10.8 Within-tree distribution of Candidatus *Liberibacter asiaticus*

[1]**Gottwald T.** [2]Parnell S., [1]Taylor E., [1]Poole K., [1]Hodge J., [1]Ford A., [1]Therrien L., [1]Mayo S. and [3]Irey M.

[1]USDA, ARS, US Horticultural Research Laboratory, Fort Pierce, Florida, USA; [2]Biomathematics and Bioinformatics, Rothamsted Research, Harpenden, UK, and [3]Southern Gardens Citrus, US Sugar Corp., Clewiston, Florida, USA.

Introduction:
One characteristic of HLB infection that has not been intensely investigated is the within-tree distribution of *Candidatus* Liberibacter asiaticus (Las). Recently there have been some investigations of the distribution of Las in small greenhouse grown seedlings and grafted trees that have demonstrated incomplete to more systemic variability of bacterial distribution in the vascular system depending on duration of time post-infection when sampled, cultivar, and other horticultural aspects (W. Dawson, personal communication). However, the within-tree distribution of Las in field trees has been incompletely investigated due to the difficulty and complexity of doing so and the multitude of assays required. When a tree displays only minimal symptoms, it is unknown what other portions of the tree harbor the Las bacteria and if these other portions have sufficient titer to be transmissable by psyllid vectors. The goal of this study was to dissect minimally symptomatic field trees to gain an initial understanding of Las within-tree distribution and to attempt to quantitate this distribution where possible.

Materials and methods:
Two Valencia citrus trees which were visually but minimally symptomatic for HLB were selected for PCR analysis. Both were expressing minimal initial HLB symptoms restricted to only one to two branch tips. Based on the PCR results, two trees (approximately 4 years old) were chosen for study. Analysis of leaf/petiole samples was performed via Applied Biosystems 7500 Real-time (RT) PCR using Li primers, probe and thermal cycling parameters (Li et al., 2006). Tree A (harvested November 2007 at 5 years of age) was selected because all initial leaf samples were considered positive via RT-PCR, while tree B (harvested April 2008 at 7 years of age) was selected because only 20% of its initial leaf samples tested positive. Each tree, including the root system, was removed and transported to a covered facility for dissection. Each tree was dissected over a 3-day period, during which time the individual tree sections were labeled, sketched, and photographed for reference purposes, and to allow for precise recording of branch distances and orientation with regard to one another. Beginning at the tree base, branches were removed from the trunk, consecutively numbered and stored at minus 20C. Segments within branches (named "nodes") were dissected from the main and labeled, followed by their corresponding sub-branches (Figure 1). Representative samples from the branches and nodes consisted of 0.180g leaf vein and/or petiole tissue whenever possible. If no leaves were present on a segment, cambium was collected (0.180g). Thus all dissected pieces and the entire tree was assayed. The DNA was isolated using a modified SDS/ KOAc extraction method (De Paulo and Powell, 1995) and was analyzed as above via real-time PCR (using FAM/TAMRA chemistries with ROX dye as internal positive control). Based on the literature, cycle threshold (Ct) values of less than or equal to 30 were considered positive for the presence of HLB bacteria. (Li et al., 2006).
PCR data, section length, and node designations for each diseased aboveground and root piece were entered into a spreadsheet for analysis and a subsequent data file created. Analysis was performed via a C^{++} computer program written specifically for the task by the

second author. The program was used to calculate vascular distances between PCR+ sections, by reconstructing the tree's vascular pathway utilizing unique node designations of

