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Starch Content in Citrus Leaves: A Test with Seasonal Accuracy

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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 detail both frequentist and Bayesian statistical approaches to predict HLB using starch levels in citrus leaves. Real-time PCR detection of the presumptive causing agent *Candidatus* Liberibacter asiaticus was used as a proxy for HLB status, but we also present a competing analysis that does not use PCR as a gold standard. 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. Further, assuming PCR possibly has error, the total probability of misclassification for HLB status could be controlled using an "uncertain" classification. Analysis of the biotic conditions surrounding HLB in terms of bacteria life cycle and the plant development provides insights into these patterns.

Citrus huanglongbing (*syn.* HLB 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). There are several species identified worldwide (Kim et al., 2009), but only *Candidatus* Liberibacter asiaticus (*C*Las) is found in Florida (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.

There are no specific symptoms for HLB-affected trees. Although some symptoms such as yellow shoots, leaf blotchy mottle, and lopsided fruits with color inversion and aborted seeds are quite typical, they are not always present 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). A notable characteristic of HLB-affected citrus trees is the massive accumulation of starch in photosynthetic cells and other parenchymatous tissues of nonreproductive aerial parts (Etxeberria et al., 2009; Folimonova and Achor, 2010; Schneider, 1968). Under natural conditions (Goldschmidt and Koch, 1996) or in the presence of other diseases (Gonzalez et al., 2011), 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 (Gonzalez et al., 2011).

The elevated levels of leaf starch resulting from *C*Las infection has been utilized as a general indication of HLB-association with citrus trees (Etxeberria et al., 2007). In fact, due to its simplicity, perceived reliability, rapidity, and cost efficiency, iodine-based starch tests have been used as visual field tests for HLB presence in many other locations (Onuki et al., 2002; Taba et al., 2006; Takushi et al., 2007). These tests are based on the ability of iodine to bind starch, resulting in a blue/purple-colored solution (McGrane et al., 1998), and could be used as the foundation for the development of a quantitative, statistically-based system aimed at HLB detection. Therefore, starch concentrations over a threshold level could indicate the presence of HLB. 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). Based on this assumption, we

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aimed at finding a threshold level of starch content in citrus leaves that would indicate HLB association.

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, Carlsbad, 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 OD determined at 595 in a BioRad micro-plate reader Model 680. Starch content 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 prior to 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 COXp (Li et al., 2006). The internal probe COX-p was labeled with 6carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE) 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 screen-house 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 Ctvalue between 32 and 36 was put in the category of "resample" for the purposes of this study based upon the recommendations to growers using the Florida Extension HLB Diagnostic Lab at SWFREC. The recommendation to growers with samples generating these values is to resample the tree for a second analysis since growers were basing tree removal upon PCR positive results. The second analysis or "opinion" was recommended since tissue having these values would be asymptomatic and contain 300–30 bacteria per reaction (data not published).

Data processing. The data used in this study are comprised of two datasets: 1) the samples brought to SWFREC diagnostic lab for testing by commercial scouting organizations (Immokalee), and 2) samples collected from seemingly healthy trees (controls). The former dataset consists of leaf samples that were assumed to be positive because they were collected from trees exhibiting HLB symptoms, and the latter dataset consists of leaves that are assumed to be negative because they were collected from apparently healthy trees. Because the datasets consist of samples that were collected independently of each other, the combination of the two datasets (the full data used in the study) does not itself constitute a random sample from the population of all citrus trees. Although estimates of incidence and prevalence of HLB from these data are not possible, various classification techniques are available to predict PCR/HLB status of a tree. The number of observations in the controls and Immokalee datasets, by month, is given in Table 1.

For each tree, between three and eight leaves were analyzed for starch content. However, PCR analyses were performed by combining all leaves for each tree to obtain a single PCR result for individual trees. Consequently, only the maximum starch value observed for each tree is used in the analysis, since even one HLB positive leaf would make the PCR result positive.

Because there appeared to be a temporal effect on starch content, we defined a new variable: season. Season takes two values, "warm season" (the months June–November, 6–11) and "cool season" (the months December–May, 12–5). Starch values tended to be higher in the warm season months than the cool season months. Tables 2 and 3 show the number of observations after the creation of the season variable.

Statistical analysis. As established from the histogram in

Table 1. Number of observations from the controls and Immokalee datasets by month (1 = January; 12 = December).

Month	1	2	3	4	5	6	7	8	9	10	11	12
Controls	29	80	20	99	25	59	42	97	0	0	0	0
Immokalee	187	50	58	4	9	0	0	0	0	56	235	56

Table 2. The number of observations from the controls and Immokalee datasets by season.

	Warm season	Cool season
Controls	198	253
Immokalee	291	364

Table 3. The number of observations that are PCR negative and PCR positive by season.

