Near-infrared spectroscopy for discrimination of huanglongbing-infected citrus leaves from uninfected leaves

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Introduction

Citrus greening, also called Huanglongbing (HLB) or yellow dragon disease, is one of the more serious diseases of citrus. The disease which is caused by the bacteria *Candidatus Liberibacter asiaticus* is primarily spread by the psyllid insect *Diaphorina citri* which acts as a vector in spreading the disease.[1–3] Citrus greening disease is a threat to the U.S. and world citrus industries.[1–3] The most characteristic symptoms of citrus greening are a blotchy leaf mottle and vein yellowing, that develop on leaves attached to shoots showing the overall yellow appearance. An infected tree produces fruit that is unsuitable for sale as fresh fruit or for juice. The only definitive method of diagnosis of trees suspected of infection by citrus greening pathogens is by analysis of DNA.[4] The purpose of the research was to investigate the use of near infrared spectroscopy to differentiate HLB infected leaves from uninfected leaves.

Experimental

Seventy-four and 79 leaf samples that showed symptoms of HLB were collected in July and November 2008, respectively from orange and grapefruit trees on the US Horticultural Research Laboratory's farm. These symptoms included: green islands, yellow dragon, blotchy, light chlorosis, heavy chlorosis, and visually negative for HLB. Leaves ($N=30$) were also collected from trees in a controlled quarantined greenhouse and were used as a "negative" control. The mid-rib veins of the leaves were excised and analysed for the citrus greening pathogens DNA by real-time polymerase chain reaction (PCR). In the technique used in this paper, a dye is attached to the DNA probe, along with a quencher. The amount of fluorescence is measured at the end of each cycle, and plotted with fluorescence on the y-axis, and cycle number on the x-axis. Once the fluorescence exceeds a user defined threshold, the number of cycles needed to get that sample to

that point is recorded as the "critical threshold" (*Ct*) for that sample. The higher the *Ct* value, the lower the concentration of the DNA in the sample. A *Ct* value above 30 is considered negative for citrus greening disease, whereas, below 30 is considered positive. However, *Ct* values between ~30 and 32 are considered a "grey" area in which it is difficult to say with certainty that the sample is positive, or negative for HLB. The remaining leaves were dried in a 1250 W microwave oven for 3 min at one-half power and then ground. Spectra were collected from 400 nm to 2500 nm using a Foss XDS Rapid Content Analyser. WinISI was used for multivariate analysis. The range and *SD* of the *Ct* values for infected and uninfected leaves were 21.1 to 27.4 ± 1.0 and 35.2 to 40.0 ± 1.1, respectively. The sample sets were combined *(N* = 183) and 66% were selected at random for calibration. The remaining samples (*N* = 63) were used for validation. Partial least squares (PLS) regression was used to regress HLB *Ct* values on log (1/reflectance) spectra of ground leaves. Full one-out cross validation was used with PLS regression.

Results and discussion

HLB infected and uninfected leaf spectra from the July harvest were transformed with a 1st derivative (10 nm gap) and averaged. The spectra for the uninfected leaves showed the normal spectral response in the blue (400 – 500 nm) and red (600 – 700 nm) regions for healthy plants[5] (Figure 1).

The primary difference between the HLB negative and the HLB positive leaves was that the peaks associated with chlorophyll absorption decreased for the infected leaves.

The most notable differences between infected and uninfected leaves in the NIR region can be seen due to the absorption of carbohydrate and cuticle wax (Figure 2).

The difference in cuticle wax was evident for the first overtone of the C-H stretching vibration around 1700 nm and the C-H combination band near 2300 nm. Carbohydrate differences are

