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 pathological 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 (*C*Las) is the only species found in Florida thus far (Albrecht and Bowman, 2009). *C*Las 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 inversion 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 HLB-affected 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 the 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 *C*Las infection have been associated with HLB symptoms in citrus trees (Etxeberria et al., 2009; Onuki et al., 2002; Taba 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-mm^2 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 H_2O. Homogenization was carried out in two 40-s cycles for a total of 80 s using a Precellys 24 Tissue Homogenizer (Bertin Technologies, France). The homogenate

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., Bartlesville, 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 *C*Las (HLBas/HLBr 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′-dimethoxyfluorescein 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 screenhouse 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.

Statistical analysis. All analyses were conducted in R 3.0.1 (R Development Core Team, 2013).

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 through November 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:

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

where y_{ijk} is the (log) starch value from the *k*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), µ is the overall mean (log) starch value, β_i is the *i*th season effect, γ_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 ε_{ijk} are independent and identically normally distributed with mean 0 and variance σ^2 [$N(0,\sigma^2)$].

Tests classifying trees as HLB-positive or HLB-negative are generally characterized by their sensitivity and specificity. A test's sensitivity is the (conditional) probability of classifying a sample that is from an HLB-infected tree as being HLB-positive, and the specificity is the (conditional) probability of classifying a sample from an HLB-free tree as being HLB-negative. The receiver operating characteristic (ROC) curve represents the tradeoff between correctly classifying samples that are positive for HLB and those that are negative for HLB. Specifically, an ROC curve is defined by the graph of sensitivity vs. (1 – specificity) over all possible values of the threshold parameter, the level of starch used to separate HLB-positive from HLB-negative samples. Although the use of ROC curves is traditionally associated with analyses involving a gold standard, a growing body of research uses ROC curves in the absence of a gold standard (Beiden et al., 2000; Choi et al., 2006). Our frequentist analytic approach has been informed by the work of Choi et al. (2006) who considered Bayesian inferences for ROC curves with and without a gold standard.

As noted earlier, in HLB-affected trees, starch accumulates for some time before a tree tests positive using PCR. Thus, the distribution of starch for HLB-negative trees partially overlaps the distribution of starch for HLB-positive trees. This partial overlap is reflected in the standard set of Ct values for which HLB status is inconclusive (between 32 and 36) when using PCR (Turechek et al., 2009).

Just as it takes time for starch to accumulate in the leaves, the likelihood of selecting a leaf that will test positive for *C*Las when the tree is HLB-positive increases as the titer of *C*Las increases. Therefore, PCR classification of HLB-positive and -negative trees has some error that is not fully accounted for by the category when Ct values are between 32 and 36, an inherent degree of uncertainty common to all diagnostic tests (Turechek et al., 2009). Thus, although PCR is considered to be a standard here, it is assumed to have some error and is thus not an accepted authoritative standard.

The distributions of starch values from HLB-positive and HLB-negative trees are each considered to be normally distributed. Let μ_1 and σ_1 denote the mean and SD of HLB-negative trees and μ_2 and σ_2 denote the mean and SD of HLB-positive trees. The observed starch values are then a random sample of observations from a mixture of the normal distributions of HLB-positive and HLB-negative starch values. The proportions of HLB-negative and HLB-positive trees are then p and ($1 - p$), respectively.

An ROC curve can be created for each season. Suppose $c \in (-\infty, \infty)$ is the cutoff value used in the test. The sensitivity of the test, the probability of correctly classifying an HLB-positive sample, is estimated by $(1 - \Phi((c - \hat{\mu}_1)/\hat{\sigma}_1))$, where Φ is the cumulative distribution function of the standard normal distribution and $\hat{\mu}_1$ and $\hat{\sigma}_1$ 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 $\Phi((c - \hat{\mu}_2)/\hat{\sigma}_2)$ where $\hat{\mu}_2$ and are $\hat{\sigma}_2$ the estimated mean and SDs, respectively, of the HLB-negative starch values. To produce the ROC curve, pairs of (1 – specificity) and sensitivity are plotted, that is,

$$(1 - \Phi((c - \hat{\mu}_2)/\hat{\sigma}_2), 1 - \Phi((c - \hat{\mu}_1)/\hat{\sigma}_1)),$$

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 uninfected

or HLB-infected. Perfect classification corresponds to an AUC of 1.0, whereas an AUC of 0.5 corresponds to randomly guessing. The AUC will allow comparisons between classification schemes based on identified cutoffs.

The EM algorithm (Robert and Casella, 2004) is used to find the maximum likelihood estimates (MLEs) of the means and SDs of the two normal distributions as well as the mixing proportions for the warm-season and the cool-season data.

Instead of classifying all samples as HLB-positive or HLB-negative, the focus turns to establishing a zone of indecision lying between the classification zones for leaves from HLB-positive and HLB-negative trees. That is, when starch levels are low, the tree would be classified as HLB-negative. As the starch increases, the classification is inconclusive; and as it increases still more, the classification becomes HLB-positive. The goal is to determine a cutoff value c that minimizes the Total Probability of Misclassification (TPM) (Johnson and Wichern, 2007). A TPM curve plots the estimated TPM values vs. c. Specifically, the TPM curve estimate at a cutoff point c is given by

$$\widehat{TPM}(c) = \hat{p}\left[1 - \Phi\left(\frac{c - \hat{\mu}_1}{\hat{\sigma}_1}\right)\right] + (1 - \hat{p})\Phi\left(\frac{c - \hat{\mu}_2}{\hat{\sigma}_2}\right),$$

where $\hat{p}$ is the estimated proportion of HLB-negative trees. The zone of uncertainty is that region of the overlap in the distributions of HLB-negative and HLB-positive trees that minimizes the distance between the two thresholds while constraining the TPM to be less than a prespecified value. This process was implemented in R using the R function uniroot.

