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Identification of Citrus Huanglongbing Disease at the Pre-Symptomatic Stage Using Polarized Imaging Technique

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Abstract: Huanglongbing (HLB), also known as yellow shoot or citrus greening, is a devastating disease, associated with '*Candidatus* Liberibacter asiaticus (*C*Las).' Unfortunately, there is no effective cure for this disease, and since a tree is infected, it will eventually die. HLB has negatively affected citrus industries in many countries in Africa, Asia, and South America. In 2005, HLB was confirmed in a groove in South Florida, and after a few years, the entire state of Florida got infected. Timely detection and removal of HLB-affected trees are substantially necessary for managing the disease. Accumulation of starch that appears as yellow/light-green islands on the citrus leaf is known as an early diagnostic symptom of HLB. A polarized imaging system was used to follow the hyper-accumulation of starch in leaves of graft-inoculated citrus plants. Time-lapse images of citrus leaves were acquired in a weekly manner after plants were grafted with a disk from another HLB-affected leaf to follow the variation in the images' gray values at different locations on a leaf. An image analysis algorithm was developed to highlight the regions of citrus leaf samples with an increase in average gray value as an indication of starch accumulation in the pre-symptomatic stage. The proposed method was able to detect successfully any local increase in the image gray values which associates with hyper-accumulation of starch, and consequently, the existence of *C*Las.

Keywords: Citrus greening, Disease diagnosis, Image analysis, Starch, Polarization, Sensing.

1. INTRODUCTION

Huanglongbing (HLB), transmitted by an insect vector called Asian citrus psyllid (*Diaphorina citri*), is a bacterial disease of citrus. Unfortunately, no effective treatment for this disease has been found yet (Pourreza et al., 2014a) and infected trees eventually die. Hodges and Spreen (2012) reported that between 2006 and 2012 HLB cost $3.63 billion in lost revenue and 6,611 jobs in Florida. Neupane et al. (2016) analyzed various aspects of citrus yield loss caused by HLB and found that the chance of production loss in the majority of Florida counties is over 90%. This likelihood shows the failure of the current HLB disease control and management procedures mainly because no efficient management measures exists. National Research Council (2010) recommended eliminating HLB-affected trees to protect the rest of the groves. However, a rapid and large-scale diagnosis method is required to detect and localize all HLB-affected trees (Pourreza et al., 2014b) especially those at early pre-symptomatic stages so that the disease can effectively be controlled.

Keremane et al. (2015) employed loop-mediated amplification technology (LAMP) to develop a detection kit for field testing of psyllids for '*Candidatus Liberibacter asiaticus*' (*C*Las) that associates with HLB. Their method can be an alternative to polymerase chain reaction (PCR) test for testing both psyllids and plant DNA extractions. It took 30 minutes using their method to test six samples as well as a negative and positive control which was not repaid comparing to real-time methods. Wu et al. (2016) used an SE-Quant Tube Scanner to develop an improved real-time fluorescence loop-mediated isothermal amplification (RealAmp) for the purpose of quantitative and rapid detection of Las. Li et al. (2015) analyzed multispectral satellite image acquired by WorldView-2 to assess the feasibility of HLB detection in a large-scale. They achieved an overall accuracy of 81% using a Mahalanobis distance classifier. In another study, Wetterich et al. (2016) combined a machine learning technique and fluorescence imaging spectroscopy (FIS) to identify two citrus diseases, HLB, and citrus canker, and differentiate them from other conditions such as zinc deficiency and citrus crab. Their results showed their recommended technique was able to detect zinc deficiency from HLB symptoms with an accuracy of 95%. Although some field conditions have been put forward in this study, their method was primarily designed for laboratory experiment, while working under field conditions is necessary for a rapid and large-scale screening approach. Additionally,

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the diagnosis method should be cost-effective enough to be used in a high spatial and temporal resolutions.

Grafting is the process of joining parts of two separate (but compatible) plants such that they unite and grow into a plant with shared characteristics (Hartmann and Kester, 1975). In horticulture, grafting has been used for centuries as a means to develop plants with improved horticultural properties. Also, grafting can also be used to transfer communicable diseases from one plant to another for research and epidemiological studies. Conventional grafting methods (e.g. inserting a young branch or leaf base into the bark of another plant) can be very efficient in transferring diseases, yet the appearance of symptoms cannot be accurately predicted. For citrus, disc grafting, the replacement of a leaf disc in a healthy leaf with a disc of a diseased leaf (Blue et al., 1976), offers the advantage that development of symptoms is rapid and can be monitored in pre-selected leaves. Given the benefits provided by disc grafting, we proceeded to monitor the progression and accuracy of HLB disease by polarized narrow band imaging analysis using starch as an indicator (Pourreza et al., 2013).