	Warm season	Cool season
PCR negative	175	251
PCR positive	314	366

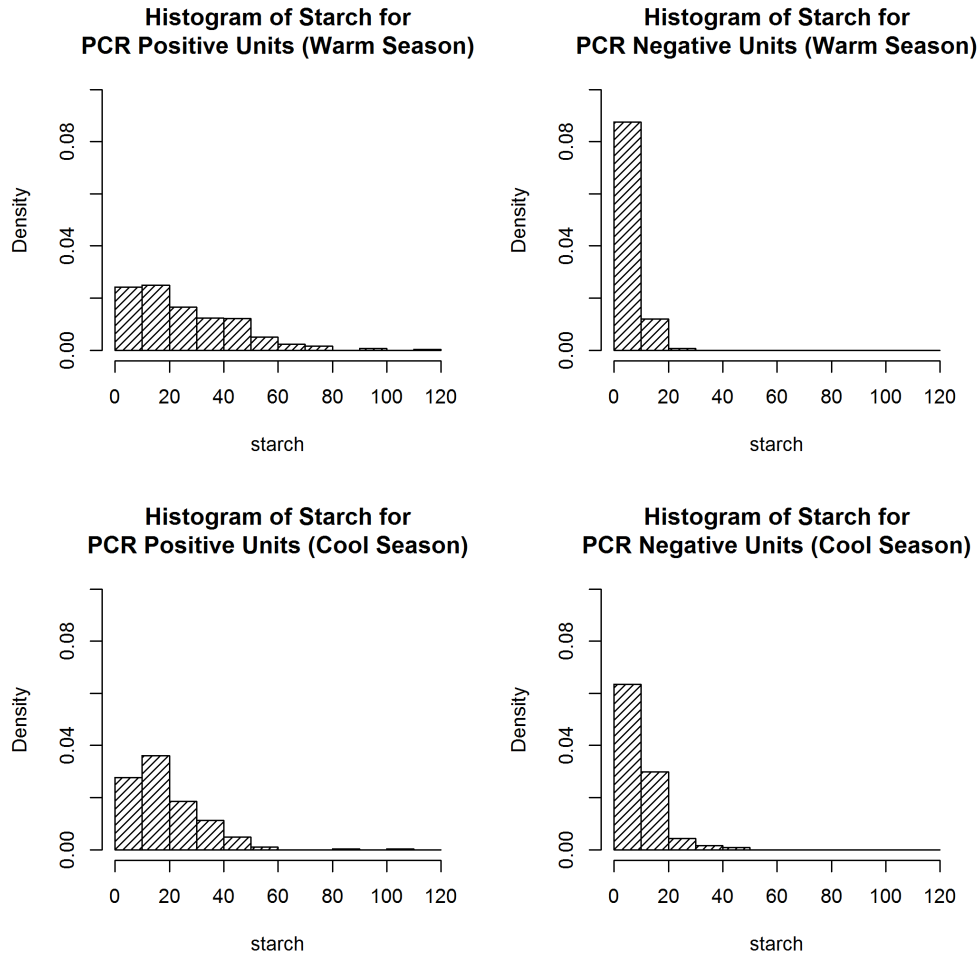

Fig. 1. Histograms of (untransformed) starch values for the PCR × season groups.

Figure 1, the distribution of starch is not normal. The Box-Cox transformation suggests that natural log was the appropriate transformation. A linear model was also fit which gave more evidence for the appropriateness of the natural log transformation. Figure 2 shows the histogram of log-transformed starch values which appears to be normally distributed. Hereafter, all references to starch refer to the logtransformed starch variable.

To achieve our goal of using leaf starch content as a predictor of HLB status, we used three analytic techniques: linear discriminant analysis (LDA), threshold optimization classification (TOC), and receiver operating characteristic (ROC) curves. TOC and ROC curves were performed under four conditions: with (1) and without (2) PCR as a gold standard for HLB, and from a frequentist (3) and Bayesian (4) perspective. The LDA was performed only with PCR as a gold standard for HLB and from the frequentist perspective.

Linear discrimination analysis. LDA is a method for classification that seeks to separate observations into two or more classes by minimizing the total probability of misclassification (TPM). The TPM is the total proportion of observations misclassified. Because we have two observed classes (PCR positive, PCR negative), LDA will only classify observations as being positive or negative. To identify an uncertainty region between the positive and negative regions, a different approach is needed.

