Figure 1. Culture plants infected with Spiroplama citri *(left), and mild (middle) and severe (right) strains of* citrus tristeza virus.

Are Early HLB Detection Methods Impacted by Citrus Stubborn and Citrus Tristeza Diseases in California?

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Detection of huanglongbing (HLB) or citrus greening disease prior to the development of visible symptoms in the tree (i.e., early detection) can give growers more options for managing the disease or its insect vector, the Asian citrus psyllid (ACP). Several HLB early detection methods have been developed and are being tested in greenhouse trials in California and in field settings in Florida and Texas. While field testing in Florida and Texas is critical, these two citrus growing areas do not have citrus stubborn disease, and the isolates of *citrus tristeza virus* (CTV) are different than those found in California.

Citrus stubborn disease is caused by *Spiroplasma citri,* a bacterium that inhabits the phloem of the plant much like the bacterium that is associated with HLB, '*Candidatus* Liberibacter asiaticus' (CLas). CTV occurs in phloem-related cells and impacts the phloem of the plant. HLB early detection methods did not appear to be impacted by the presence of CTV in field

trials (Aksenov et al. 2014). However, HLB early detection methods have not been tested in trees that already have been infected with either *S. citri* or CTV or both, and then infected with CLas. This project seeks to determine the impact of previous *S. citri* and/or CTV infection on the ability of some presumptive HLB early detection methods to detect CLas or HLB.

The impact of mixed infections of plant pathogens on CLas or HLB early detection methods will be examined by graft inoculation of small (four- to six-month old) Washington navel trees (on Carrizo rootstock) with combinations of the pathogens and assaying the plants for CLas or HLB using early detection methods. Early detection methods include metabolomics analysis of plant tissue, volatile organic compound analysis, small plant RNA analysis, microbiome analysis and droplet digital polymerase chain reaction (ddPCR). Plant pathogens to be used in the mixed infections include *S. citri* (isolate from Southern California), two CTV isolates that are common in California (a mild isolate and a stem-pitting isolate) and CLas (isolate from Hacienda Heights, California).

All of the plant pathogens are being introduced to the test plants by grafting. The treatments (i.e., plant pathogen combinations) and controls are listed in **Table 1**. Ten Washington navel trees are being subjected to each treatment. Sampling will occur at approximately monthly intervals and continue until CLas-infected plants test positive using the USDA-APHIS quantitative PCR (qPCR) based assay. This study is being conducted in the University of California, Davis Contained Research Facility, a Biosafety Level III-Plant quarantine, to ensure environmental safety.

Figure 2. Experimental Washington navel trees ready for graft inoculation.