It is still unclear how early the HLB disease can be diagnosed after bacteria transferred to a healthy plant. Lack of such knowledge is a major problem because there is no measure to evaluate the efficiency of an HLB diagnosis method at the incubation stage in the disease cycle. It was shown that starch accumulation in the HLB-affected citrus leaf is an early diagnostic symptom of this disease (Pourreza and Lee, 2014). The primary goal of this study was to identify the earliest pre-symptomatic stage of the HLB disease when it can be diagnosed using the polarized imaging technique based on faster rates of starch accumulation. Specific objectives were to (1) develop an infection protocol using disk graft-inoculation technique, and (2) monitor the hyper-accumulation of starch by acquiring time-lapse polarized images of HLB-infected citrus leaves.

2. MATERIALS AND METHODS

2.1 Graft Inoculation

For this study, we used five 2-year-old 'Valencia' (*Citrus sinensis* L. Osbeck*)* trees on Swingle rootstock kept in a psyllid protected greenhouse. Graft inoculation was conducted on healthy plants, so that we were able to follow the hyper accumulation of starch on the leaves since the beginning of infection.

Fig. 1.Valencia citrus leaf grafted with the tissue disc Method. A leaf disc of a healthy leaf is replaced with a similar leaf disc of an infected tree. Tissue is kept in place by Scotch tape on both sides.

As HLB-infected source tissue, we used HLB positive certified trees grown in the groves at the Citrus Research and Education Center in Lake Alfred, FL. A paper hole-punch of the midrib section of a healthy mature leaf was replaced with a similar disc from an HLB-infected leaf. As shown in Fig. 1, the hole-punch disc was kept in place with Scotch tape on both sides of the leaf (Blue et al., 1976). Grafted trees were kept in a greenhouse with automatic watering and frequent fertilization. Four leaves on each plant were grafted, so in total twenty grafting was conducted for five citrus plants.

2.2 Polarized Imaging

The vision-based sensor, which was developed previously for in-field HLB diagnosis (Pourreza et al., 2015a), was modified and employed for acquiring time-lapse polarized images of citrus leaves. Starch has a unique optical property of rotating the polarization plane of light (McMahon, 2004). The optimal wavelength in which the maximum polarization rotation occurred was near 600 nm as indicated in our previous study (Pourreza et al., 2014c). The vision sensor had a sensitive camera at 591 nm (DMK 23G445, TheImagingSource, Bremen, Germany), as well as ten high-intensity LEDs (LS4-00A100, LED Engin, San Jose, California). The LED panel was equipped with a linear polarizing film (visible linear polarizing laminated film, Edmund Optics, Barrington, New Jersey) and another linear polarizer with a perpendicular direction of the LEDs' polarizing film was mounted in front of the camera (Pourreza et al., 2015b). A dark box (13 × 13 × 22 cm) was created and attached as an extension to the front side of the sensor (Fig. 2a). The purpose of adding a dark box extension was to block the environmental light and also to keep a uniform imaging distance of 22 cm for all the leave samples and the weekly repetitions of image acquisition. The dark box extension had a removable cap on the end in which the leaf sample could be placed to be imaged without being removed from the stem (Fig. 2b). The polarized images of the same leaf samples on citrus plants were acquired using the vision-based sensor on a weekly basis.

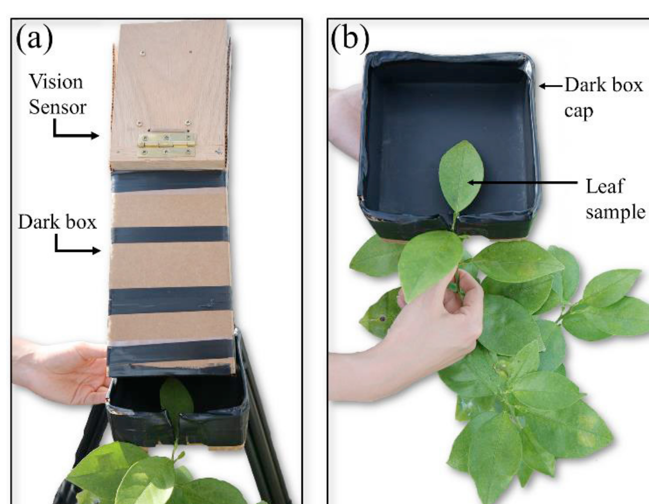

Fig. 2. The polarized imaging procedure: (a) the vision sensor and the dark box extension; (b) citrus leaf was placed on the dark box cap while it was attached to the plant stem.