Threshold optimization classification. We propose a new method called TOC that will identify an uncertainty region if the TPM is greater than a specified threshold while controlling for TPM. TOC seeks to identify two cutoffs (between the uncertainty and negative regions and between the positive and uncertainty regions, d_1 and d_2, respectively). This is equivalent to minimizing $d_1 - d_2$ subject to TPM = γ, where γ is the chosen threshold for the misclassification rate. In this work, we have

$$\text{TPM} = p \int_{R_2} f_1(x)dx + (1-p) \int_{R_1} f_2(x)dx$$

(where f is a normal density function) because of the normality described above. This equation relies on five unknowns: μ_1, μ_2, σ_1, σ_2, and p (the means of negative and positive samples, the standard deviations of negative and positive samples, and the proportion of negative samples, respectively), but these can be estimated.

Receiver operating characteristic curves. While TPM relies on an estimate of p, ROC curves do not. This is beneficial for our study because the data come from two independent samples, Controls and Immokalee, and not a single random sample that would allow us to estimate the prevalence of HLB. Furthermore, ROC curves can be easily compared using the area under the curve (AUC). AUC typically is between 0.5 and 1.0, with values closer to 1.0 considered desirable. While the use of ROC curves is traditionally associated with analyses involving a gold standard,

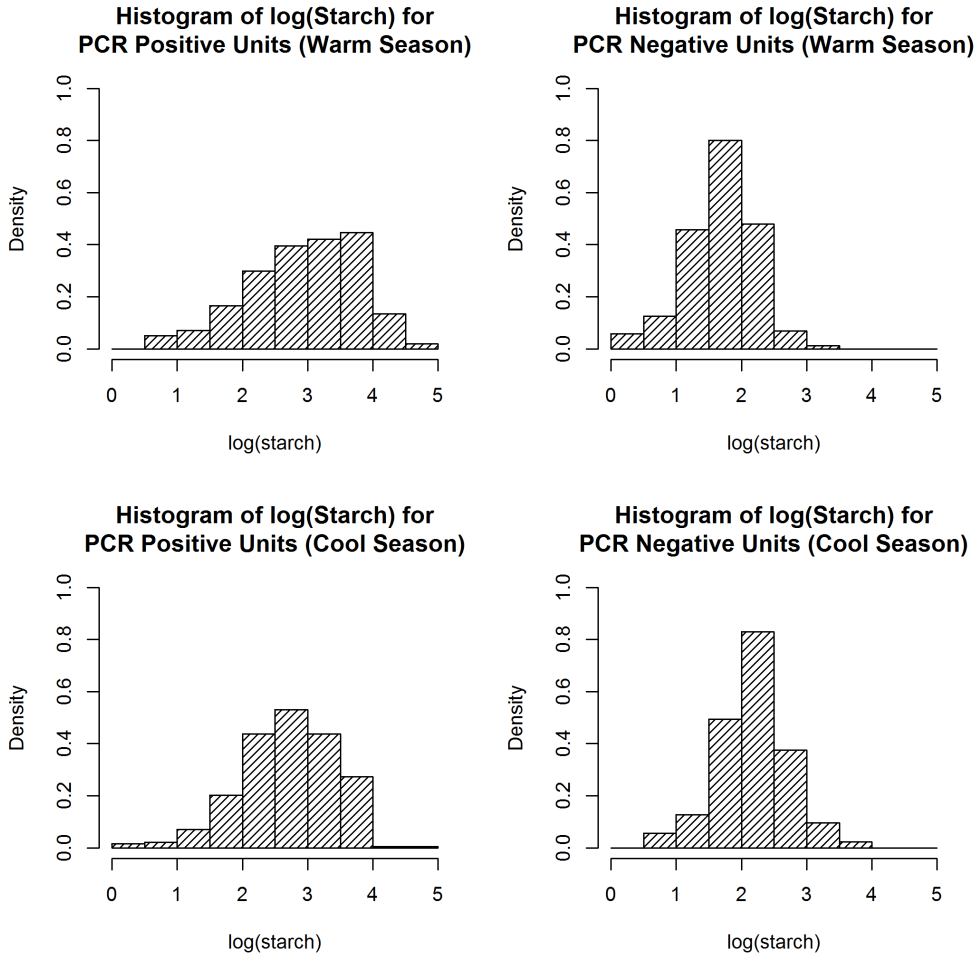

Fig. 2. Histograms of (log-transformed) starch for the PCR × season groups.

a growing body of research employs ROC curves in the absence of a gold standard (Beiden et al., 2000; Choi et al., 2006).

Estimation of parameters. In the methods described above, we need to estimate at most five parameters: μ_1, μ_2, σ_1, σ_2, and p. We estimate these parameters from both a frequentist and a Bayesian perspective, and with and without PCR as a gold standard for HLB. Using PCR as a gold standard is an appropriate assumption because it is presently used as a de facto gold standard for HLB. Viewing PCR as not being a gold standard may be more appropriate based on the current scientific understanding of the disease. All analyses are performed separately for the different values of season (warm season and cool season).

