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COMPARISON OF VISUAL ASSESSMENT AND POLYMERASE CHAIN REACTION ASSAY TESTING TO ESTIMATE THE INCIDENCE OF THE HUANGLONGBING PATHOGEN IN COMMERCIAL FLORIDA CITRUS

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Abstract. **Huanglongbing (HLB = citrus greening) is one of the most serious diseases of citrus worldwide. The disease is caused by species of bacteria in the genus *Candidatus* Liberibacter and was discovered for the first time in the United States in Florida in August, 2005. To date, the Asian form of HLB, caused by *Candidatus* Liberibacter asiaticus, is the only form of HLB that has been found in Florida. Molecular detection protocols are available for the detection of HLB but due to the uneven distribution of the bacterium in trees, the serological variability of the pathogen, and perceived limitations of testing methodology, molecular testing generally has been limited to the confirmation of visible infections and not for detection of infections in non-symptomatic plants. In this study, visual surveys were compared to the testing of trees with a real-time polymerase chain reaction-based assay (real-time PCR). The incidence of infection based on real-time PCR testing may be up to two times the incidence of infection estimated by visible symptoms alone.**

Citrus huanglongbing (HLB = citrus greening) is one of the most serious diseases of citrus caused by a vectored pathogen (Halbert and Manjunath, 2004). The disease is caused by different strains of *Candidatus* Liberibacter species, *Ca.* Liberibacter asiaticus (*Las*), *Ca.* L. africanus, and *Ca.* americanus (*Lam*), and is vectored by two insect psyllid vectors (Capoor et al., 1967; McLean and Oberholzer, 1965). The disease is widespread in Asian countries, the Indian Subcontinent, and southern Africa (da Graça, 1991; da Graça and Korsten, 2004; Halbert and Manjunath, 2004). Prior to 2004, HLB had never been reported from the American continent. Early in 2004, leaf and fruit symptoms of a disease that was similar to HLB but that did not yield disease products when assayed with primers specific for the causal bacterium were observed in citrus from São Paulo, Brazil (Teixiera et al., 2005a). Since then, both *Las* and *Lam* have been reported in Brazil (Teixiera et al., 2005b). In September 2005, the presence of HLB was confirmed for the first time in the United States (USDA press release 9/2/2005). The disease was detected first in Dade County, south Florida, in multiple locations and has since been reported in many counties in Florida. To date, only *Las* has been detected in Florida.

Since the first detection of HLB in Florida, intensive survey efforts have been undertaken to attempt to determine the extent of infection within the state. The survey has been based largely on the presence of visual symptoms with confirmation using molecular approaches. The non-specific nature of foliar symptoms makes the disease difficult to distinguish from nutritional deficiencies or other plant diseases based on symptomatology alone. This makes it necessary to confirm tentative field identifications with some type of laboratory testing. Multiple PCR-based methods have been used to detect and differentiate *Ca.* Liberibacter species of the HLB pathogens (Jagoueix et al., 1996; Hocquellet et al., 1999; Hung et al., 1999; Li et al., 2006; Teixiera et al., 2005a, b; Tian et al., 1996). Although PCR methods are sensitive and specific, consistent detection of HLB pathogens in infected plants is generally thought to be problematic, presumably because of the low concentration and the uneven distribution of the pathogens in host plants (McLean, 1970). It is generally accepted that the most reliable PCR results are obtained from samples collected from symptomatic tissue. It is unclear as to the utility of PCR testing in asymptomatic trees. The present study was conducted to determine if PCR testing could be used more effectively than visual surveys alone to attempt to determine infection levels in field plantings.

Materials and Methods

Visual surveys and sample collection. Visual surveys of a Hendry County, Fla. grove which was naturally infected with HLB, were conducted using field crews trained to recognize the symptoms of HLB. Two blocks, each ca. 4 ha (10 ac) in size, containing 4- to 5-year-old Valencia sweet orange trees on Swingle citrumelo rootstock, were visually surveyed for the presence of HLB on a tree by tree basis in late January and early February, 2006. Blocks consisted of 14 rows of approximately 105 trees per row planted on two-row beds. Survey crew personnel were trained to identify symptoms of LB HHLB (Fig. 1) prior to the survey by supervisors with prior experience in the identification of HLB. The actual surveys were conducted by walking each row and visually inspecting each tree. Suspect trees were flagged with marking tape and the row and tree numbers recorded. The list of suspect trees was then given to the experienced supervisors who then re-examined the suspect trees. If the supervisors concurred with the identification by the scouts, the suspect the trees were left flagged as possible HLB infected trees. If the supervisors did not concur with the identification by the field scouts, the marking tape was removed.

