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Development of Symptom Expression and Presence of *Candidatus Liberibacter asiaticus* in Recently Infected, Mature Orange Trees

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There have been limited studies and documentation of how long a mature Florida orange tree can remain commercially viable after expressing initial symptoms of the HLB disease. This study focuses on understanding the distribution and spread of HLB symptoms on newly symptomatic, mature trees and the association of these symptoms with the presence of the *Candidatus Liberibacter asiaticus* (Las) bacterium in the various sectors of a tree. The investigation of this process will lead to a better understanding of how long HLB-infected trees can survive under conventional grove practices and, possibly, to better management decisions on tree elimination.

Materials and Methods

Three trees were selected amongst the 6,566 planted acres at Southern Gardens Citrus' Southern Grove. The trees used for this ongoing study are located in Irrigation Zone 110, block 2F. Trees are Valencia on Swingle rootstock, planted in June of 1990. The trees were initially identified as HLB infected by Southern Gardens' team of dedicated HLB scouts and selected for study on the basis that initial symptoms were found during the most recent HLB inspection (9/21/2010) and were present on one small branch only. Initial symptoms consisted of 3 to 5 symptomatic leaves. After the study trees were selected symptomatic leaves were sampled and taken to the Southern Gardens Diagnostic Lab where they were analyzed using real time PCR and confirmed to have been infected with the HLB disease.

A total of 17 sampling sites were established on each tree (Fig.1). The canopy was divided into eight sectors labeled northwest high, southwest high, southeast high, and northeast high for the four sectors more than 5 feet above ground level; and northwest low, southwest low, southeast low, and northeast low for the four sectors less than 5 feet above ground level. Within each sector two sampling sites were established on separate branches, e.g. northwest low 1 (abbreviated as NWL1) and northwest low 2 (abbreviated as NWL2); the highest point of the canopy was also sampled and labeled "Apex" making the total of 17 sampling sites. The initial symptom was located within one of the sectors and included as one of the original sampling sites.

Samples consisted of 4 hardened leaves taken randomly from the designated branch in the case of asymptomatic branches. If symptomatic leaves were present they were used for sampling. Sample collection started October 12, 2010, and trees were sampled on a bimonthly basis thereafter. On each sampling date the trees were inspected carefully for new symptoms; when new symptoms were discovered the symptomatic branch was flagged and labeled, and the location in relation to other branches being studied was logged. Leaf samples for PCR analysis were collected from new symptoms on the date of discovery and all subsequent sampling dates.

All leaf samples were taken to the Southern Gardens Diagnostic Lab where they are analyzed using Real Time PCR (qPCR) to test for the Las bacterium. A Ct value <30 was considered positive for the presence of Las; a Ct between 30 and 32 was considered questionable; and a Ct >32 was considered negative.

In February of 2012, root samples were collected from the soil below each tree, within four quadrants designated NW, SW, NE, and SE. Root samples were analyzed using qPCR by Jim Graham and Evan Johnson at the University of Florida Citrus Research and Education Center in Lake Alfred, FL.

Results and Discussion

The initial HLB symptom on Tree 1 was found on the SWL2 branch. From the first set of samples collected, only the initial symptom tested positive for the presence of Las; all other samples collected were negative. PCR results for the initial symptom remained positive throughout the entire study sampling period, from 10/12/2010 to 12/20/2012. Within a span of 26 months Tree 1 expressed new visual symptoms in six additional locations all of which tested positive for the presence of Las. Five of six new symptoms discovered over time appeared on the same secondary branch as the initial symptom. A comparison of initial and final distribution of HLB symptoms in Tree 1 can be seen in Figures 2 and 3 (the distribution of HLB symptoms is represented as red in the figures).

The initial HLB symptom on Tree 2 was found on the SEL1 branch. From the first set of samples collected, only the initial symptom tested positive for the presence of Las; all other samples collected were negative. PCR results for the initial symptom remained positive throughout the entire study sampling period, from 10/14/2010 to 12/20/2012. Within a span of 26 months Tree 2 expressed new visual symptoms in nineteen additional locations all of which tested positive for the presence of Las. Eleven of the nineteen new symptoms discovered over time appeared on the same secondary branch as the initial symptom. Of the eight new symptoms that were not on the same secondary branch, a majority were still on the eastern side of the tree. A comparison of initial and final distribution of HLB symptoms in Tree 2 can be seen in Figures 4 and 5.