In the frequentist case with PCR as a gold standard, we use the maximum likelihood estimates (MLEs) $\hat{\mu}_1$, $\hat{\mu}_2$, $\hat{\sigma}_1$, $\hat{\sigma}_2$, and calculate $\hat{p}$ from the proportion of PCR negative observations. In the frequentist case without PCR as a gold standard, we view the problem as a mixture of normal distributions and estimate all five parameters using an EM algorithm (Robert and Casella, 2004). In the Bayesian case with PCR as a gold standard, we use the following (non-informative) priors for the means and precision parameters: $\mu_1 \sim N$ (mean = 0; variance = 100), τ_1~inverse Gamma (shape = 0.001, rate = 100), $\mu_2 \sim N$ (mean = 0; variance = 100), and τ_2~ inverse Gamma (shape = 0.001, rate = 100), and calculate $\hat{p}$ from the proportion of PCR negative observations. In the Bayesian case without PCR as a gold standard, we use the priors as before but with a Beta ($shape_1 = 2$, $shape_2 = 2$) prior for the proportion. We present analyses for the frequentist case with PCR as a gold standard and the Bayesian case without PCR as a gold standard, as they are the most different from each other; the other analyses reach the same overall conclusion.

Results

Frequentist approach with PCR as a gold standard for HLB. The MLEs of the parameters for the warm season and cool season data are given in Table 4. The LDA was performed for

Table 4. Maximum likelihood estimates of the mean and variance parameters μ_1, σ_1^2, μ_2, and σ_2^2, the proportion of negative leaves p, and 95% lower (LCL) and upper (UCL) confidence limits for μ_1 and μ_2 for the warm season and cool season data with PCR as a gold standard.

	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	1.70	0.26	0.36	1.62	1.78	2.94	0.70	2.85	3.03
Cool season	2.18	0.28	0.41	2.12	2.25	2.71	0.50	2.63	2.78

Table 5. Leave-one-out cross-validation estimates of the classification probabilities for the warm season and cool season data with PCR as a gold standard.

	Warm season		Cool season	
	True neg	True pos	True neg	True pos
Pred neg	0.27	0.10	0.20	0.13
Pred pos	0.09	0.54	0.21	0.47

each season: warm season and cool season. Leave-one-out cross-validation estimates of classification probabilities are employed using the lda() function in the MASS package (Venables and Ripley, 2010) and are given in Table 5. Specifically, individual leaves are predicted to be either HLB positive or negative based on LDA classification performed on a training data set consisting of all other leaves not being classified. This process is repeated for all leaves in order to obtain the leave-one-out cross-validation estimates of classification probabilities. According to results displayed in Table 5, for the warm season data, the TPM estimate is 0.09 + 0.10 = 0.19, the sensitivity estimate is 0.54/(0.10 + 0.54) = 0.84, and the specificity estimate is 0.27/(0.27 + 0.09) = 0.75. Based on the sensitivity and specificity estimates for the warm season data, note that the LDA classifier better predicts positive leaves than negative leaves. For the cool season data, the TPM estimate is 0.21 + 0.13 = 0.34, the sensitivity estimate is 0.47/(0.13 + 0.47) = 0.78, and the specificity estimate is 0.20/(0.20 + 0.21) = 0.49. Similar to the warm season data, the LDA classifier performs better when predicting HLB positive leaves than HLB negative leaves for the cool season data. Note the substantial decrease in sensitivity and specificity for the cool season data. Figure 3 shows the LDA binary classification of the leaves for the warm season and cool season data using PCR as a gold standard. The ROC curves for the warm season and cool season data are shown in Figure 4, based on the estimates given in Table 4. In this case, the AUC estimate is 0.9 for warm season and 0.72 for cool season.