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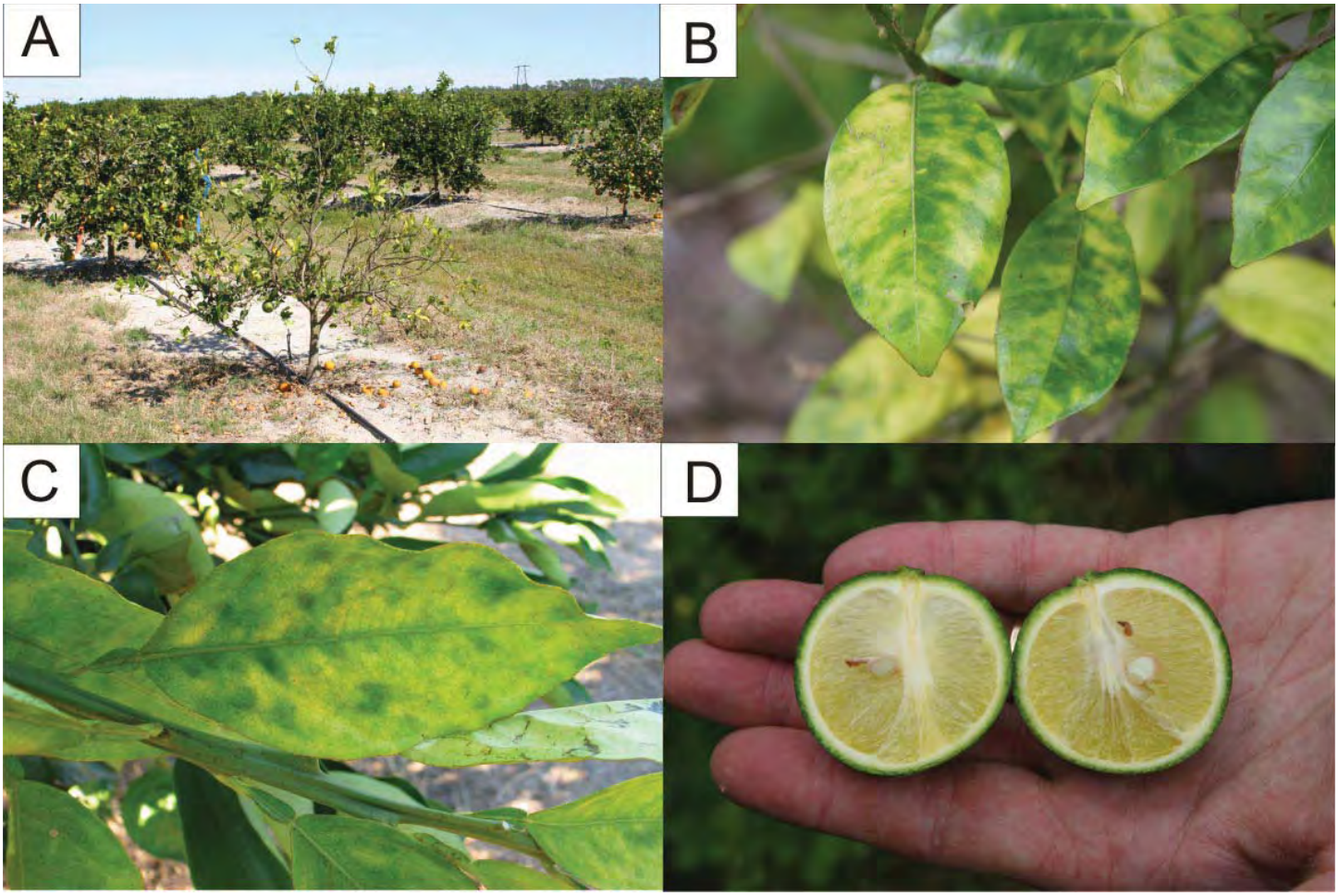

Fig. 1. Symptoms of HLB infection in sweet orange trees in Florida. Declining young tree (A), mottled leaves (B), leaves with green and yellow islands (C), distorted fruit with aborted seeds (D).