The initial HLB symptom on Tree 3 was found on the NWL1 branch. From the first set of samples collected, only the initial symptom tested positive for the presence of Las; all other samples collected were negative. Over the course of the study, the primary and secondary branches on which the initial symptom was located became 100% symptomatic, and eventually died. PCR results for the initial symptom were consistently positive, but titer as determined by qPCR began to fluctuate about eight months before the branch died. On the final two sampling dates, 2/17/2012 and 4/17/2012, the initial symptoms tested negative for the presence of Las. After this date there was no material on this branch available for sampling. Within a span of 26 months Tree 3 expressed new visual symptoms in twenty-two additional locations all of which tested positive for the presence of Las. There was a second area of spread of symptoms in Tree 3 in the upper portion of the canopy. This second symptomatic area shared the same codominant leader as the initial symptom. A comparison of initial and final distribution of HLB symptoms in Tree 3 can be seen in Figures 6 and 7 (distribution of HLB symptoms is represented as red in the figure; branches that had declined and died after originally exhibiting symptoms and testing positive for HLB are represented in blue).

An estimate was made of the percentage of the canopy of each tree that was affected by HLB disease and decline after 26 months based on visual HLB symptoms and tree appearance. Tree 1 was only 5% affected by HLB, while Tree 2 and Tree 3 were 40% and 45% affected, respectively. A higher percentage of samples from Trees 2 and 3 tested positive for Las over time than samples Tree 1. The percentage of samples from each study tree that tested positive for the presence of Las over time can be seen in Figure 8.

Roots were analyzed using real time PCR in Feb of 2012. Las bacterium was detected in the NW quadrant of the roots in Tree 1, in the SW and NE quadrants in Tree 2, and the SW quadrant in Tree 3.

Conclusion

There is a clear relationship between symptom expression, pathogen detection, and spread from the initial symptom. A concentration of Las bacteria is found within branches surrounding initial symptoms. There is a definite pattern to the spread of HLB bacterium and symptoms, it moves systemically along main scaffolding branches. There may be movement of Las to sectors other than that of the initial symptom as shown by PCR positive results on other scaffolding branches, although reinfection by Asian Citrus Psyllids at a new site cannot be ruled out.

After initial symptom expression the trees decline at varying rates, possibly, due to environmental conditions. Trees 2 and 3 are declining at a faster rate than Tree 1. Tree 1 is in an area of the block where trees are generally healthier and more vigorous indicating better soil quality, an observation that is consistent with Florida growers' widely-held belief that stress or suboptimal conditions accelerate the decline of HLB-infected trees. There appears to be a relationship between the rate of decline of a given tree and the percentage of samples that test positive for the presence of Las in that tree over time.

Root analysis reveals that Las bacterium is also found in the roots confirming that HLB disease is indeed systemic.

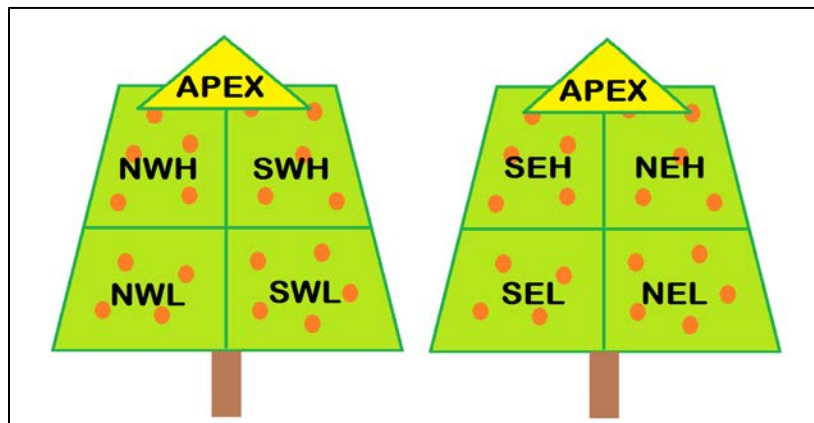


Figure 1: Schematic diagram of sampling procedure showing the west and east side of a tree