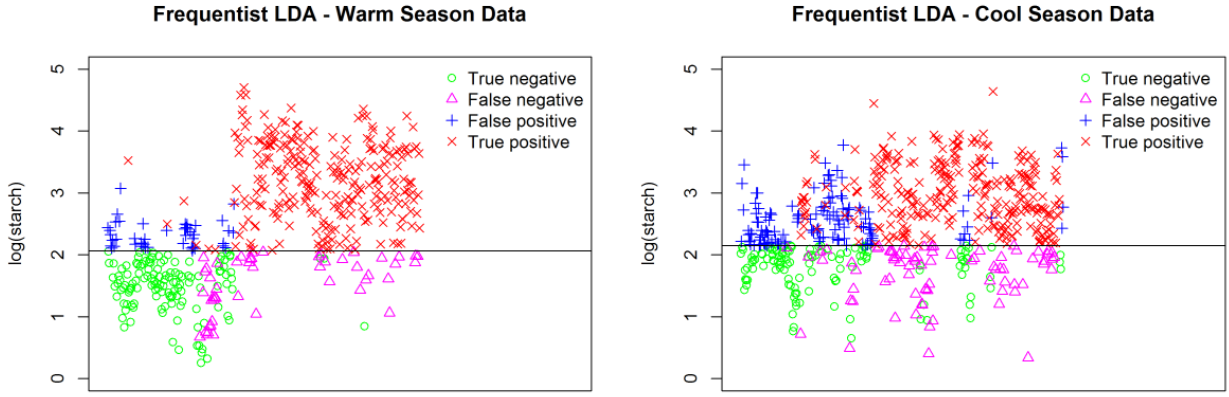

Fig. 3. LDA binary classification of the leaves for warm season and cool season data using PCR as a gold standard. Observations above the line are classified as positive: "true positives" are PCR positive and are represented by a red "x" and "false positives" are PCR negative and are represented by a blue "+." Observations below the line are classified as negative: "true negatives" are PCR negative and are represented by a green "○" and "false negatives" are PCR positive and are represented by a purple "Δ."

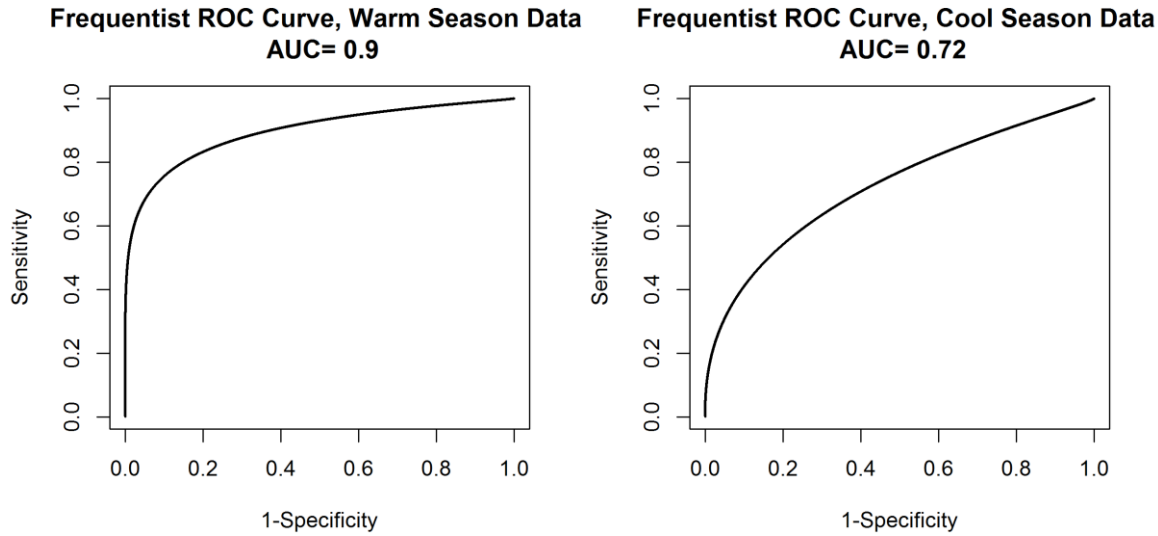

Fig. 4 . Frequentist ROC curves for the warm season and cool season data with PCR as a gold standard. The AUC estimates are 0.9 for warm season and 0.72 for cool season.

Table 6. Classification probability estimates of the frequentist TOC method for the negative, uncertain, and positive groups for the warm season and cool season data with PCR as a gold standard. The TPM estimate for the warm season data is 0.04 + 0.01 = 0.05 and for the cool season data is 0.04 + 0.02 = 0.06.

	Warm season		Cool season	
	Negative	Positive	Negative	Positive
Negative	0.13	0.04	0.04	0.04
Uncertain	0.21	0.15	0.34	0.35
Positive	0.01	0.45	0.02	0.20

The LDA classifier is not able to reliably differentiate between HLB positive and negative leaves based on starch content. This motivates us to use the TOC method for classification in order to control TPM below the nominal level of $\gamma = 0.05$. Classification probability estimates of the TOC method for the negative, uncertain, and positive groups for the warm season and cool season data are given in Table 6. Note that, as expected, the TOC method controls the TPM. Specifically, the TPM estimate for warm season is 0.04 + 0.01 = 0.05 and the TPM estimate for cool season is 0.04 + 0.02 = 0.06. Figure 5 shows the estimated TPM curves displaying the uncertainty regions, and Figure 6 shows the classified leaves based on TOC for the warm season and cool season data.