Within the two blocks surveyed, five 14-row by 14-tree areas (grids) were selected for testing using a real-time polymerase chain reaction (real-time PCR) assay designed to detect *Las* (Li et al., 2006). Samples were collected for the real-time PCR assays beginning on 26 Jan 2006 and ending on 7 Mar 2006. Samples consisted of four leaves collected from three of the four sides of each tree. The leaves that were selected for sampling were fully expanded leaves that originated from the growth of one of the two most recent flushes. Typically these leaves were on branches that were angular in cross section and whose bark was not visibly suberized. Unless visual symptoms were present, the leaves were collected arbitrarily. If visual symptoms were present, one or two symptomatic leaves were collected and the remaining leaves of the sample were collected arbitrarily.

Preparation of nucleic acid extracts. DNA from leaf samples was extracted using a modified SDS-potassium acetate protocol similar to that used by Garnsey et al. (2002) for the detection of citrus viroids (DePaulo and Powell, 1995). A 0.25g fr wt sample of leaf petioles and midribs was macerated in a Kleco Tissue Pulverizer (Kinetic Laboratory Equipment Co., Visalia, Calif.) in 2.5 mL of extraction buffer. The extraction buffer was 0.1M Tris, pH 8.0, which contained 50 mM EDTA, 500 mM NaCl and 10 mM 2-mercaptoethanol. After maceration, 1.5 mL of extract was removed to a new tube and 0.1 mL of 20% SDS was added. The resulting mixture was incubated 30-60 min at 65°C. After incubation, 0.5 mL of 5 M potassium acetate was added and mixed thoroughly. After a 20-min incubation on ice, the mixture was centrifuged for 10 min at 15,000 rpm in a microfuge and 0.5 mL of the resulting supernatant was transferred to a fresh tube. One-half mL of ice cold isopropanol was added to the supernatant, mixed thoroughly and centrifuged for 10 min at 15,000 rpm in a microfuge. After centrifugation, the pellet was washed with 1.0 ml of 70% ethanol. After washing, the pellet was vacuum dried and resuspended in 0.1 mL of distilled water. Extracts were stored at -20°C until assayed.

Real-time PCR amplification. Real-time PCR was performed in a total volume of 25 µl (23 µL master mix + 2 µL sample) using an ABI7500 (Applied Biosystems, Foster City, Calif.) real-time PCR instrument as per the manufacturer's instructions. The primers used were HLBas, HLBr, and HLBp as described by Li et al. (2006). The forward and reverse primers were used at a concentration of 0.3 µM and the labeled probe was used at 0.15 µM in TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, Calif.). Following initial denaturation at 95°C for 10 min, PCR was carried out for 40 cycles of 95°C for 15 sec and 58°C for 60 sec. The data were analyzed using Sequence Detection Software Version 1.2.2 (Applied Biosystems, Foster City, Calif.). A sample was determined to test positive for HLB if it produced a FAM Ct value of 30 or less.

Spatial analyses. The incidence data were partitioned into four tree (2 by 2) quadrats using a Visual Basic EXCEL macro (T. R. Gottwald, unpublished) to determine the presence of aggregation. When data were expressed as disease incidence, the beta-binomial distribution provides the best adjustment for random conditions (Hughes and Madden 1993). Randomness within a quadrat was thus assessed via beta-binomial analysis. The beta-binomial index of dispersion *D* was used to test for the presence of randomness of HLB-symptomatic trees (Madden and Hughes, 1995). For the beta-binomial index, a large *D* (>1) combined with a small *P* (<0.05) suggests aggregation of symptomatic trees.

A preliminary nearest neighbor analysis was performed to estimate the proportion of the population of PCR-HLB positive trees that occurred at various distances from visual HLB positive trees. This analysis was much the same as used to interpret citrus canker data and to determine possible eradication distances and proportions of the population of canker-positive trees captured at various distances (Gottwald et al., 2002). In this case, the intention was to describe the proximity of PCR-HLB positive trees to known visual HLB positive trees.

Results

Comparison of visual surveys and PCR analyses. Five 14 row by 14 tree grids were compared by visual assessment and real-time PCR for the presence of HLB. The incidence of symptomatic trees in the five grids ranged from 1.0% to 13.8% whereas the incidence of trees that were positive for the presence of *Las* by real-time PCR ranged from 2.0% to 23.3% in the same blocks (Table 1). Over all five grids, visual assessment and real-time PCR testing agreed 94.9% of the time as either negative/negative or positive/positive for visual and real-time PCR assessments (Table 2). In all grids, real-time PCR testing allowed the identification of asymptomatic trees as being infected with *Las*. Over all grids, nearly as many asymptomatic trees were identified as being infected (4.1%) by real-time PCR as the number of trees showing visual symptoms (5.5%). In many instances, the asymptomatic trees that were detected by real-time PCR as being infected with HLB were next to or in close proximity to trees that showed visual symptoms of HLB (Fig. 2). Although more infected trees were detected by real-time PCR than with visual assessments, a small number of trees (0.5 to 2.1%) were identified as being symptomatic but were real-time PCR negative in four of five grids (Table 2).