Four grafted leaves on each plant as well as six other leaves were selected randomly for image acquisition. In total, 50 leaves (ten leaves per plant) were chosen for this experiment. The image acquisition was repeated six times (every Mondays at 3 PM Eastern Time) on a weekly basis between January 25th and February 29th, 2016. Images were acquired with similar camera settings (Exposure: 1/90 sec; gain: 0) and in the same condition every week.

2.3 Image Pre-processing

To conduct a locally based analysis, time-lapse images of every single leaf were registered, so that corresponding locations on the leaf images acquired over six weeks had similar coordinates in the images as well. As a result, the time-lapse images of the same leaf acquired on a weekly basis overlapped on top of each other. To conduct such registration, leaf image that was acquired in week #1 was defined as a reference, and all other images of the same leaf (acquired in weeks #2-6) were projected on the reference image. Image registration was conducted in multiple steps. At the first step, all the images were converted into binary images in which white pixels were belonging to the leaf area, and black pixels were belonging to the background. As shown in fig. 3, the leaves were positioned vertically in the polarized images, so the lower white pixel in each binary image represented the tip of the leaf that was considered as a common point in all images of the same leaf. Then, three values of Δx, Δy, and $\Delta\theta$ were defined for each projection, where Δx represented a shift in the horizontal axis, Δy represented a shift in the vertical axis, and $\Delta\theta$ represented a rotation angle that was used to spin the second image so that it matched the reference image.

All polarized images were calibrated using customized gain multipliers so that six images of the same leaf acquired over six weeks had the same average of gray values after calibration. The following equation was used for relatively calibrating the image acquired at week w:

$$\acute{P}_w(i,j) = \frac{\sum_{r=1}^{6} m_r}{6m_w} \times P(i,j) \quad (1)$$

where, i and j were the horizontal and vertical coordinates of pixels, $P(i,j)$ was the original image, $\acute{P}_w(i,j)$ was the calibrated image at week w, m_r was the variation in gray values mean at week r, and m_w was the gray values mean at week w.

2.4 Image Analysis

The key concept behind the image analysis in this study was that the gray values in the calibrated images at different locations must not differ unless a notable gray values increase in some locations occurred. Since the average gray values in the time-lapse images of a leaf were analogs after calibration, the increase in gray values at some location would cause a decrease at other locations. The areas with increased gray values were then considered as the probable locations where starch was accumulated. To conduct such an area based analysis, the images were divided into 25×25 pixels grids, and average gray values of corresponding grids in the six images of a leaf were compared to each other. An increase intensity index was defined to illustrate an overall percentage of increase in gray values means at different grids on the leaf and in different weeks using the following equation:

$$I = \frac{\sum_{i=1}^{N_p} \Delta m_i}{256(N_p+N_n)} \quad (2)$$

where N_p and N_n denotes the number of grids with positive and negative intensity changes, respectively, and Δm_i is average intensity increase in grid i.

Nine categories of gray values mean changes were defined based on the percentage of changes in the overall intensity of each grid as shown in the legend of fig. 4. Changes less than 2% was neglected.

3. RESULTS AND DISCUSSION

3.1 Infection Efficiency

A successful grafting had a healthy look after a couple of weeks. Fig. 3 shows one successful and one unsuccessful grafting.

Fig. 3. Grafting evaluation conducted eight weeks after the leaves were grafted; left: a successfully grafted leaf; right: an unsuccessful grafting

An expert evaluated all grafted leaves and judged if the grafting was successful. Table 1 includes the number of successfully grafted leaves for each plant. For plants number 1 to 4, there was, at least, one successful grafted leaf while grafting for all four leaves in plant number 5 was unsuccessful. One grafted leaf in plant number 4 was lost during the experiment, and it was excluded from the analysis. Since there was one successful grafting for all plants except plant #5, we considered plants #1-4 as HLB infected, and plant #5 as questionable.

Table 1. Numbers of successful and unsuccessful grafting for each citrus plant.

Grafting	Number of leaves on plant				
	#1	#2	#3	#4	#5
Successful	1	1	1	1	0
Unsuccessful	3	3	3	2	4

3.3 Area-based Image Comparison

Fig. 4 shows two examples of area based image comparison for two non-grafted sample leaves. For each sample, the top rows of images show the original gray scale image and the lower rows show a color image indicating the negative and positive average gray values changes on the corresponding gray scale image.