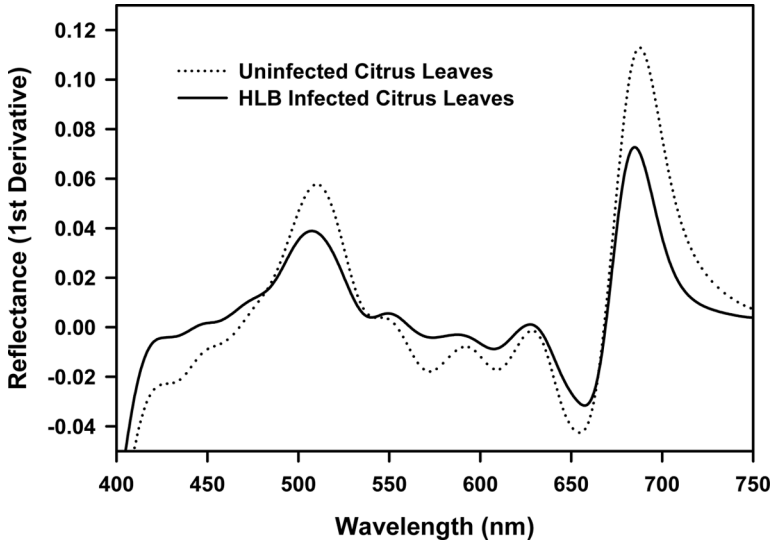

Figure 1. Average visible spectra of HLB infected and uninfected citrus leaves.

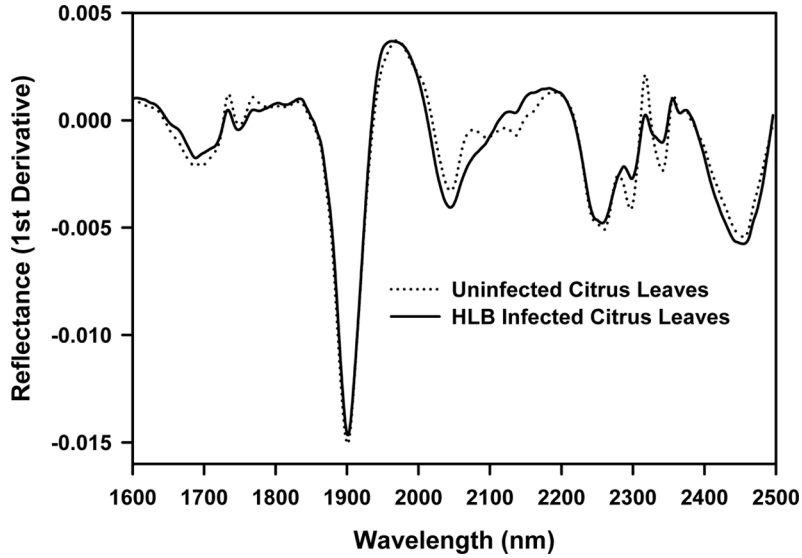

Figure 2. Average near-infrared spectra of HLB infected and uninfected citrus leaves.

obvious from 2050 nm to 2300 nm. The carbohydrates in this region were represented by similar vibrational bands and included various sugars, starches, and cellulose. Both infected and uninfected leaves have peaks due to waxes and carbohydrates, but it was clear that a chemical change occurred in either the amount, type or structure to the waxes and carbohydrates present in the HLB infected leaves.

PLS calibrations for HLB *Ct* values were developed with the visible region (400 – 750 nm), visible plus the NIR region [(VNIR) 400 – 2500 nm)] and the NIR region (1100 – 2500 nm). Calibration statistics for all spectral regions were lower for a first derivative (10 nm gap) data pretreatment compared to log 1/reflectance spectra. The standard error of cross-validation (*SECV*) and R^2 for the 6-term visible model was 3.1 and 0.84, respectively. Standard error of prediction (*SEP*) and r^2 was 2.9 and 0.85, respectively. The model correctly predicted the uninfected samples (Figure 3), but 4 infected samples were predicted as uninfected or in the gray area.

The *SECV* and R^2 for the V/NIR model were 2.4 and 0.90, respectively. The *SEP* and the r^2 were 2.6 and 0.88, respectively. The calibration and validation statistic for the V/NIR model were lower compared to the visible model and correctly predicted the infected samples with only one sample in the gray area. The *SECV* and R^2 for the 7-term NIR model was 2.4 and 0.88, respectively. Validation results are shown in Figure 4 and the SEP was equal to the visible/NIR model.