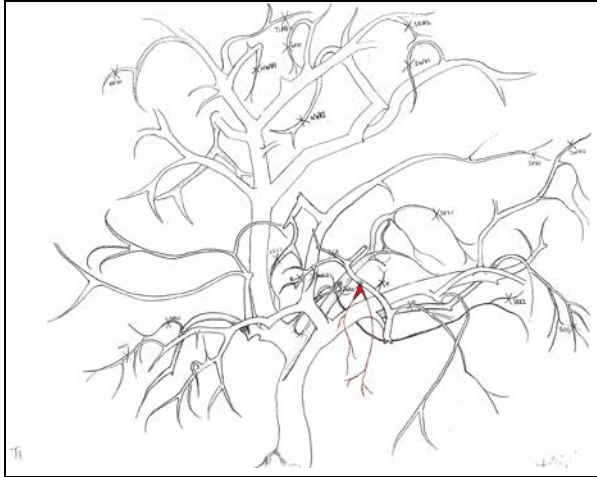


Figure 2: Location of Tree 1 initial symptom on 10/12/2010

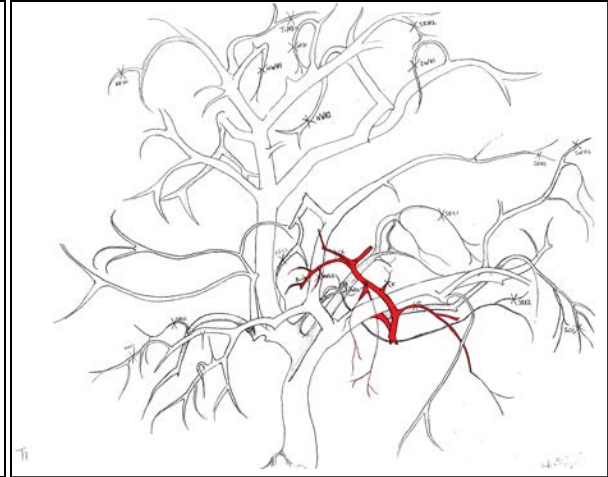


Figure 3: Tree 1 progression of spread of HLB from initial 12/20/2012

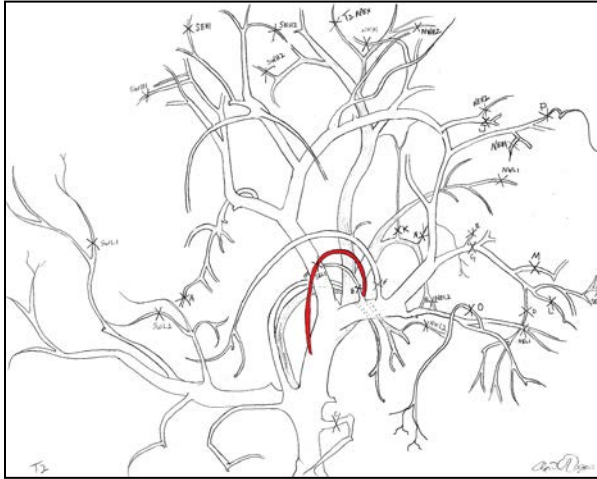


Figure 4: Location of Tree 2 initial symptom on 10/14/2010

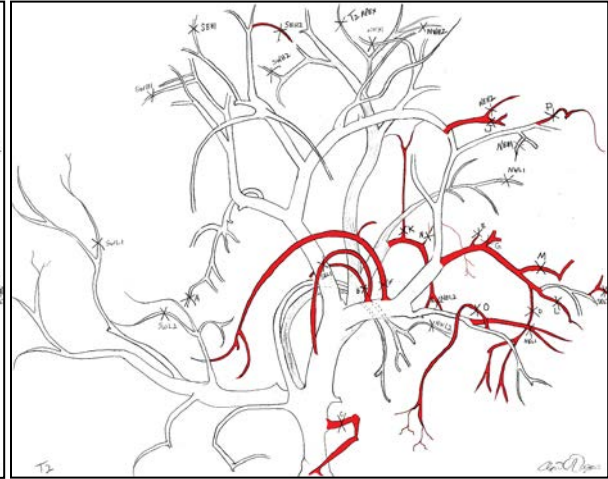