Sample #1 in Fig. 4 had an area of increased intensity on the right side of the primary vein that can be considered as a location with accumulated starch. Sample #2 also showed an increased intensity area on the left side of the primary vein that mostly appeared after week #5. For grafted leaves, this comparison was conducted only on the potion of leaves which were not covered by the scotch tape.

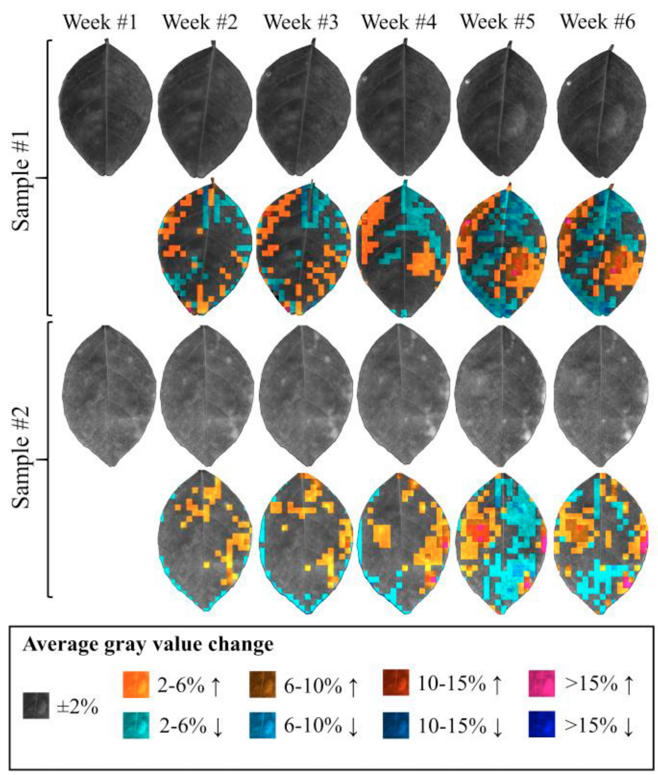

Fig. 4. Two examples of area based image comparison; sample #1 was a non-grafted leaf from plant #1, and sample #2 was another non-grafted leaf from plant #4.

Table 2 shows average intensity increase for each plant and in each week. Plant #1 had the maximum intensity increase comparing to other plants.

Table 2. Average intensity increases indices for each plant in each week and also for all weeks.

	Week				
Plant	All	#2	#3	#4	#5
#1	1.45%	1.76%	1.66%	1.26%	1.11%
#2	0.69%	0.85%	0.68%	0.69%	0.55%
#3	0.49%	0.53%	0.55%	0.51%	0.40%
#4	0.64%	0.56%	0.65%	0.71%	0.71%
#5	0.82%	0.87%	0.73%	0.71%	0.84%

Also, averages of the intensity increases indices for weeks #2-6 were shown in fig. 5. As the time passed from the grafting date, the average of the intensity increase indices decreased (except week #5) and in week #2 it was at its maximum of 0.92%.

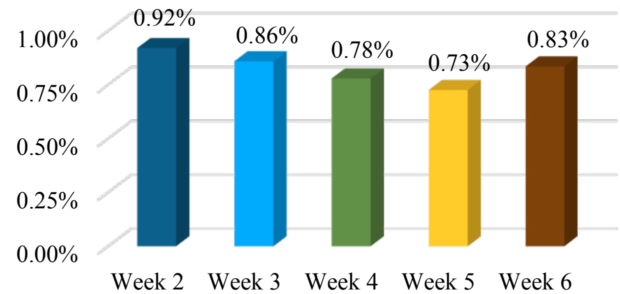

Fig. 5. Average of intensity increase indices for week #2-6.

The intensity increase indices in the images of all leaves acquired between week #2 and week #6 were shown in fig. 6.

Leaves on plant #1 illustrated higher percentages of intensity increase comparing to other plants (fig. 6). The weekly intensity increase percentages presented in Table 2 also shows that the maximum changes happened in weeks #2, but the percentages of the average of intensity change for plant #1 decrease gradually in the following weeks. It can be inferred that there is a higher chance that plant #1 got a severe HLB infection. Plant #5 had three leaves with overall intensity increase indices bigger than 5% (fig. 6). The image analysis results showed a higher infection probability for plant #5 comparing to plants # 2, 3 and 4, although, all four grafting in plant #5 were unsuccessful. The average of intensity increase percentages (all weeks) for plant #5 was 0.82% (Table 2) which was more than the same values for plants #2, 3 and 4; however, in plants #2, 3, and 4 there was, at least, one successful grafting. The leaf samples should be examined by a polymerase chain reaction (PCR) test to make an accurate judgment about their infection status; however, it can be inferred that the results of the subjective inspection for grafting successfulness might not provide enough information to judge about bacteria transmission to the grafted plants.