The range of *Ct* values for the infected leaves was greater for both the visible and the V/NIR model than the NIR model. The visible and the V/NIR PLS models loading factor 1 had a large absorption band at 692 nm, which appeared to be a measure of the "greenness" of the leaves. Scores from the first component of both the visible and V/NIR models had correlations of 0.59 and 0.61, respectively with *Ct* values, and were the dominant components. Therefore, the more chlorotic a leaf is, the lower the predicted *Ct* value. Being that most plant stressors will induce a change in chlorophyll absorption due to a decrease in chlorophyll production, it is unlikely that

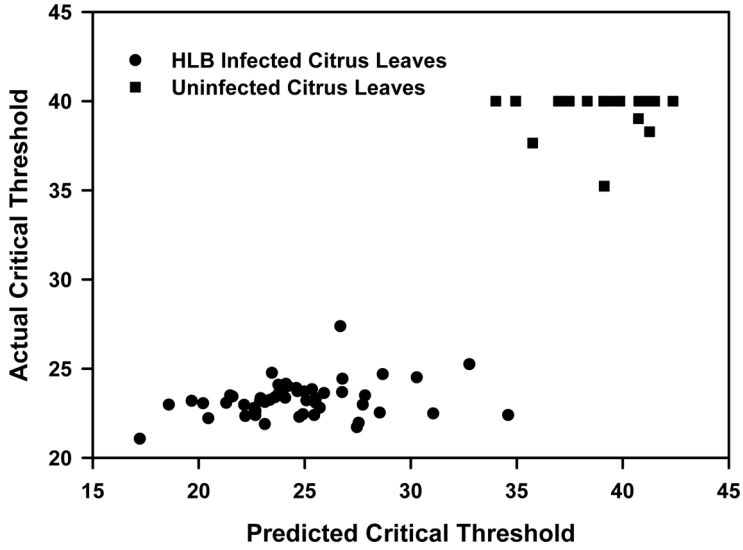

Figure 3. Validation results for citrus green PCR critical threshold using model developed with visible spectra.

the visible portion of the spectrum alone or in combination with the NIR region will be able to differentiate HLB infected plants from plants that are compromised by some other biostressor.

Scores for components 1–3 for the NIR model had correlations of 0.17, 0.44, and 0.21, respectively with *Ct* values. The shape of the plot for the second component had large intensities at wavelengths related to the absorbance of carbohydrates and cuticle wax.

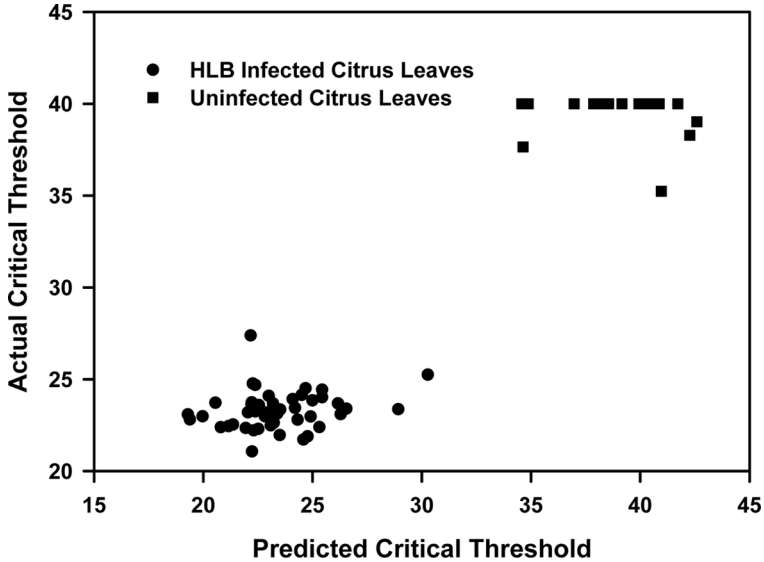

Figure 4. Validation results for citrus green PCR critical threshold using model developed with NIR spectra.

Conclusions

All PLS models correctly predicted 100% of the leaves that were negative for HLB or uninfected. The visible, VNIR, and NIR models correctly predicted 94%, 9%, and 98%, respectively of the leaves that were HLB infected. The PLS loadings for the visible and VNIR models indicated absorption bands related to chlorophyll. The PLS loading for the NIR model indicated absorption bands related to carbohydrate and wax. The results indicated that NIR spectroscopy is a useful, rapid, and inexpensive tool compared to PCR, in identification of citrus greening disease.

References

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