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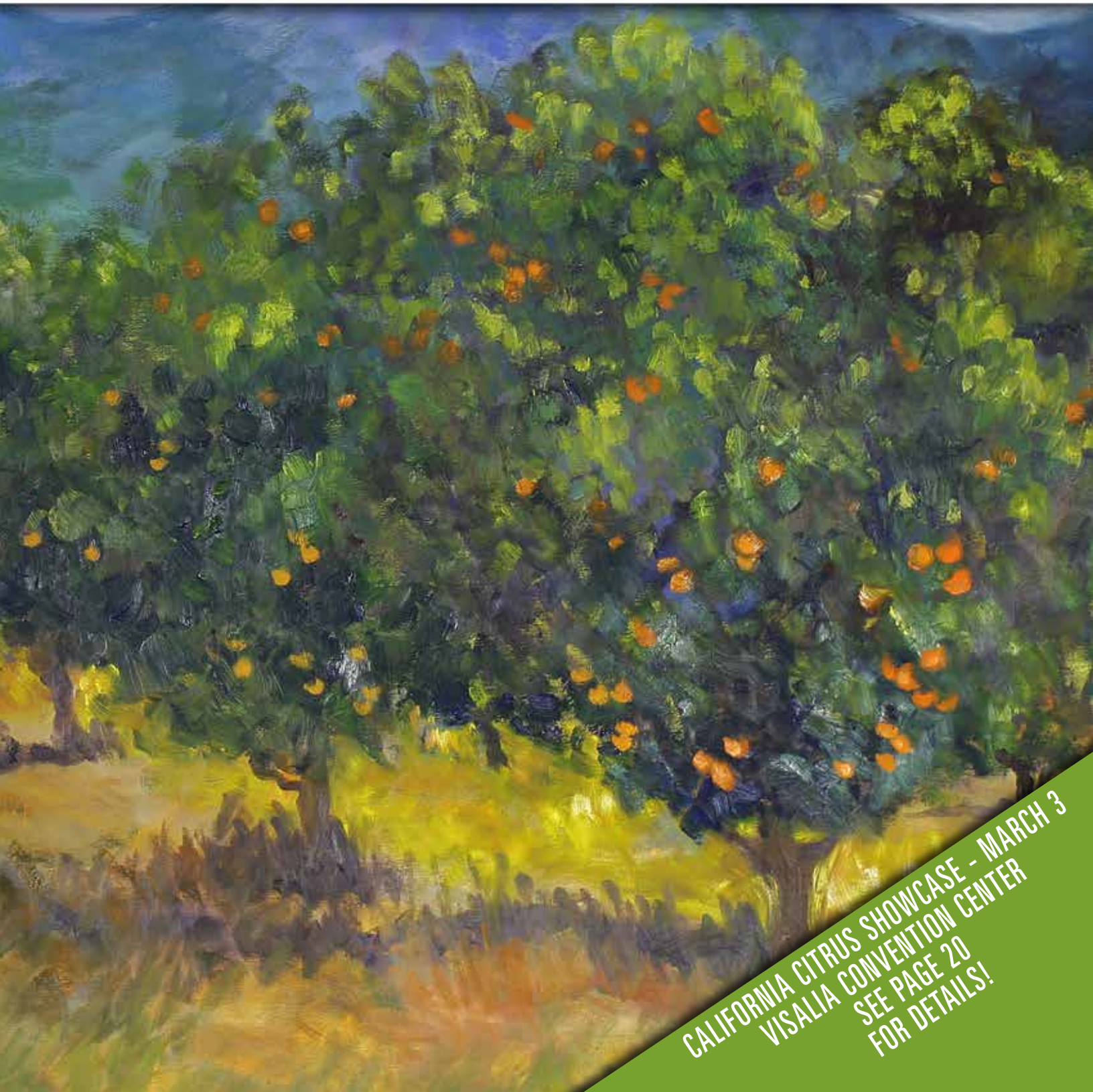

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Figure 1: Jackson grapefruit trees differentially impacted by HLB. The tree to the left (A) looks healthier than the tree to the right (B). The trees are genetically identical and of the same age, so it may be suggested that one of several factors accounting for the different appearances of these trees is that a beneficial microbiota is protecting tree 'A' from CLas-induced symptoms, whereas tree 'B' is succumbing to CLas. Pictures were taken at the USDA Fort Pierce research station in Florida (P.E Rolshausen photos).