Bayesian approach without PCR as a gold standard for HLB. Posterior estimates of the parameters were obtained using Markov Chain Monte Carlo (MCMC) techniques. The MCMC simulation is run in JAGS (Plummer, 2003). We also use the R-packages rjags (Plummer, 2013), R2jags (Su and Yajima, 2012), and coda (Plummer et al., 2013) to call the JAGS software from R environment and to process the data from the MCMC samples in R. After removing the first 10,000 (burn-in) samples, we used 10,000 draws from the JAGS MCMC sampler to approximate the Bayes estimates. Posterior estimates are given in Table 7; note that these estimates differ appreciably from those given in Table 4.

Table 8 gives the Bayes estimates of LDA classification probabilities using the estimates given in Table 7. For the warm season

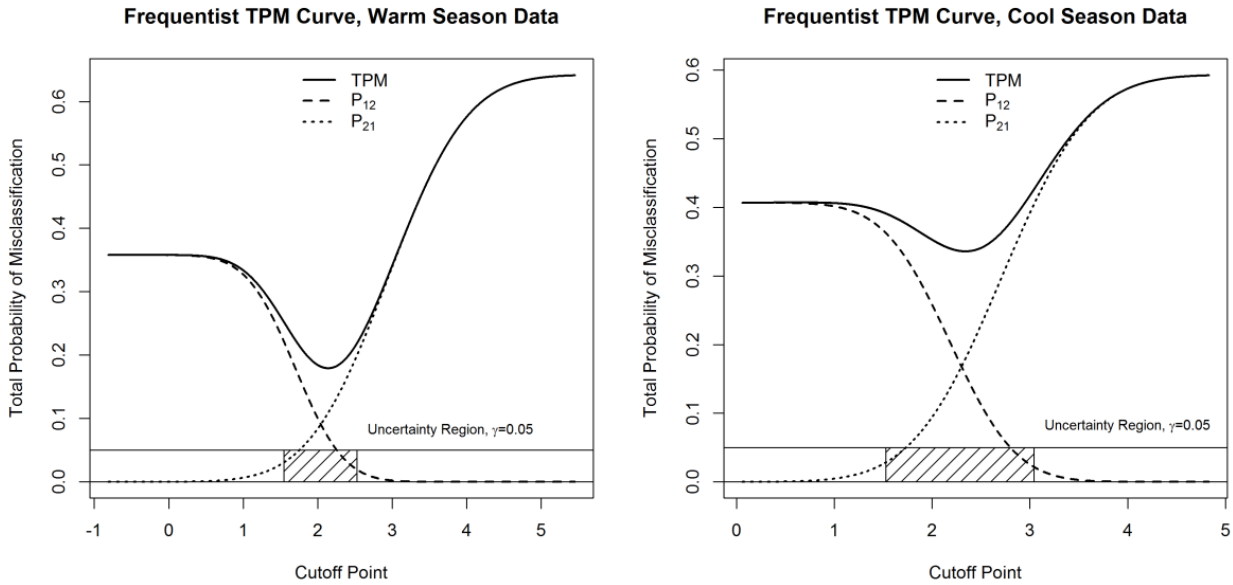

Fig. 5. Frequentist TPM curves for warm season and cool season with PCR as a gold standard.

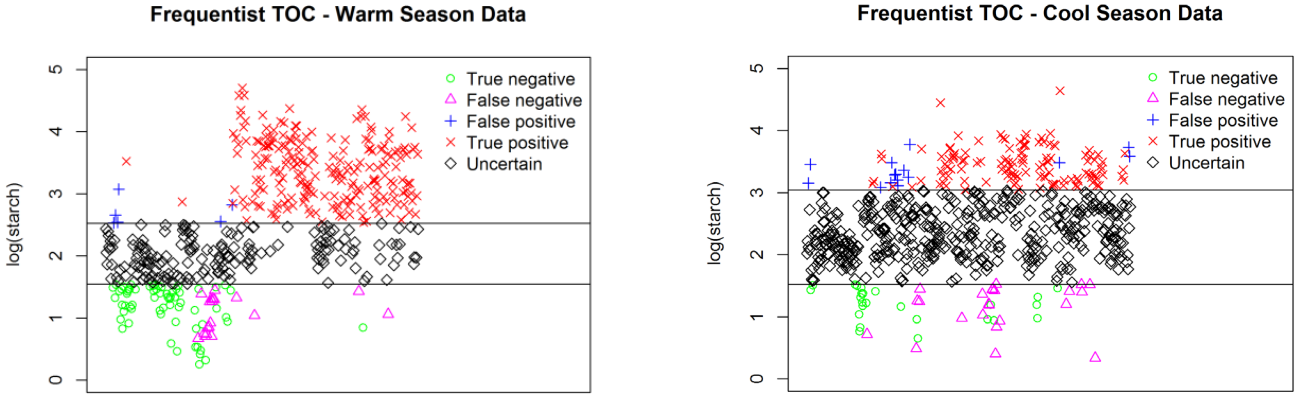

Fig. 6. Classified leaves using frequentist TOC for warm season and cool season data using PCR as a gold standard. Observations above the top line are classified as positive: "true positives" are PCR positive and are represented by a red "x" and "false positives" are PCR negative and are represented by a blue "+." Observations below the bottom line are classified as negative: "true negatives" are PCR negative and are represented by a green "○" and "false negatives" are PCR positive and are represented by a purple "△." Observations between the lines are classified as "uncertain" and represented by a black "◇."

