Starch Content of Citrus Leaves Permits Diagnosis of Huanglongbing in the Warm Season but Not Cool Season

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Abstract. One of the most prominent characteristics of Huanglongbing (HLB or citrus greening)-affected citrus trees is the abundant starch accumulation in photosynthetic cells and all other remaining parenchyma cells of aerial parts. Under natural conditions, citrus leaves store very low levels of starch and detectable amounts are only seen as a result of zinc deficiency or accidental girdling of branches. Therefore, leaf starch concentrations over a threshold level should indicate the presence of HLB. In this report, we detailed a comprehensive statistical analysis of starch levels in citrus leaves and compared them with real-time polymerase chain reaction (PCR) detection of the presumptive causal agent Candidatus Liberibacter asiaticus. Starch content was found to reliably predict the PCR results (the proxy for HLB presence) during the “warm season” (June through November) but not in the “cool season” (December through May). During the cool season, starch levels for HLB-positive trees tend to be lower, and 43% of samples were incorrectly classified using Linear Discriminant Analysis (LDA). In contrast, during the warm season, only 8% were misclassified. Furthermore, assuming PCR possibly has error, the total probability of misclassification for HLB status could be controlled using an “uncertain” classification. The temporal pattern of leaf starch is consistent with our understanding of seasonal changes in plant development and bacterial titer.

Citrus huanglongbing (or citrus greening) is a highly destructive, fast-spreading disease of citrus worldwide. Its presumed pathogenic agent, Candidatus Liberibacter spp., is a fastidious Gram-negative, obligate parasite, phloem-limited α-proteobacterium (Garnier et al., 1987; Jagoueix et al., 1994) not yet cultured to purity, although recent attempts have resulted in partial or mixed cultures of the organism (Davis et al., 2008; Parker et al., 2014; Sechler et al., 2009). Of the several species identified worldwide (Kim et al., 2009), Candidatus Liberibacter asiaticus (CLas) is the only species found in Florida thus far (Albrecht and Bowman, 2009). CLas is vectored by the phloem feeding psyllid Diaphorina citri (Halbert and Manjunath, 2004) and transmitted into the phloem stream of citrus leaves during the feeding process.

In affected citrus trees, specific HLB symptoms do not exist. Although some symptoms such as yellow shoots, leaf blotchy mottle, and lopsided fruits with color inver- sion and aborted seeds are quite typical, they do not always occur together in the same tree. Furthermore, these symptoms can be distorted or masked by other diseases or induced by conditions other than HLB such as zinc deficiency (Bové, 2006). Another notable characteristic of CLas-infected citrus trees is the massive accumulation of starch in photosynthetic cells and other parenchymatous tissues of non-reproductive aerial parts (Etxeberria et al., 2009; Folimonova and Achor, 2010; Schneider, 1968). In fact, the notorious accumulation of starch in chloroplasts contributes to the discoloration of chlorophyllous tissue (Schaffer et al., 1986) and to the appearance of blotchy mottle (Achor et al., 2010; Etxeberria et al., 2009) as well as to the corky texture of symptomatic leaves.

Under natural conditions or in presence of other diseases, citrus leaves store very low levels of starch (Goldschmidt and Koch, 1996), and detectable amounts are only seen as a result of zinc deficiency or accidental girdling of branches (Gonzalez et al., 2011). Concurrent with starch accumulation in aerial parts, the depletion of carbohydrate reserves from the root system not only reflects a general disturbance in carbohydrate metabolism, but is also believed to be a main reason for HLB-associated tree senescence (Achor et al., 2010; Etxeberria et al., 2009).

The elevated levels of leaf starch resulting from CLas infection have been associated with HLB symptoms in citrus trees (Etxeberria et al., 2009; Onuki et al., 2002; Tabas et al., 2006; Takushi et al., 2007). These tests are based on the binding of iodine to starch, resulting in a blue/purple-colored solution (McGrane et al., 1998), and the results could be used as the foundation for a quantitative, statistically-based system for HLB detection. The suitability of a starch-based test is contingent on being able to accurately classify HLB-positive and -negative trees as measured by agreement with PCR analysis, the industry standard test (Li et al., 2008; Teixeira et al., 2005). The goal of this research was to determine whether tests of starch can provide an inexpensive, rapid alternative to PCR as an HLB predictor.