Figure 5: Tree 2 progression of spread of HLB from initial 12/20/2012

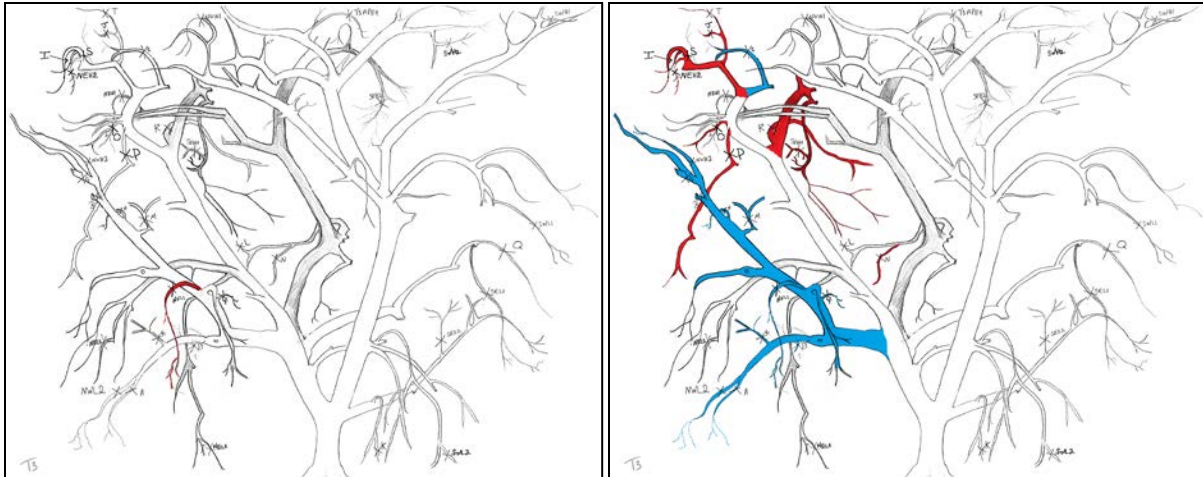


Figure 6: Location of Tree 3 initial symptom on 10/14/2010

Figure 7: Tree 3 progression of spread of HLB from initial 12/20/2012

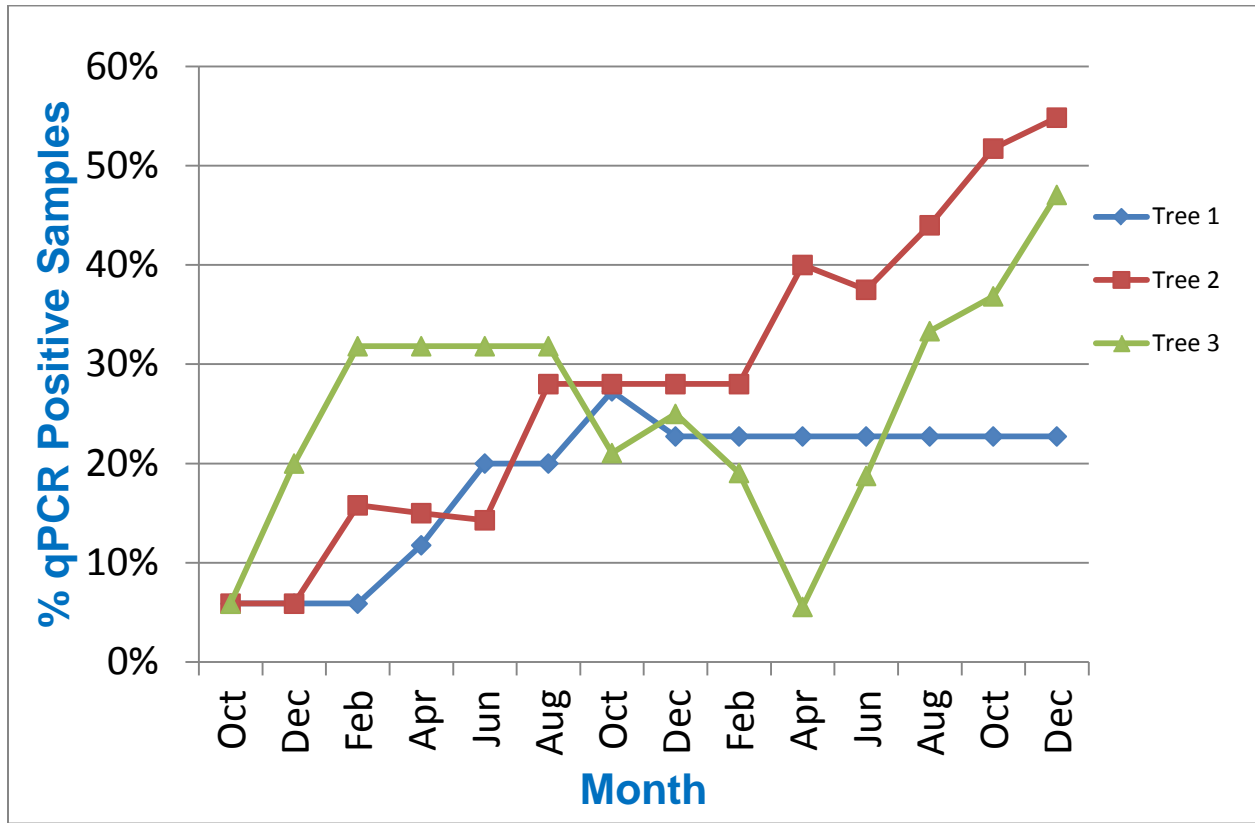


Figure 8: Percentage of samples tested positive for Las by month