A MICROBIOTA-BASED APPROACH TO CITRUS TREE HEALTH

Johan Leveau and Philippe Rolshausen

PROJECT SUMMARY

The goal of this CRB-funded research project is to describe, by DNA-based technology, the microbial communities (also known as microbiota) that associate with healthy and huanglongbing (HLB)-affected citrus trees from different plant tissues, geographical locations and under different management regimes. We will be mining this database for correlations between microbiota and metadata to incorporate into models, experiments and possibly products aimed at increased HLB tolerance, earlier diagnosis and/or more efficient management. In this first of a series of articles, we provide a primer on:

- *plant microbiota,*
- *complex relationships between HLB and the citrus tree microbiota, and*
- *objectives, expected outcomes and preliminary first-year results of our research project.*

In subsequent articles, we will provide updates on the project's progress.

HLB is one of the most destructive diseases of citrus worldwide. In the United States, it results from phloem colonization by the bacterium '*Candidatus* Liberibacter asiaticus' (CLas) and is vectored by the Asian citrus psyllid (ACP) *Diaphorina citri*. Since its discovery in Florida in 2005, HLB has significantly impacted the economic vitality of the Florida citrus industry and quickly spread to the western U.S. and Mexico. No cost-effective methods for early detection or cure of the disease have been discovered yet, and this remains a major hurdle in combatting HLB. In our project, we use DNA-based methodology to establish principles of how trees impacted by HLB may be recognized early, and possibly remedied, based on their associated microbiota.

PLANT MICROBIOTA

Plants and trees are not sterile: they host large and diverse communities of microscopically small organisms (bacteria, fungi and viruses) on (epiphytically) and in (endophytically) their leaves, stems, roots and other tissues. We refer to these communities as the plant-associated microbiota, plant microbiome or phytobiome. The most familiar and best-studied representatives of these communities are the pathogens, i.e., microorganisms such as CLas that have evolved to infect their host, impede normal plant functioning and cause disease. Other members of the plant microbiota include those that are beneficial to their host, for example, root-associated mycorrhizal fungi that sequester phosphate from the soil and share it with their host, or rhizobacteria that fix atmospheric nitrogen or stimulate plant defenses against pathogens.

Despite their potential to seriously impact plant health and function, pathogens and beneficials combined make up a relatively small proportion of a typical plant-associated microbiota. The large majority of microorganisms that colonize plants is composed of commensals. They exploit the plant as a substrate (as a habitat to attach to and thrive in and as a source of nutrients), but in doing so are not demonstrably harmful or helpful to the plant.

New technologies, especially those based on DNA profiling of host-associated microbial communities, have greatly facilitated the analysis of plant microbiota in terms of their composition and function. Such analyses are rapidly accumulating in the scientific literature and have led to interesting and novel insights. For example, it is becoming increasingly clear that the host plant plays an active role in selecting specific microbes from soil or air to colonize its roots, leaves or other plant parts. Another driving factor of plant microbiota composition is the environment, either as a source of microorganisms (e.g. soil and air) or as a modifier of microbial activity, whether it is natural (rain, temperature) or human-imposed (irrigation, fertilization). Possibly yet another

Figure 2. Symptoms of HLB on Jackson grapefruit showing characteristic blotchy mottle leaves. Pictures were taken at the USDA Fort Pierce research station in Florida (P.E Rolshausen photo).

driver of plant microbial community structure is plant disease, or in broader terms, infection with a plant pathogen. We are interested in exploiting this phenomenon in the context of HLB and to develop microbiota-based diagnostics for early (pre-symptom) detection of CLas. Also, because microbial community structure may impact the establishment of pathogens in or on plants to the point that disease is delayed or prevented, we are interested in knowing what such a 'protective' microbiota would look like and use it as a major point of departure for finding practical solutions to manage HLB (**Figure 1A**).