Materials and Methods

Plant material. Leaf samples were collected randomly throughout the state by two different personnel groups. HLB-symptomatic leaves from 714 sweet orange trees were gathered by commercial scouts and processed at the Florida Extension Huanglongbing Diagnostic Laboratory at the University of Florida’s Southwest Florida Research and Education Center (SWFREC) in Immokalee, FL. These samples, consisting of three to five leaves, were specifically selected for having evident HLB-related symptoms. A second group of leaf samples was collected randomly from 479 seemingly healthy trees throughout the state. Care was taken to collect leaves devoid of symptoms of any kind or having physical or insect damage. In both cases, time of year and citrus growing region were recorded.

Starch analysis. From each leaf, a 27.3-mm2 leaf disc was obtained using a paper hole puncher. Each disc was placed in a 2-ml capped tube with four metal beads (2.36 mm diameter) (Mobio Laboratories, CA) and 0.5 ml H2O. Homogenization was carried out in two 40-s cycles for a total of 80 s using a Precells 24 Tissue Homogenizer (Bertin Technologies, France). The homogenate

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volume was brought up to 1 mL with water, boiled for 10 min, and allowed to cool before addition of 25 μL of 2% iodine. The resulting colored solution was allowed to stabilize for 20 min and O.D. determined at 595 nm in a BioRad microplate reader Model 680. Starch content (μg·mm−2 leaf surface area) was estimated from a standard curve using rice starch (S-7260; Sigma, St. Louis, MO).

PCR analysis. Total genomic DNA was extracted from 100 mg of petiole tissue using the Promega Wizard® 96 DNA Plant isolation kit (Promega, Madison, WI). Tissues were lyophilized before bead beating using a Mini-bead beater (Bio Spec Products Inc., Bartsville, OK) to a fine powder. Samples were processed as per manufacturer’s instruction, and DNA was eluted in 50 μL AE buffer and stored at −20 °C.

Primers and Taqman probes were obtained based on Li et al. (2006) for CLas (HLB/HLB and HLBp) and for an internal control, cytochrome oxidase, COX gene [COXf/COXr and COX-p; Li et al. (2006)]. The internal probe COX-p was labeled with 6-carboxy-4’, 5’-dichloro-2’, 7’-dimethoxysulforesence reporter dye at the 5’-terminal nucleotide and with BHQ-2 at the 3’-terminal nucleotide. Controls were as follows: DNA from HLB-positive citrus trees located in the SWFREC grove and DNA from known HLB-negative citrus trees grown under greenhouse conditions and tested annually as negative for the HLB pathogen (SWFREC).

Real-time PCR reactions were performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using TaqMan® Fast Advance PCR Master Mix (Applied Biosystems) in a 20 μL reaction. The standard amplification protocol was initial denaturation at 95 °C followed by 40 cycles of reactions (95 °C for 3 s, 60 °C for 30 s). Data were analyzed using Applied Biosystems 7500 system SDS software Version 1.2. For the purpose of analysis, Ct values greater than 36 were considered negative and samples with Ct values less than or equal to 32 were considered positive for HLB. Any sample with a Ct value between 32 and 36 was put in the category of “resample” for the purposes of this study based on the recommendations to growers using the Florida Extension HLB Diagnostic Laboratory at SWFREC. The recommendation to growers with samples generating these values is to resample the tree for a second analysis because growers were basing tree removal on PCR-positive results. The second analysis or “opinion” was recommended because tissue having these values would be asymptomatic and contain 300 to 30 bacteria per reaction (data not published).

Data processing. The data were obtained from two sources: the samples brought to the SWFREC diagnostic laboratory for testing by commercial scouting organizations and the samples randomly collected from seemingly healthy trees. The date of sample collection was available for all samples. Although the samples from the healthy trees had the county of data collection associated with them, only the region (North, Central, Southeast, and Southwest) of collection was available for samples collected by commercial scouting organizations and brought to SWFREC.

Each tree under inspection had between three and eight leaves analyzed for starch content, but PCR analyses were performed on all leaf samples from a single tree combined to obtain a single PCR result for that tree. Consequently, only the maximum starch value observed for each tree was used in the analysis because even one HLB positive leaf would make the PCR result positive. Data from trees with missing values for starch content were excluded resulting in a final data set composed of 1106 observations.

Starch content in healthy citrus leaves is highest during the cooler months and declines during times of rapid vegetative and reproductive growth taking place during spring and summer months (Monerri et al., 2011; personal unpublished data). Thus, we identify samples collected during December through May as “cool-season” samples and those collected during June throughNovember as “warm-season” samples. There were 489 warm-season samples and 617 cool-season samples.

