilization was applied to all treatments: 120 N, 20 P, 15 K, 23.3 Ca, and 15.6 Mg (g plant^{-1} year^{-1}). The annual fertilization rate was divided into 20 applications, according to the recommendations of Atta *et al.* (2020).

Reflectance measurement

The reflectance of mature Persian lime leaves, including those infected with HLB, was measured. These samples were randomly selected among specimens that did not show symptoms of yellowing or chlorosis caused by nutritional deficiencies or other diseases (*e.g.*, wood pocket). A spectrometer with a 0.45 nm resolution was used for the analysis, which was conducted within the 450-800 nm range. The spectrometer was coupled to a QP600-025-VIS optical fiber and a 74-UV series collimating lens fixed 5 cm away from the module's base. The data was captured with the Oceanview® software. A reflectance module, mounted in dark conditions with two 90-W halogen lamps, was used as the primary light source. The equipment was calibrated with a white polytetrafluoroethylene panel.

Chlorophyll quantification and severity of symptoms in leaves

The chlorophyll was quantified at the end of the experiment, on 100 mg of leaves without midribs, based on the methodology reported by Das *et al.* (2019). The severity of the symptoms was determined using the scale proposed by Gottwald *et al.* (2007) and Shokrollah *et al.* (2011).

Data analysis

Patterns or clusters in individual leaf reflectance data were identified through a dimension reduction analysis (UMAP) using RStudio v. 4.3.1, considering the visible (VIS) and near-infrared (NIR) range, according to López-Collado *et al.* (2024). Box-Cox transformation and correlation structures were used to meet statistical assumptions. The other variables were subjected to an analysis of variance (ANOVA) for linear mixed models, followed by Tukey's multiple comparison test ($p \leq 0.05$).

RESULTS AND DISCUSSION

Reflectance of healthy and HLB-infected Persian lime leaves

Significant variations were observed when reflectance was analyzed under natural light. These variations are associated with changes in cloudy conditions and reduced light input in the greenhouse. To address this variability, readings of the sampled leaves were taken in a reflectance module with halogen light set up in the laboratory (Figures 1A and B). The reflectance of diseased plants was significantly higher than the reflectance of healthy plants ($p \leq 0.0001$) at 550 nm (VIS range). In the case of NIR, the data set showed a similar response between both groups, but a higher reflectance ($p = 0.0397$) was observed in healthy plants than in diseased plants, in the mean separation at 790 nm (Figures 1C and D).

The comprehensive analysis of the data confirmed the VIS differences through dimension reduction (UMAP), revealing a clear separation between plant groups. However, in the NIR, no distinct clustering patterns were found between groups (Figures 1E and F). Overlap was also observed in some individual values, indicating considerable variability within each group, possibly due to heterogeneity and low symptom intensity. This phenomenon would explain the similarity between plant groups in the NIR. This lack of differences could be caused by the variability in carbohydrate accumulation, a correlation previously reported by Weng *et al.* (2018). While the equipment clearly differentiated between leaves of healthy and diseased plants at 550 nm, the variability in leaves with mild symptoms of HLB hinders the application of this method as an early detection tool. This situation is particularly noticeable with spectrometers that operate exclusively in the visible and near-infrared spectrum (400-800 nm) and whose software has not been trained to increase their accuracy. These results match the findings of Deng *et al.* (2019), who faced difficulties in the identification of asymptomatic leaves, even with hyperspectral reflectance. However, this challenge was addressed through the implementation of machine learning algorithms. After pre-training, the algorithm was able to differentiate between healthy plants without symptoms and plants with symptoms of HLB with a 96% accuracy.

The reflectance pattern of Persian lime is consistent with the findings reported by He *et al.* (2002) regarding Navel oranges: leaves from plants without HLB symptoms had slightly higher reflectance in the VIS than leaves from healthy plants, but lower than leaves with noticeable HLB symptoms.