3. CONCLUSION

In this study, the hyper-accumulation of starch as an early indication of HLB disease on citrus leaves was followed by a time-lapse polarized imaging technique. Five citrus plants were graft-inoculated with HLB-infected source tissues. A previously developed vision based sensor was modified to acquire polarized images of citrus leaves on a weekly basis. The time-lapse images were first calibrated and registered; then their gray values were compared with each other in an area-based manner. The appearance of starch accumulation symptoms on several citrus leaves can be considered as a success sign of graft-inoculation technique. The results also showed that the HLB symptomatic areas with an elevated level of starch on the citrus leaves might be detected using this method as soon as one week after grafting. Further starch measurement and/or PCR test need to be conducted to confirm the infection level in each citrus plant.

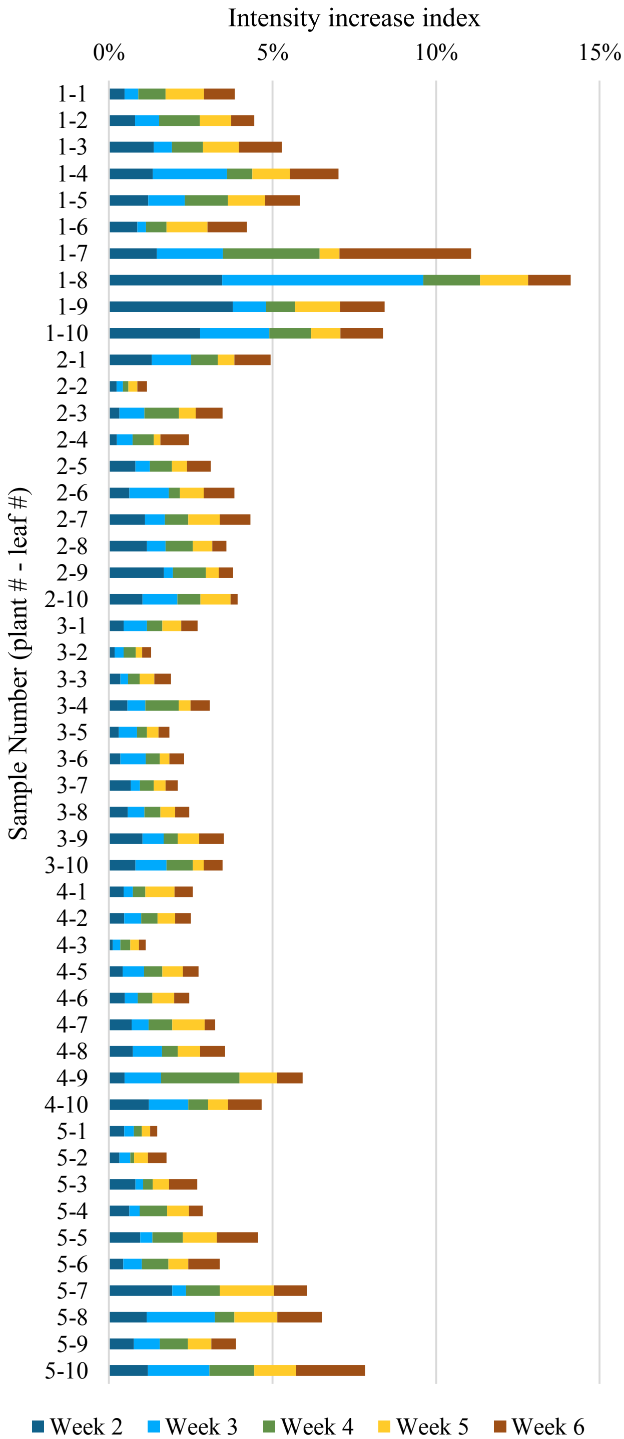

Fig. 6. Accumulative intensity increase indices (percentages) for all samples in each week. The first digit in the sample numbers (in the vertical axis) indicates the plant number and the second digit indicates the leaf number. Leaf number 1-4 in each plant were grafted and leaf number 5-10 were randomly selected for image acquisition.

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