So that the assumption of normality was more nearly met, all starch values were transformed using $y = \log(x)$, where $x$ is the observed starch value, $y$ is the corresponding transformed response, and $\log$ is the natural logarithm. The transformed starch values are used in all statistical analyses, although the term “starch” is used in the discussion of the methods.

To assess the potential effect of season and PCR on starch, a two-factor factorial linear model was fit to the starch values:

$$y_{ijk} = \mu + \beta_i + \gamma_j + (\beta\gamma)_{ij} + \epsilon_{ijk}$$

where $y_{ijk}$ is the (log) starch value from the $i$th tree with the $j$th PCR status ($j = 1$ for negative and $j = 2$ for positive) in the $i$th season ($i = 1$ for warm and $i = 2$ for cool), $\mu$ is the overall mean (log) starch value, $\beta_i$ is the $i$th season effect, $\gamma_j$ is the $j$th PCR status effect, $(\beta\gamma)_{ij}$ is the interaction between the $i$th season and the $j$th PCR status, and $\epsilon_{ijk}$ denote the mean 0 and variance $\sigma^2$ of the cumulative distribution function of the standard normal distribution and $\Phi(\cdot)$, where $\Phi(\cdot)$ is the cumulative distribution function of the standard normal distribution and $\mu_1$ and $\sigma_1$ are, respectively, the estimated mean and SD of the HLB-positive starch values. Similarly, the specificity of the test, the probability of correctly classifying an HLB-positive sample, is estimated by $\Phi(c - \mu_1)/\sigma_1$, where $\Phi(\cdot)$ is the cumulative distribution function of the standard normal distribution and $\mu_1$ and $\sigma_1$ denote the mean and SD of HLB-positive trees.

The ROC curve can be created for each season. Suppose $c \in (−\infty, \infty)$ is the cutoff value in the test. The sensitivity of the test, the probability of correctly classifying an HLB-positive sample, is estimated by $\Phi\left((c - \mu_2)/\sigma_2\right)$, where $\Phi(\cdot)$ is the cumulative distribution function of the standard normal distribution and $\mu_2$ and $\sigma_2$ denote the estimated mean and SD of the HLB-negative starch values.

The area under a ROC curve (AUC) generally ranges between 0.5 and 1 with larger values indicating greater predictive ability. In biological terms, the ROC curves illustrate our ability to classify samples as either unaffected...
PCR status was found to be highly significant in the model, the interaction between season and variety was in evidence. Only sweet orange samples were used in this study. These included mostly ‘Valencia’ and ‘Hamlin’ oranges, but ‘Pineapple’, ‘Navels’, and ‘Temple’ (a tangor) varieties were included as well. From the two-factor linear model, the interaction between season and PCR status was found to be highly significant ($F_{1,1102} = 69.76, P < 0.001$). This is consistent with the assumption that the difference in starch for the positive and negative samples changes with season. Therefore, separate analyses were conducted for the warm and cool seasons.

The real-time PCR results were represented as a Ct value for each sample. The cutoff value (Ct value 32 or less) for a positive sample was determined from work done by Li et al. (2006) and further substantiated by Turechek et al. (2009). Turechek and colleagues demonstrated that the Li primers can produce greater sensitivity without compromising specificity if a Ct value cutoff of 36 was applied for confirmation of an HLB-infected sample using real-time PCR. However, to increase the stringency of the existing detection method, a Ct value of 32 is used as the cutoff for positive samples and values above 32 but less than or equal to 36 are deemed for the purposes of management recommendations (tree removal) to be inconclusive. Samples above a Ct value of 36 are deemed negative. Real-time PCR analysis for HLB classified 631 samples as positive, 66 as negative, and 17 as resample (uncertain) for the first set of 714 sweet orange tree samples obtained from the HLB diagnostic facilities and 67 samples as positive, 384 as negative, and 28 as resample (uncertain) for the second set of 479 visually healthy tree samples throughout the state. Figure 1 shows the classification of samples according to their starch content and their PCR analysis during the two time periods.

Statistical analyses. The log-transformed values were closer to normality than the observed values. Therefore, subsequent analyses were conducted based on the transformed values and the assumption of normality.