Table 7. (MCMC) Bayes estimates of the mean, variance, and mixing probability parameters μ_1, σ_1^2, μ_2, σ_2^2, and p, and 95% lower (LCL) and upper (UCL) credible limits for μ_1 and μ_2 for the warm season and cool season data without PCR as a gold standard.

	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	0.44	0.66	1.78	2.18	3.52	0.22	3.29	3.72
Cool season	2.07	0.02	0.15	2.01	2.14	2.54	0.56	2.46	2.62

Table 8. (MCMC) Bayes estimates of LDA classification probabilities for the warm season and cool season data without PCR as a gold standard.

	Warm season		Cool season	
	True neg	True pos	True neg	True pos
Pred neg	0.59	0.08	0.00	0.00
Pred pos	0.07	0.26	0.15	0.84

Table 9. (MCMC) Bayes estimates of TOC classification probabilities for the warm season and cool season data without PCR as a gold standard.

	Warm season		Cool season	
	HLB neg	HLB pos	HLB neg	HLB pos
Negative	0.59	0.03	0.00	0.01
Uncertain	0.04	0.05	0.15	0.28
Positive	0.02	0.27	0.04	0.52

data, the Bayes estimate of TPM is 0.07 + 0.08 = 0.15, the Bayes estimate of sensitivity is 0.26/(0.08 + 0.26) = 0.76, and the Bayes estimate of specificity is 0.59/(0.59 + 0.05) = 0.92. For the cool season data, the Bayes estimate of TPM is 0.15 + 0.00 = 0.15, the Bayes estimate of sensitivity is 0.84/(0.84 + 0.00) = 1.00, and the Bayes estimate of specificity is 0.00/(0.00 + 0.16) = 0.00. Table 9 shows the Bayes estimate of the TOC classification probabilities for the warm season and cool season data without PCR as a gold standard using the estimates given in Table 7. By design, the TPM for warm season and cool season is 0.05. Bayesian ROC curves for the warm season and cool season data are given in Figure 7. The Bayes estimates of AUCs are 0.97 for warm season and 0.73 for cool season. Figure 8 shows the TPM curves displaying the uncertainty region determined by the TOC method.

Discussion

The approaches presented in this communication suggest that starch has predictive ability for HLB, but that the predictive ability is higher in the warm season months (June–November) than in the cool season months (December–May). This is evidenced by the differences in the ROC curves (Figs. 4 and 7) and the size differences in the uncertainty regions determined by the TOC method (Figs. 7 and 8). Tables 4 and 7 show that the means for the negative and positive groups are more separated in the warm season than in the cool season. Moreover, TOC was able to control the TPM for both the warm season and cool season data through the establishment of an uncertainty region.

A detailed analysis of the biotic conditions surrounding HLB in terms of bacteria life cycle and the plant development reveals a rational explanation for two types of misclassifications when PCR is a gold standard: 1) false negatives (low starch but PCR positive) and 2) 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 with PCR as a gold standard.

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 Schneider (1968), Etxeberria et al. (2009), and Folimonova and Achor (2010), visible symptoms of starch accumulation (indicating high starch content) only arise