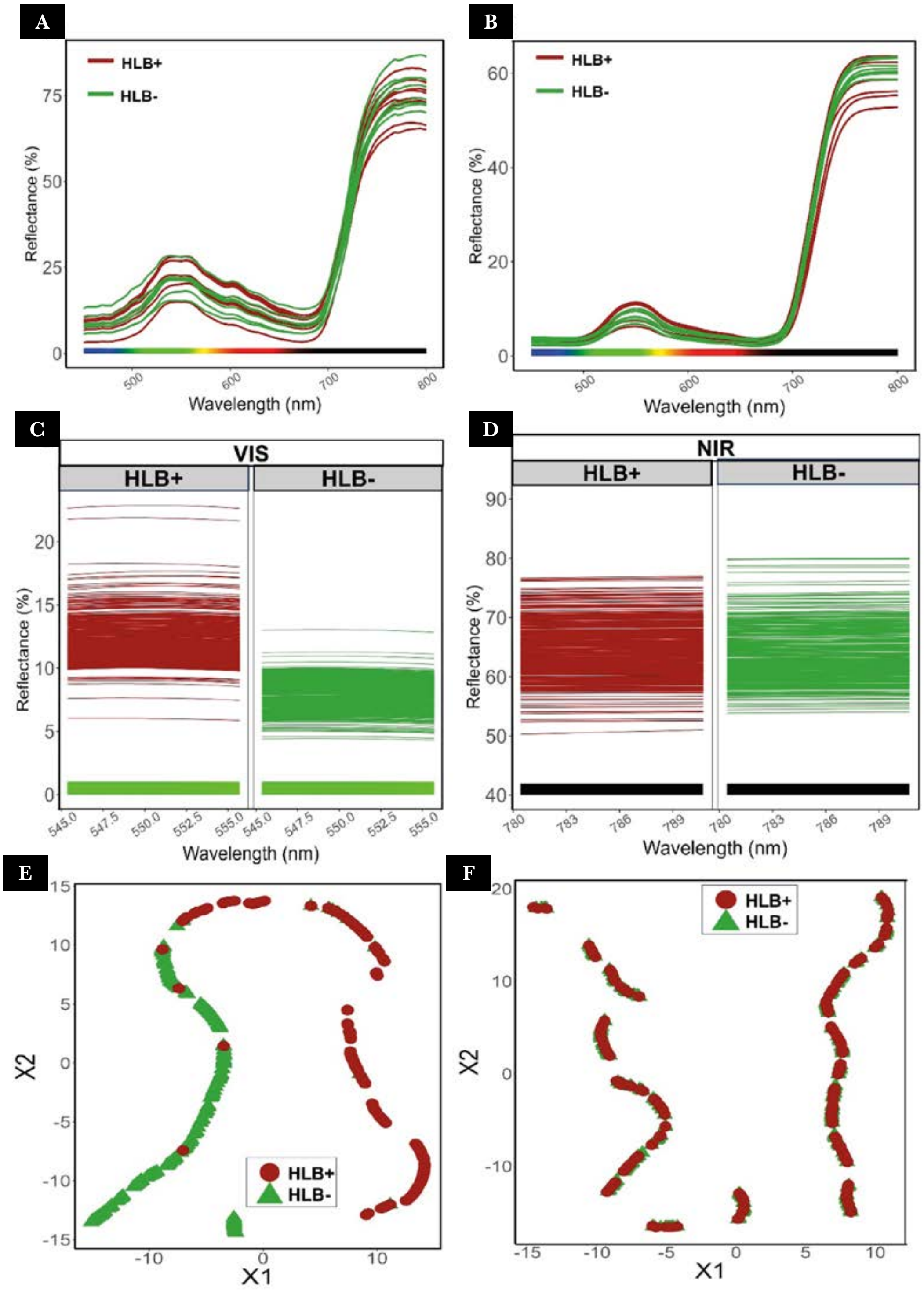

Figure 1. A) Leaf reflectance spectrograms with sunlight (higher variability) and B) with halogen light. C) Spectrograms with individual leaf data in the VIS and D) NIR (n=500) ranges. E) UMAP analysis with separation between healthy and diseased plant groups in the VIS, F) but not in the NIR range.