The estimated parameters for the distributions of HLB-negative and HLB-positive as well as the estimated proportions $P$ of HLB-negative samples in the warm- and cool-season data sets are displayed in Table 1. Because our data were collected from the trees that were symptomatic and asymptomatic for HLB separately and not through random sampling, these proportions are not estimates of the incidence or prevalence of HLB. For the warm-season data, the estimate of the proportion of HLB-negative responses $p$ is $p = 0.66$, whereas it is $p = 0.49$ for the cool-season data. Again, these proportions are not expected to be the same because the data were not collected to have the same proportion of HLB-positive and -negative trees in each of the seasons. The ROCs (Fig. 2) illustrate the diagnostic performance of this classifier for the warm and cool seasons. The AUCs corresponding to the ROCs given in Figure 2 provide a basis of comparison of our ability to classify leaves as HLB-positive or HLB-negative. In the warm season, the AUC is 0.97, indicating high predictive ability. However, the AUC of 0.6 in the cool season is little better than the 0.5, associated with a random choice between HLB-positive and HLB-negative for each sample.

The estimated parameters were used to find the zone of uncertainty with a specified error rate of 0.05 (Fig. 3). That is, the cutoff values were calculated so that only 5% of samples would be incorrectly classified. For the warm season, the starch values for the zone of uncertainty were 1.54 and 2.53 on the log scale or 4.66 and 12.55 on the scale of measurement; for the cool season, the starch values for the zone of uncertainty were 1.52 and 3.04 on the log scale or 4.57 and 20.91 on the scale of measurement. Based on the estimates of misclassification based on the MLEs of the distributions of HLB-positive and HLB-negative, the probabilities of classification for warm- and cool-season data based on a LDA classifier (Johnson and Wichern, 2007) are shown in Table 2. Allowing for only a 5% misclassification rate resulted in an estimated 85% of the samples being classified as inconclusive during the cool season, but only 7% of the warm-season data are classified as uncertain during the warm season.

Discussion

High starch content in citrus leaves has been regularly used as a provisional indication of HLB presence in citrus trees. Visual comparisons of leaf starch between leaves from HLB-affected and HLB-unaffected trees made with a 2% iodine solution present a clear contrast between these two perceived

![Figure 1](image-url)  
**Fig. 1.** The polymerase chain reaction (PCR)-based classification—positive or negative—and log starch values of the 1106 samples separately for the months of June through November (warm season, **A**) and December through May (cool season, **B**).
Table 1. The natural log of starch content (log starch) from 1106 trees [including healthy and huanglongbing (HLB)-symptomatic trees] was used for classifying trees as being HLB-positive or HLB-negative. a

<table>
<thead>
<tr>
<th>Season</th>
<th>μ1</th>
<th>σ1²</th>
<th>p</th>
<th>LCL (μ1)</th>
<th>UCL (μ1)</th>
<th>μ2</th>
<th>σ2²</th>
<th>LCL (μ2)</th>
<th>UCL (μ2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warm</td>
<td>2.37</td>
<td>0.49</td>
<td>0.49</td>
<td>2.29</td>
<td>2.45</td>
<td>2.61</td>
<td>0.44</td>
<td>2.54</td>
<td>2.68</td>
</tr>
<tr>
<td>Cool</td>
<td>1.97</td>
<td>0.43</td>
<td>0.66</td>
<td>1.90</td>
<td>2.05</td>
<td>3.53</td>
<td>0.21</td>
<td>3.46</td>
<td>3.60</td>
</tr>
</tbody>
</table>

Classification was determined separately for the months of June through November (warm season) and December through May (cool season) and did not use polymerase chain reaction as a gold standard. For the warm season data (Table 1), the AUC estimates of the means of the positive and negative groups are clearly more separated than for the cool season data (Table 2), allowing for a zone of uncertainty. The increased predictability of starch for HLB detection is higher in the warm season than in the cool season as evidenced by the AUC of 0.97 in the warm season and 0.6 in the cool season (Fig. 2), and the zone of uncertainty is considerably smaller in the warm season than in the cool season (Fig. 3). Furthermore, the estimates of the means of the positive and negative samples are clearly more separated for the warm season than for the cool season (Table 1).