Results

Sample selection and categorization. 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 Turecek et al. (2009). Turecek 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 $\hat{p} = 0.66$, whereas it is $\hat{p} = 0.49$ 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
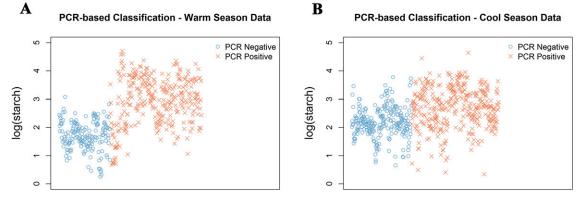

Fig. 1. The polymerase chain reaction (PCR)-based classification—positive or negative—and log starch values of the 1106 samples separately for the months of June through November (warm season, **A**) and December through May (cool season, **B**).

circumstances (Etxeberria et al., 2007). However, although our initial observations reflect an unmistakable accumulation of starch in HLB-affected trees (Achor et al., 2010; Etxeberria et al., 2007, 2009; Folimonova and Achor, 2010; Schneider, 1968), developmental 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 interested) per sample could not be achieved.

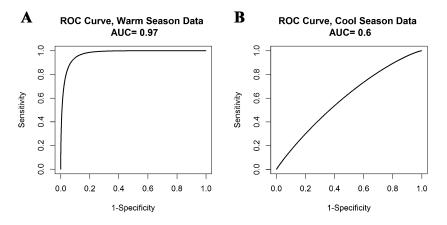Table 1. The natural log of starch content (log-starch) from 1106 trees [including healthy and huanglongbing (HLB)-symptomatic trees] was used for classifying trees as being HLB-positive or HLB-negative.[z]

	μ_1	σ_1^2	p	LCL (μ_1)	UCL (μ_1)	μ_2	σ_2^2	LCL (μ_2)	UCL (μ_2)
Warm season	1.97	0.43	0.66	1.90	2.05	3.53	0.21	3.46	3.60
Cool season	2.37	0.49	0.49	2.29	2.45	2.61	0.44	2.54	2.68

[z]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 and cool seasons, the expectation-maximization algorithm was used to calculate maximum likelihood estimates (MLEs) of the following parameters are given: means of the log starch values for negative and positive samples (μ_1 and μ_2, respectively), variances of the log starch values for negative and positive samples (σ_1^2 and σ_2^2, respectively), and the proportion p of HLB-negative trees in the sample. MLE-based 95% lower (LCL) and upper (UCL) confidence limits for μ_1 and μ_2 are also presented.

Fig. 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. Classification was determined separately for the months of June through November (warm season, **A**) and December through May (cool season, **B**) and did not use polymerase chain reaction as a accepted authoritative standard. The areas under (AUC) receiver operating characteristic (ROC) curves are used to compare the diagnostic ability of the starch-based classification system in the warm season to the cool season. An AUC value of 1.0 indicates perfect classification, whereas an AUC value of 0.5 indicates classification based on essentially random guessing. The AUC estimates are 0.97 for the warm season and 0.6 for cool season.

The predictive ability 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 the cool season (Fig. 3). Furthermore, the estimates of the means of the positive and negative groups are clearly more separated for the warm season data than for the cool-season data (Table 1).

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
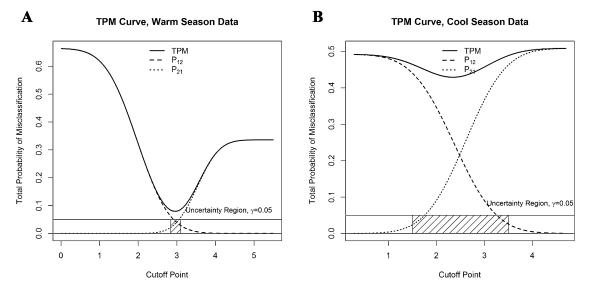

Fig. 3. The natural log of starch content (log starch) from 1106 trees [including healthy and huanglongbing (HLB)-symptomatic trees] was used for classifying trees as being HLB-positive or HLB-negative. Classification was determined separately for the months of June through November (warm season, **A**) and December through May (cool season, **B**) and did not use polymerase chain reaction (PCR) as an accepted authoritative standard. Misclassification rates were controlled by setting the total probability of misclassification (TPM) to 0.05 (5%). As displayed in the TPM curves, the size of the middle region consisting of log starch values for which no classification is provided (indicated by the hatched regions) is calculated to control the TPM.

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.[z]

	Warm season		Cool season	
	True-negative	True-positive	True-negative	True-positive
Predicted negative	0.61	0.03	0.26	0.20
Predicted positive	0.05	0.31	0.23	0.31

[z]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 *C*Las. 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 *C*Las 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, *C*Las 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 *C*Las-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 *C*Las-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.

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