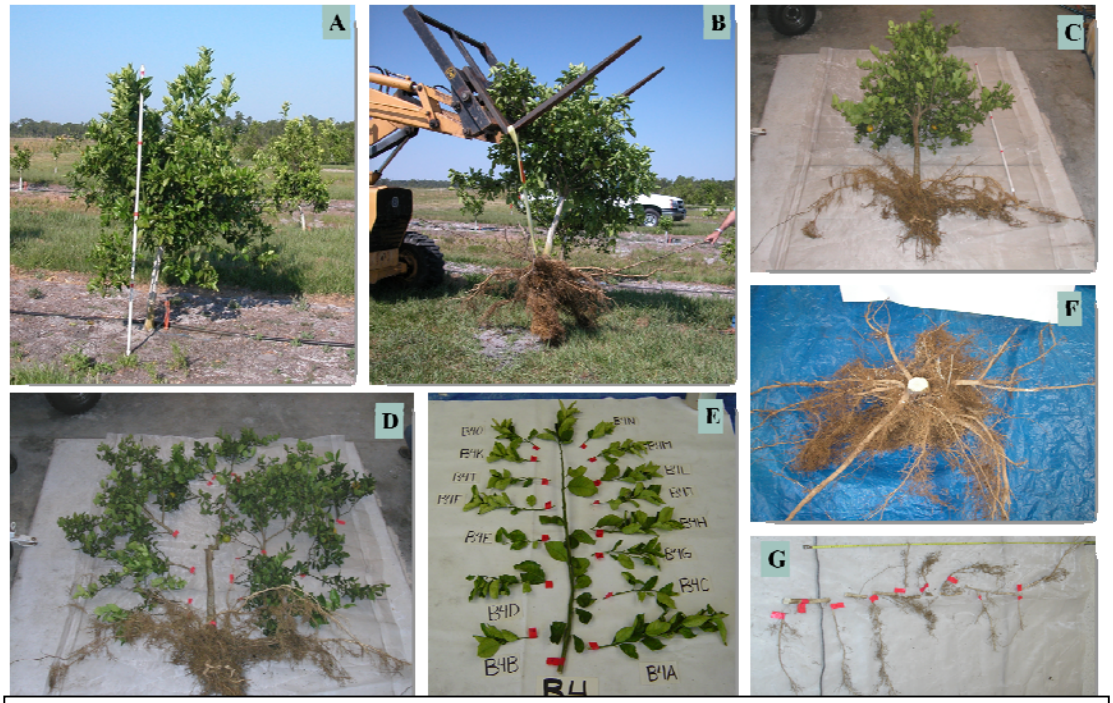

Figure 1. A) Valencia Tree B in the field, B) Tree after removing it from soil, C) Entire tree prepared for dissection, D) Initial dissection of above-ground portion, E) Individual scaffold branch dissected, F) root system, and G) individual root piece dissected.

each diseased piece (Figure 2). Frequency histograms of distances between all PCR HLB+ segments were then calculated. The algorithm assumes a priori knowledge of the physical tree structure which is acyclic. A deterministic search between a predefined 'start' and 'end' segment is performed. The search identifies the unique minimum path between the given segments which is fully determined by the connections between all lower and upper nodes in the tree. The distance of the path travelled (i.e. the sum of the lengths of each segment in the path) is recorded and represents the vascular distance between the start and end segments. Frequency histograms of distances between all PCR HLB+ segments were then calculated.

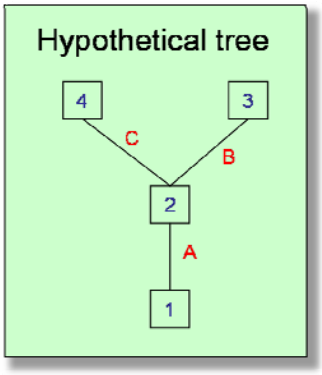

Figure 2. Diagrammatic representation of data matrix for identifying individual nodes and diseased tree sections used to reconstruct and analyze the tree's vascular system connections and Las distribution.

Results and Conclusion:

For both field trees examined, only one branch displayed visual symptoms of HLB. However, for the aboveground portion of the tree, both the main scion trunk and the main rootstock trunk were both PCR-positive for Las. Of the main scaffold branches, greater than 76 percent had at least some portion that assayed as PCR-positive, i.e., at least one of the terminal branches or individual shoot pieces extending from the subtending main branch were PCR-positive. Of these main scaffold branches that were PCR-positive, Las detections via PCR were found in 5 to 100 percent of the population of distal sections. This range in the proportion of PCR-positive detections in main scaffold branches and distal branches and

shoot pieces is consistent with the often incomplete distribution of HLB symptoms seen in individual branches as disease symptoms develop. For the root systems, 26 percent of the main root scaffolds were also PCR-positive for Las. A lesser proportion of the distal root pieces extending from the main root scaffolds were PCR-positive compared to the aboveground distal pieces extending from the main scaffold scion branches, however, there was still a detectible population of 5-10 percent of the distal root pieces that tested PCR-positive for Las.

Quantitative analyses of the distribution of PCR-positive segments is shown in Figure 3. Only the analysis for the above-ground portion of the tree is shown. The results show the frequency distributions labeled that represent the frequency of occurance of distance estimations between PCR-positive sections to the nearest 20cm. An intriguing aspect of these frequency distributions is that there are repeating peaks of approximately 200, 400, 800, 1000, 1200, and 1300 cm and these occur for both trees A and B (Figure 3 A, B). This