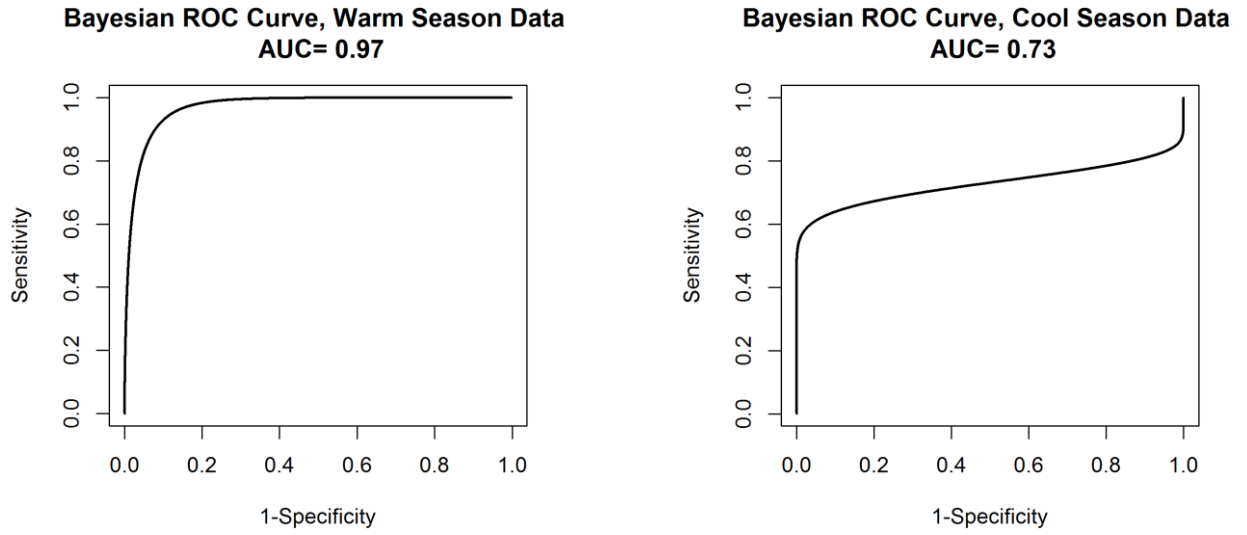

Fig. 7. Bayesian ROC curves for the warm season and cool season data without PCR as a gold standard. The Bayes estimates of AUC are 0.97 for warm season and 0.73 for cool season.

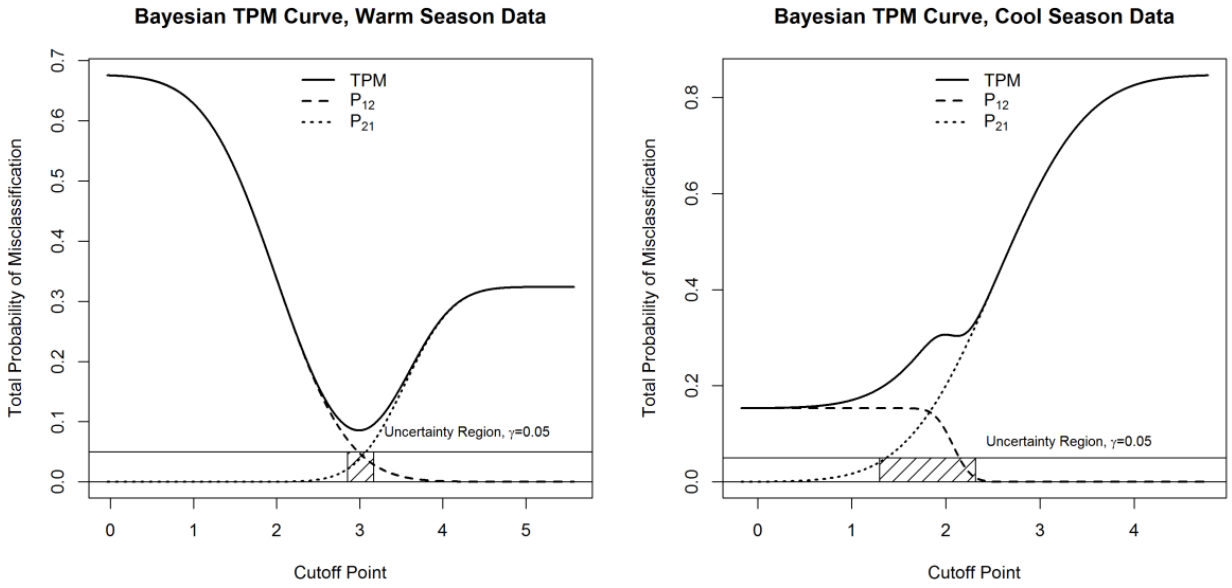

Fig 8. Bayesian TPM curves for the warm season and cool season data without PCR as a gold standard.

after phloem plugging. During the process of phloem plugging and eventual collapse, *C*Las concentration declines, resulting in diminished DNA fingerprinting and lack of PCR detection (Folimonova and Achor, 2010). 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 also results in the accumulation of starch in leaves acropetally from the initial *C*Las infected leaf 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 a gold standard.

Limitations. The data collection scheme that was followed does not support the creation of universal starch threshold levels for the classification of citrus trees as negative, uncertain, or positive. Further research into the biological relationship between starch, HLB, and time is needed before such cutoff points could be established and grounded in the literature. Our results are based on three variables: starch, PCR status, and season. Had other variables (e.g., symptomatology) or longitudinal data been available, a more comprehensive understanding of the relationships between starch and HLB for the purpose of diagnosis may have been possible.

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