Several factors determine leaf reflectance, including: photosynthetic pigment content, anatomical structure, morphological characteristics, biochemical properties, and even water content (Mishra *et al.*, 2012; Kior *et al.*, 2021). The photosynthetic pigment content is closely related to the reflectance in the visible spectrum (VIS), which depends mainly on the content of chlorophyll and pigments (*e.g.*, anthocyanins and xanthophylls). The latter are involved in light dissipation and protection of the photosynthetic apparatus. A gradual loss of pigments is recorded under stress, causing a shift in spectral peaks and alterations in the balance between photosynthetic energy consumption processes and changes associated with photosynthetic stress (Kior *et al.*, 2021). These processes are particularly altered in HLB-infected citrus plants, even in asymptomatic cases (Weng *et al.*, 2020). Cell death has been reported in the sieve cells and companion cells of asymptomatic young leaves, which shows that the effects of the disease are already present before visible symptoms appear (Ma *et al.*, 2022). These results are consistent with the findings of this study, which show chlorophyll loss and reflectance differences between healthy and diseased leaves, even in plants with mild symptoms.

Reflectance of Persian lime leaves with fertilization treatments

In both summer and winter, leaf reflectance at 550 nm was similar between treatments with healthy and diseased plants. During autumn, the application of micronutrients to the soil (B1) exhibited a significantly higher reflectance ($p \leq 0.05$) in diseased plants, which was associated with an increased bacterial count in the leaves (Figure 2A and D), but not with a lower amount of chlorophyl. At 790 nm, differences in reflectance were only observed during the winter period, with B1 treatment also recording the highest reflectance (Figure 2B).

On average, diseased plants exhibited higher reflectance in the VIS range, but no differences in the NIR, compared to healthy plants ($p=0.0118$ and 0.1872, respectively). In addition, chlorophyll concentration was significantly lower in infected than in healthy plants, with no differences between fertilization treatments in each group (Figure 2C). These findings match previous reports documenting decreased chlorophyll content in Persian lime plants infected with HLB (Flores-De la Rosa *et al.*, 2021). Following the conclusion of the experiment, a negative correlation was observed between reflectance at 550 nm and chlorophyll content in healthy plants, but not in diseased plants (Figure 2C). The degradation of chloroplast thylakoids in HBL-infected plants could explain this phenomenon, which is attributed to starch accumulation (Jin *et al.*, 2017). Additionally, at the initial stage of infection, variability in starch accumulation and photosynthetic pigments among leaves could contribute to the lack of correlation between reflectance and chlorophyll content in diseased plants.

The number of bacteria increased at the end of the experiment, both in the treatments in which micronutrients were added to the soil and in those treatments in which no nutrients were added. A similar trend was observed between the number of bacteria and the severity of symptoms (%) at foliar level. However, no significant differences were detected between the various fertilization treatments (Figure 2D). These results match previous studies (Atta *et al.*, 2020), suggesting that fertilization is essential for HLB management, since it helps to