HUANGLONGBING

The '*Candidatus*' label of CLas identifies this bacterium as an unculturable or 'yet-to-be-cultured' organism. The inability to grow CLas in the lab has greatly hampered the conclusive demonstration by classical methods that CLas is the causative agent of HLB. However, consistent association between disease symptoms and CLas presence (so-called Koch's first postulate) has been demonstrated using different culture-independent, DNA-based methods. For example, metagenomic sequencing exposed CLas to be the most abundant bacterial species in phloem tissue from Florida citrus trees with HLB symptoms.

The HLB disease cycle starts with the feeding of a CLas-infected ACP on young citrus leaves, thus introducing CLas into the phloem of the tree. The bacterium also may be introduced by graft inoculation of CLas-infected material onto a healthy tree. CLas resides and replicates in the phloem, and moves through the vascular system site of infection to other parts of the plant, including the root system. From there, CLas may move back into the foliage, where it becomes available for pick-up by psyllids and spread to another tree. Characteristic of HLB is the long latency period between the time of infection and the

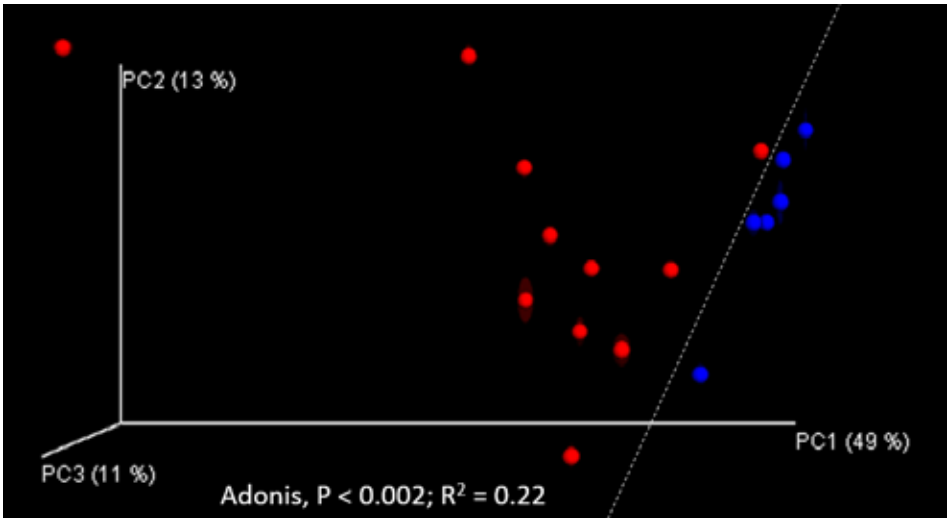

Figure 3. Principle Coordinate Analysis (PCoA) plot showing the differences in microbial community composition among leaf surface samples from Lisbon citrus trees grown in the Contained Research Facility (CRF) at UC Davis. Each data point represents a sample, and the closer two points are to each other, the more similar their microbial community composition is. The data points are colored by the inoculation status of the tree from which the samples came: blue is uninoculated, red is CLas-inoculated (by graft). Data and figure are courtesy of Nilesh Maharaj (Leveau lab).

appearance of CLas-induced symptoms, which include the blotchy mottling of leaves (**Figure 2**) and the development of small, misshapen poorly-colored and bitter fruit and eventually, death of the tree (**Figure 1B**).