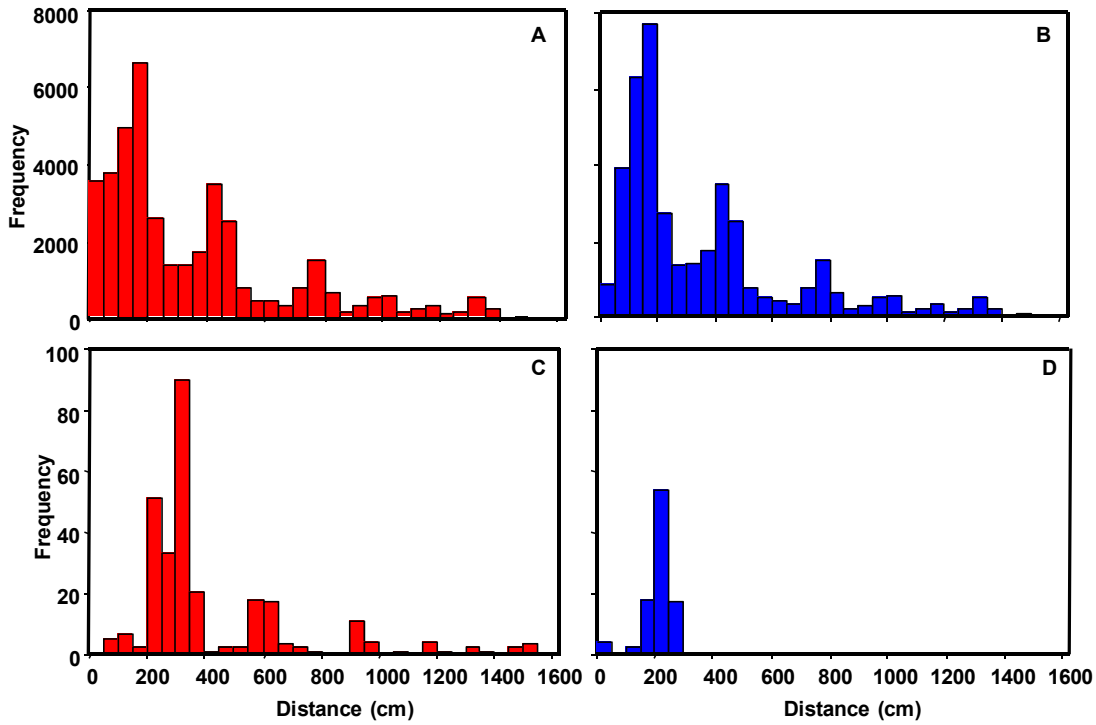

Figure 3. Tree A (A, C) and Tree B (B, D) – A and B) frequency of distances (cm) between all possible paires of infected segments. C and D) frequency of distances (cm) between the tree base and all infected segments.

demonstrates repeating distances between PCR-positive portions of the tree and may point to slight zones of inhibition between areas of the phloem that are being colonized.

A second portion of the analysis generated frequency distributions for the distance from the base of the tree (ground level) to all above-ground PCR-positive segments (Figure 3 C, D). Although the graphs look quite different, it is because of a difference in the scale of the horizontal axis. Tree A had a peak from 200 to 400cm, whereas, Tree B had a similar peak corresponding to 200-250cm. Note also that the frequency peak related to Tree B is less extensive in magnitude and reflects a smaller number of PCR-positive segments in general near to the base of the tree. Obviously no two trees will have the exact same infection or distribution of bacteria and thus such differences in magnitude of frequency are expected.

An overall conclusion is that for trees that are displaying only very few HLB symptoms, there is still a widely detectible and systemic distribution of Las throughout the trees.

However, via PCR assay, this systemic infection is not necessarily complete. This was interpreted to indicate that it is likely that most portions of the tree have Las infection, i.e., the infection is completely or nearly completely systemic, but the bacterial titer in individual portions of the tree may be below the threshold of PCR detection. This also has implication for psyllid transmission. In trees with only few HLB symptoms, Las is still present in PCR detectable titer levels throughout much of the tree, and thus may be available to be aquired by psyllid vectors for additional transmission and spread. The transmission rate of Las from asymptomatic but PCR-positive citrus tissues continues to be a point of discussion by entomologists and vector biologists.

Literature Cited:

De Paulo JJ, Powell CA. 1995. Extraction of double-stranded RNA from plant tissues without the use of organic solvents. Plant Dis. 79:246-248

Gottwald TR, daGraça JV, Bassanezi RB. 2007. Citrus Huanglongbing: The pathogen, its epidemiology, and impact. Plant Health Progress doi:10.1094/PHP-2007-0906-01-RV.

Li W, Hartung J, Levy L. 2006. Quantitative real-time PCR for detection and identification of *Candidatus* Liberibacter species associated with citrus Huanglongbing. Journal of Microbiological Methods 66:104-115