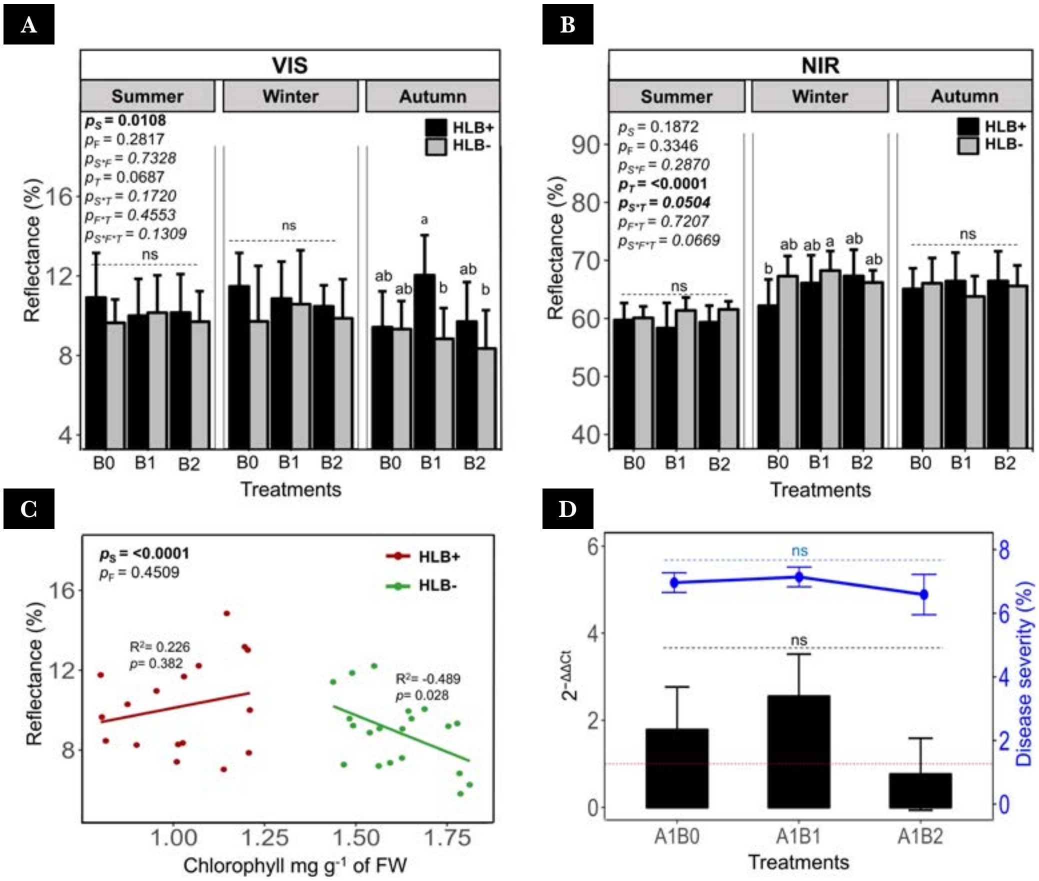

Figure 2. Effect of HLB and micronutrient levels on percent of reflectance in A) VIS range (550 nm) and B) NIR range (790 nm) at three sampling times (mean ± SD). C) Relationship between chlorophyll content and reflectance in leaves of healthy and diseased plants. D) Relative quantification of the 16S rDNA gene of CLas in midrib (mean ± error propagation) and its relationship with the severity of symptom in leaves at the end of the experiment (mean ± SD).

maintain the vigor of diseased plants. However, this phenomenon does not appear to have a direct effect on bacterial titer reduction as previously reported by Gottwald *et al.* (2012) and da Silva *et al.* (2020). However, recent research has indicated a decrease in *C*Las titers, following the application of four times the recommend dose of manganese (Zambon *et al.*, 2019). Further studies are required to accurately determine the role of some micronutrients in the variability of *C*Las titers.

Fertilization treatments did not show any significant effect on the percentage of reflectance, even in the absence of micronutrient supplementation. This finding contrasts with the observations of Ayala-Silva and Beyl (2005), who noted that deficiencies of macro- and micronutrients (*e.g.*, nitrogen and magnesium) are associated with lower chlorophyll concentrations and higher reflectance values in the VIS and NIR ranges. Consequently, the impact of fertilization on reflectance should be assessed over an extended period, using multispectral cameras.

Despite the variability observed in the VIS and NIR ranges between leaves, plants, and sampling times, reflectance enables the differentiation between healthy and diseased plant leaves, even in asymptomatic stages and under different management practices, such as fertilization. Using equipment with a broader spectrum of wavelengths or multispectral cameras —combined with machine learning tools— holds significant promise for an enhanced accuracy of asymptomatic leaf detection, even under field conditions.

CONCLUSIONS

Spectral reflectance in the visible range has proven to be an effective tool for differentiating leaves of healthy plants and leaves with mild symptoms of HLB, recording a higher reflectance and lower chlorophyll content in diseased leaves. In contrast, near-infrared reflectance showed a reverse trend, although it was less consistent. The impact of fertilization treatments on reflectance, chlorophyll content, severity of symptoms, and bacterial content was not significant.

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