Much of the ongoing HLB research is aimed toward a better mechanistic understanding of the interactions between CLas, ACP and the citrus tree, in order to come up with practical strategies to manage the disease through prevention, early detection and/or intervention. The current gold standard for CLas detection is based on the polymerase chain reaction (PCR) using CLas-specific primer pairs. However, the spotty distribution of CLas in a single tree may make it easy to miss CLas, resulting in false-negative PCR outcomes. Several alternatives to PCR are in the works, which are based not on the (direct) detection of the bacterial pathogen, but on measuring the (indirect) effects of CLas on its host. For example, CLas infection of citrus trees has been shown to induce changes in the genes that are expressed in the tree (the transcriptome), the proteins that are synthesized (the proteome), and the chemicals that are produced (the metabolome). Some of these alternative detection methods, for example, those that quantify volatiles emitted from the tree foliage, appear to perform better than the traditional PCR-based method because they suffer much less from the problem of false-negatives.

While most of the citrus tree microbiota are unlikely to interact directly with phloem-limited CLas, we can definitely predict the existence of indirect interactions. As an example, the microbial community structure on or in plants may alter in response to the changes in the plant transcriptome, proteome and metabolome after initial infection. Some evidence already exists for a change in composition and function in the microbial communities on roots of CLas-infected versus uninfected trees. A descriptive and quantitative appreciation for these interactions may reveal new and complementary methods of not only early disease detection, but also the identification of specific members of the leaf and root microbiota that prevent or mitigate infection with or establishment of the HLB pathogen.

YEAR ONE PROJECT PROGRESS

We are using DNA-based methodology to survey the epiphytic and endophytic microbiota that associate with the leaves, roots and stems of citrus trees from greenhouse and field environments located in California, Texas and Florida. Specifically, we are mining the variation in those microbial communities as a function of time, location, management practices and disease symptoms, with the goal to extract from these data consistent associations that have practical use. This effort is generating a database that will be minable by researchers and citrus growers for links between microbiota, tree and environment in the context of orchard management. We also hope to identify organisms that could be used as biomarkers for HLB diagnosis and potential biocontrol agents that could be used to deter the establishment of the disease.

In the first year of the project (2014-2015), we received and processed hundreds of citrus tree samples (leaf, root, budwood). Of these, 100+ samples came from the UC Davis Contained Research Facility (CRF), more specifically, from the tail end of a collaborative, CRB-funded experiment aimed at detecting alterations in the transcriptome, metabolome and microbiome of greenhouse-grown citrus trees that were experimentally inoculated with CLas by grafting. Preliminary analysis of the data on bacteria and fungi from these samples revealed several important and interesting insights. A key observation was that the microbiota of HLB-inoculated and uninoculated trees were different (**Figure 3**) and that this difference correlated with the presence/absence of single microbial taxa such as *Burkholderia* and *Aspergillus*, which would make very promising candidates as biomarkers for Clas infection. We are currently verifying these findings and will link them to microbial data that will be collected from field samples.

In addition, about 200 California samples originated from AgOps at UC Riverside, the Lindcove Research and Extension Center, the Citrus Clonal Protection Program and from two commercial orchards located in the Central Valley. The remaining samples came from Texas A&M Kingsville Citrus Center, Paramount Citrus orchards in Texas and the USDA

research center in Florida. We identified several endophytic fungal (e.g. *Alternaria, Fusarium, Rhizoctonia*) and bacterial (e.g. *Bacillus, Streptomyces, Pseudomonas*) taxa associated with roots and vascular tissues of citrus trees.

Overall, our preliminary results suggest that citrus tissue type, sampling location and disease status influenced microbial community composition. As we collect more samples and accumulate more data, we will be able to build correlations between different variables, such as CLas titer, and abundance, presence/absence of individual taxonomic group. This approach will help identify organisms that compose the disease-informative and/or protective microbiota and could be utilized for HLB management.

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