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Starch content cannot reliably predict the PCR results during the cool season. Using the LDA classifier, 43% were misclassified (Table 2), allowing for a zone of uncertainty resulted in nearly 85% of the samples falling in this zone, and the AUC of 0.6 indicates low predictive ability of starch (Fig. 2) during the cool season. The increased predictability using starch values during the warm season and other biotic and abiotic factors also affect leaf starch content during the course of HLB/citrus tree association, therefore adding a degree of uncertainty. Concurrently, PCR, the official test for HLB in Florida, has proven inconsistent at times as a result of several factors outlined by Gottwald (2010). Furthermore, in real-time PCR, the Ct value represents the point at which fluorescence passes a given threshold, a level set above the baseline or background fluorescence and within the exponential growth of the amplification curve. Lower Ct values correspond to higher levels of the PCR amplified template, and a one-cycle difference in Ct value represents a 2-fold increase in starting template (assuming that the efficiency of the amplification is 100%); hence, Ct values are logarithmic in nature. In the detection method used, no standard curve was generated within each plate or generated at any stage; and thus, absolute or relative quantification of the bacteria (gene of interest) per sample could not be achieved.

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Table 2. The natural log of starch content (log starch) from 1106 trees [including healthy and Huanglongbing (HLB)-symptomatic trees] was used for classifying trees as being HLB-positive or HLB-negative.∗

<table>
<thead>
<tr>
<th></th>
<th>Warm season</th>
<th></th>
<th>Cool season</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>True-negative</td>
<td>True-positive</td>
<td>True-negative</td>
<td>True-positive</td>
</tr>
<tr>
<td>Predicted negative</td>
<td>0.61</td>
<td>0.03</td>
<td>0.26</td>
<td>0.20</td>
</tr>
<tr>
<td>Predicted positive</td>
<td>0.05</td>
<td>0.31</td>
<td>0.23</td>
<td>0.31</td>
</tr>
</tbody>
</table>

∗Classification was determined separately for the months of June through November (warm season) and December through May (cool season) and did not use polymerase chain reaction (PCR) as an accepted authoritative standard. The expectation-maximization algorithm was used to calculate maximum likelihood estimates of linear discriminant analysis (LDA) classification probabilities for the warm- and cool-season data without PCR as an accepted authoritative standard. The misclassification probabilities are larger for the cool season (0.23 + 0.20) than for the warm season (0.05 + 0.03).

is likely influenced by the lower natural starch levels at this time of the year (Monerri et al., 2011). Lower natural starch content decreases the background values, thus improving statistical validity.

A detailed analysis of the biotic conditions surrounding HLB in terms of bacteria life cycle and the plant development reveals a rational explanation for the two types of potential misclassifications when using starch to detect HLB: false-negatives (low starch but PCR-positive) and false-positives (high starch but PCR-negative). Given the widespread presence of HLB and the prolonged latency period between infection and symptomatology (Gottwald, 2010), it is evident that a great number of seemingly healthy (asymptomatic) leaves are already infected with CLas. Although these leaves may in fact give a positive signal in the PCR test, starch levels still remain below the threshold levels, thus leading to false-negative results in a starch-based test when the results are compared with real-time PCR results.

Central to the second type of misclassification (false-positive, i.e., high starch but PCR-negative) is the bacteria’s life cycle and the anatomical changes occurring in the leaf as a consequence of CLas infection. As noted by Etxeberria et al. (2009), Folimonova and Achor (2010), and Schneider (1968), visible symptoms of starch accumulation (indicating high starch content) only arise after phloem plugging. Furthermore, during the process of symptom development, CLas is not evenly distributed within the vascular system (Gottwald, 2010) and its titer levels fluctuate throughout the year (Irey, 2008).

At some point, this situation would result in CLas-infected leaves with high levels of starch but no PCR-positive signal. Furthermore, plugging of phloem tissue along the stem (Etxeberria et al., 2009) also results in the accumulation of starch in leaves acropetally from the initial CLas-infected leaf likely resulting in symptomatic leaves without DNA signal above the initial infection. This would be identified as a false-positive in a starch-based test with PCR as an accepted authoritative standard.

Although the rationale for not considering PCR as an accepted authoritative standard is strong, it should be noted that for an analysis assuming PCR is an accepted authoritative standard, the AUC during the warm season would be 0.9, again indicating good predictive ability. However, the probability of having an inconclusive result would increase to 0.36. The predictive ability during the cool season would be higher but still low (AUC = 0.72), and the probability of an inconclusive result would remain high at 0.69.

The data analysis presented in this communication supports the development of a simple, inexpensive, and highly accurate starch test for HLB detection based on starch content during the warm season (June through November) but not in the cool season (December through May). Although the misclassification rates are controlled, the percentage of samples that was classified to the uncertainty region during the cool season is too high to be applied in practice, and the predictive ability is too low.