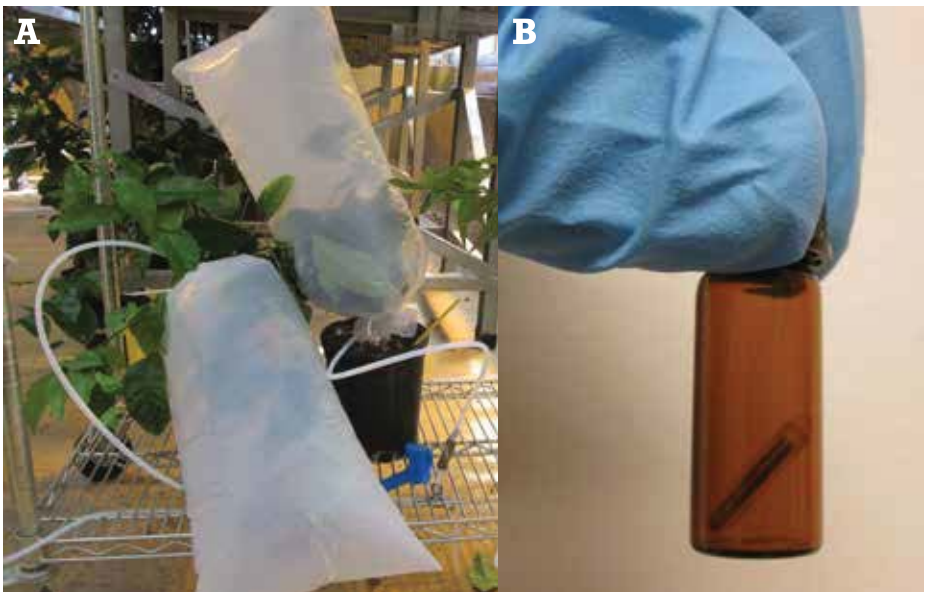

Figure 3. (A) Sampling of volatile organic compounds in a greenhouse environment requires the use of bags. (B) The Twisters used to sample the volatile organic compounds are placed in a sterile vial until the compounds are analyzed.

We established cultures of *S. citri* and two isolates of CTV (**Figure 1**) in preparation for the inoculation of the experimental plants. The first set of experimental Washington navel trees was produced and grafted with pathogens in late August to early September 2016 (**Figure 2**). Graft inoculations were done as a time series by first grafting plants with a single pathogen, then adding a second pathogen to the appropriate plants (dual infection) and grafting one set of plants with all four pathogens. Pathogen-free scion grafting treatment is being done at the same time as grafting for the additional pathogens (**Table 1**). Plants will be allowed to recover for four to six weeks following grafting.

Sampling of the plants begins about one month after the final grafts are made. To sample for metabolomic analysis, plant small RNA analysis ddPCR, and standard qPCR leaf and petiole tissues are being collected, frozen and ground to a powder in liquid nitrogen, divided into aliquots for the different early detections assays with the remaining ground tissue stored in a -80°C freezer. Volatile organic compounds are being collected using the same method as used in field sampling, by placing

Table 1. Treatments applied to greenhouse-grown Washington navels to determine the effects of mixed plant pathogen infections on early CLas and HLB detection methods. All CLas inoculations used the Hacienda Heights strain.

Treatment	Description
A	Control - no graft
B	Graft control - graft with clean scion
C	Graft-inoculated with *Spiroplasma citri*
D	Graft inoculated with CTV1, mild isolate
E	Graft inoculated with CTV2, severe isolate
F	Graft inoculated with CLas
G	Graft inoculated with *Spiroplasma citri* and CLas
H	Graft inoculated with CLas and *Spiroplasma citri*
I	Graft inoculated with CTV1 and CLas
J	Graft inoculated with CTV2 and CLas
K	Graft inoculated with *Spiroplasma citri*, CTV1, CTV2, and CLas

Figure 4. Sampling a leaf using the leaf swab method.

small Twisters® on the leaf surface (**Figure 3**). Microbiome sampling consists of swabbing the top of leaves with a special swab (**Figure 4**).

Analysis of the various samples will vary depending on which early detection method is used. For the metabolomics analysis, metabolites in the leaf and petiole tissue are extracted and analyzed with a nuclear magnetic resonance instrument as described in Chin et al. 2015. Small RNAs produced by the plant in response to CLas infection are extracted from the leaf material and analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using primers specific for three known small RNAs. Detection of CLas DNA in the plant tissue is analyzed by extraction of the nucleic acids and running either a qPCR assay (using the regulatory USDA protocol) or a ddPCR assay (a qPCR assay where the PCR reaction mix is partitioned into tens of thousands of small droplets, this method is thought to be more sensitive than the "standard" qPCR assay). The volatile organic compounds collected on Twisters are analyzed by gas chromatography and mass spectrometry. The nucleic acids from bacteria and fungi found on each leaf are extracted from the swab and sequenced to identify the genera of the bacteria and fungi found on the leaf as described in Leveau and Rolshausen (2016).

The results from these studies will provide information on whether the presence of other pathogens in the citrus phloem will interfere with methods used for early detection of HLB or CLas. The studies also may provide additional methods to detect *S. citri*, CTV and CLas when the pathogens are present alone or in combination.

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