Spatial analyses. When disease incidence is very low, <0.03, the BBD analysis software will not converge so as to give interpretable output. Therefore, for four of the 10 plot/detection method combinations, no spatial assessment could be made. However, for the remainder of the plots analyzed, the distribution of infected trees was best described by a beta binomial distribution and in all cases the dispersion index, *D*, was significantly greater than 1.0, indicating aggregation of HLB-infected trees (Table 3). For two plots, C3-100 R1-14 T20-33 and C4-100 R1-14 T61-74, when trees that were visually HLB negative but PCR positive were taken into account, the spatial pattern became more diffuse (less aggregated) as indicated by a lesser value of *D*, compared to analyses where only visually HLB positive trees are taken into account.

Nearest neighbor estimations of the proportion of the population of real-time PCR HLB positive trees that occurred at various distances from visual real-time HLB positive trees are shown in Figure 3. This preliminary analysis suggests that 38.9, 69.4, and 83.3 percent of real-time PCR HLB positive trees occurred within 7.6, 15 and 22.8 m (25, 50, and 75 ft) of known visual HLB-positive trees, respectively. These distances are equivalent to about one, two, and three rows of trees or 2, 4, and 6 tree spaces within row.

Discussion

Among the recommended control measures for HLB is the removal of trees (roguing) that express visible symptoms of HLB in order to reduce the potential inoculum reservoir. In areas where HLB is endemic, roguing has been based predominantly on the presence of visible symptoms (da Graça and Korsten, 2004). However the effectiveness of roguing has not been clearly evaluated, in part because infected trees may be overlooked or confused with nutrient deficiencies if symptoms alone are used for detection. Aubert (1990) estimated that 15-20% of the infected plants may be overlooked by nursery inspectors who rely only on visual infection. In order to evaluate the effectiveness of roguing, every effort should be made to remove all potential inoculum sources. Results from the present study also indicate that HLB infection can be substantially higher than what is readily apparent based on visual surveys alone. Thus, when evaluating the effectiveness of roguing as a potential control measure, the effect of infected but asymptomatic trees should be considered when designing

Table 1. Percentage of trees with visual symptoms and real-time PCR positive for HLB in five 14 row by 14 tree grids in two production blocks.

Grid designation (block: tree numbers evaluated)	Total number of trees evaluated	% of trees with visual symptoms	% of trees positive by real-time PCR
C3: trees 20-33	189	13.8%	23.3%
C3: trees 71-84	187	2.1%	2.7%
C4: trees 11-24	196	1.0%	2.0%
C4: trees 61-74	196	13.3%	15.8%
C4: trees 81-94	195	2.6%	4.1%

Table 2. Percentage of samples that fell into the four possible combined test result categories.

	Grid					
Possible test results	C3: trees 20-33	C3: trees 71-84	C4: trees 11-24	C4: trees 61-74	C4: trees 81-94	Mean
Visual negative/real-time PCR negative	74.6%	96.8%	98.0%	82.1%	95.4%	89.4%
Visual negative/real-time PCR positive	11.6%	1.1%	1.0%	4.6%	2.1%	4.1%
Visual positive/real-time PCR positive	11.6%	1.6%	1.0%	11.2%	2.1%	5.5%
Visual positive/real-time PCR negative	2.1%	0.5%	0.0%	2.0%	0.5%	1.0%

Fig. 2. Results of visual and real-time PCR assessments in five 14 row by 14 tree grids. Row / tree combinations highlighted in yellow were positive by real-time PCR only, combinations highlighted in gray were positive by both real-time PCR and visual assessments, and combinations highlighted in orange were positive only in the visual assessments (P = real-time PCR results, V = visual observations, X = missing trees).

roguing protocols. For instance, since infected trees were significantly aggregated and it appeared that asymptomatic but infected trees were more likely to be near symptomatic trees, it may be possible to eliminate a larger percentage of potential inoculum sources by removing the symptomatic trees and a small number of trees surrounding the symptomatic trees. The nearest neighbor analysis indicated that a substantial proportion of the visually HLB negative but real-time PCR positive trees could be removed if only a few trees within 22.8 m (75 ft) of each visually observed HLB-positive tree were also removed by roguing. However, a portion of the visually HLB negative but real-time PCR-positive trees occurred at a distance from other HLB-positive trees and was not immediately adjacent to or associated with clusters of HLB-positive trees. Therefore, additional and more detailed studies need to be conducted for a diversity of plots and situations in order to determine optimum roguing strategies.

An additional outcome of the present study was the demonstration of the utility of real-time PCR testing to detect asymptomatic HLB infections in field trees. This observation is important because it suggests that real-time PCR testing might be successfully applied to the testing of budwood sources. PCR testing for HLB is currently being applied by most regulatory agencies primarily as a method to confirm visual diagnoses. However, since more infections can be detected by combining visual diagnoses with real-time PCR testing than by either method alone, it would seem prudent to incorporate PCR testing in budwood indexing programs. Since little is known about sampling for HLB in relation to titer of the organism as a function of the time of year, location within the tree, and growth stage and age of trees, it is likely that additional improvements can be made in the real-time PCR protocols that will increase the sensitivity of the assays thereby making real-time PCR testing even more useful for indexing and field testing.

Table 3. Beta-binomial parameter and dispersion index calculations for HLB infections in five 14 × 14 tree grids.

Plot	Detection method	Disease incidence (p)	Beta-binomial parameter (θ)[a]	Dispersion index (D)[b]
			Quadrat 2 × 2	Quadrat 2 × 2
C3-100 R1-14 T20-33	PCR	0.2337	0.244326*	1.63983***
	Visual	0.1367	0.491918*	2.0438***
C3-100 R1-14 T71-84	PCR	0.0267	0	0.960401
	Visual	0.0203	0	0.993029
C4-100 R1-14 T11-24	PCR	0.0255	0	0.940663
	Visual	0.0102	0	0.989261
C4-100 R1-14 T61-74	PCR	0.1640	0.28372*	1.70954***
	Visual	0.1321	0.551909*	2.02243***
C4-100 R1-14 T81-94	PCR	0.0406	0.188162	1.41696*
	Visual	0.0254	0.148142	1.3542*

[a]Maximum likelihood estimate of the beta-binomial aggregation parameter θ. Significant departures from zero were determined by a t test, t = θ/s.e.(θ)) and indicated overdispersion. Significance is indicated by *,**,*** at respectively P = 0.05, P = 0.01 and P = 0.001.Values in italic indicate that the likelihood estimation procedure of the p and θ parameters of the beta binomial distribution has failed and that the parameter θ was calculated using the moment method but its departure from zero could no be tested.

[b]Index of dispersion (*D*) values for the indicated quadrat size by plot. Values presented for each assessment date are *D* (=observed variance/binomial variance). Tests for aggregation were performed by comparison of (N-1)× *D* with the chi-square distribution and with the C(α) test (Z statistic) as described in the text. Significance *,**,*** is indicated for the C(α) test. A large (>1) *D* and a small *P* (≤0.05) suggest rejection of H_0 (binomial distribution- random pattern of symptomatic trees) in favor of H_1 (overdispersion described by the beta-binomial).

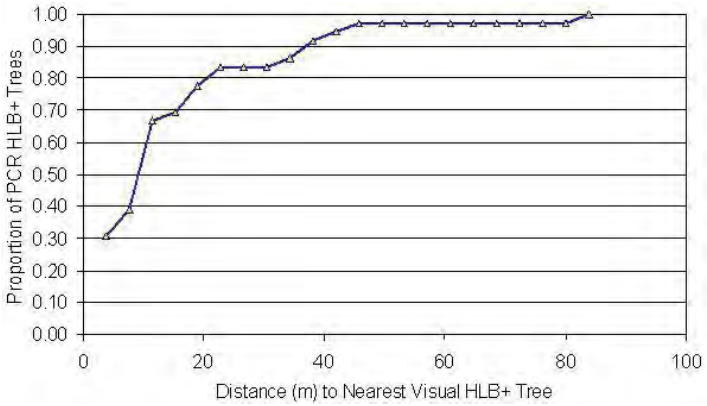

Fig. 3. Cumulative proportion of the population of HLB real-time PCR positive trees vs distance to nearest visually HLB-positive neighbor.

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