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Management of Huanglongbing and its Vector, the Asian Citrus Psyllid, *Diaphorina citri*

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Approval

The updated NAPPO discussion document 05 – **Management of Huanglongbing and its Vector, the Asian Citrus Psyllid, *Diaphorina citri*** – was approved by the North American Plant Protection Organization (NAPPO) Executive Committee. See approval dates below each signature - and is effective from the latest date below.

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Introduction

Huanglongbing (HLB) is currently recognized as the most devastating citrus disease on a global scale (Bové 2006). The disease is putatively caused by the bacterium *Candidatus* Liberibacter spp.; within the American continent, is found mainly as *Candidatus* Liberibacter asiaticus (*CLas*) and transmitted by the Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama, 1907.

Candidatus Liberibacter spp. associated with HLB includes species *Candidatus* Liberibacter asiaticus (*C*Las), *Candidatus* Liberibacter africanus (*C*Laf) and *Candidatus* Liberibacter americanus (*C*Lam), from which *C*Las is the predominant pathogen in most citrus growing regions (Wulff *et al*., 2020). *C*Las shows a certain level of heat tolerance, whereas *C*Laf and *C*Lam are sensitive to heat. *C*Laf is mainly distributed in Africa while *C*Lam has only been reported in Brazil. ACP is the most efficient insect vector of HLB. In Mexico, losses to Mexican lime production due to HLB have reached approximately 40%. In both Mexico and the United States, substantial losses due to HLB have been reported.

Management of HLB is complicated and requires a regional or an area-wide strategy for both the pathogen and its vector. Such a strategy may include the use of propagative and planting material that is free from the bacterium, early detection and removal of infected plants, and effective psyllid control. Reducing populations of pathogen-carrying vectors is an important part of integrated pest management and will assist in slowing disease spread (Pacheco *et al*., 2012). In Mexico, the Phytosanitary Epidemiological Management Areas (AMEFI for its acronym in Spanish) have contributed to damage reduction in citrus production areas and lower risk of spread to disease-free areas.

Implementing and maintaining area-wide control of ACP, especially in regions where HLB is present, is important for the following reasons:

1. ACP capability for long distance spread.
2. Constant migration of ACP into citrus orchards.
3. Difficulty in preventing primary infection caused by migrant infective ACP, even with frequent pesticide applications and
4. Difficulty in preventing multiple and repeated re-inoculations in HLB-affected trees that could potentially lead to super-infection and rapid tree decline.

Bassanezi *et al.,* 2013, reported on regional HLB management through removal of inoculum (diseased plants) and vectors (ACP) which resulted in:

1. Delays in disease onset by almost a year.
2. Considerable reduction in HLB incidence (by 90%) and disease progress rate (by 50%).
3. Decreased pesticide applications to control the vector and
4. Reduction in HLB management costs, due to fewer pesticide applications.

This management approach reduces the infective population of ACP (by 90%) from nearby groves and decreases vector populations that migrate into managed orchards (76 to 96%). Consequently, primary infection by HLB decreases.

Scope

This document describes the components involved in the operation and implementation of a program for area-wide management of HLB and its vector, the ACP. This document highlights the strategies used by NAPPO citrus-producing countries for managing HLB and can serve as a reference document for countries with no reports or recent detections of HLB.

Definitions

Definitions of phytosanitary terms used in this document can be found in NAPPO's Regional Standard for Phytosanitary Measure 5 (RSPM 5: NAPPO Glossary of Phytosanitary Terms) and in International Standard for Phytosanitary Measure 5 (ISPM 5: Glossary of Phytosanitary Terms) of the International Plant Protection Convention (IPPC).

Acronyms and Abbreviations

Acronym / Abbreviation	What it stands for
ACP	Asian citrus psyllid
AMEFI	Phytosanitary Epidemiological Management Areas (because of its acronym in Spanish)
APHIS PPQ	Animal Plant Health Inspection Service Plant Protection and Quarantine
BCA	Biological control agent
CCR	California Code of Regulations
CDFA	California Department of Food and Agriculture
CFR	Code of Federal Regulations
CHMA	Citrus Health Management Areas
CHRP	Citrus Health Response Program
*C*Las	*Candidatus* Liberibacter asiaticus
CLBV	Citrus Leaf Blotch Virus
CNRCB	National Biological Control Reference Center (because of its acronym in Spanish)
COX	Cytochrome Oxidase
CPDMC	Citrus Pest Disease Management Corporation
CPDPC	California Citrus Pest and Disease Prevention Committee
CPDPD	Citrus Pest Disease and Prevention Division
ELISA	Enzyme-Linked Immunosorbent Assay
HLB	Huanglongbing
HTS	High Throughput Sequencing
IPPC	International Plant Protection Convention
IRAC	Insect Resistance Action Committee
ISPM	International Standard for Phytosanitary Measures
NGS	Next Generation Sequencing
NPPO	National plant protection organization
NSPCP	Nursery Stock Pest Cleanliness Program
OASV	Plant Health Auxiliary Body (because of its acronym in

	Spanish)
OMRI	Organic Materials Review Institute
PCR	Polymerase Chain Reaction
PMA	Psyllid Management Area
PPDC	Plant Pest Diagnostic Center
PDR	Pest and Damage Record
QC	Quarantine Commodity
RNR	Ribonucleotide Reductase
RSPM	Regional Standard for Phytosanitary Measures
SIMDIA	*Diaphorina citri* Monitoring System (because of its acronym in Spanish)
SENASICA	National Service for Plant Health (because its acronym in Spanish)
SOP	Standard Operating Protocol
STG	Shoot-tip Micrografting
TCPDMC	Texas Citrus Pest and Disease Management Corporation
TFF	Phytosanitary Technical Facilitator (because of its acronym in Spanish)

1.0 Producing Clean Citrus Propagative Material

1.1 Background

The center of origin and diversification of citrus is continental Southeast Asia (Indochina) and Australasia (Pfeil and Crisp 2008). Citrus originally moved into new areas as seed, thereby facilitating the spread of seed-transmitted citrus diseases. When citrus began to be moved as budwood or rooted plant material, graft-transmissible diseases began to also spread around the world.

The devastating effects of some of these graft-transmissible citrus diseases, such as Tristeza (*Citrus tristeza virus*), encouraged the development of technologies to diagnose the disease pathogens and their potential vectors. These technologies are now in place in many citrus-producing countries and regions, and they are a key tool for the preservation and management of citrus health. The use of clean propagative material or nursery stock is one of the important means of preventing the spread of HLB and other graft-transmissible diseases (Krueger and Navarro, 2007). This, together with surveys to detect and remove infected trees alongside area-wide reduction of the vector population, form the basis for regional management programs for HLB and other citrus diseases. The measures described below are intended to result in the production of nursery stock that is free of all known graft-transmissible diseases.

1.2 Importation and quarantine

It is a requirement, in most citrus-producing areas, that citrus propagative material originates from pathogen-tested clean sources, where robust phytosanitary registration or certification programs are strictly followed. The movement of citrus propagative material must be highly regulated to reduce the risk of introduction of new diseases, exotic pests, and more virulent strains of pathogens into otherwise clean citrus-growing areas. To minimize these risks, national plant protection organizations (NPPOs) and regulatory agencies in individual countries use phytosanitary regulations and approved protocols to monitor the movement and confirm the cleanliness of propagative material prior to its movement. Such protocols generally apply to small-scale movement of new source material such as nursery stock or budwood, which are released only after verification of an acceptable phytosanitary status and/or a post-entry quarantine period that may include therapy and testing. However, when dealing with large-scale commercial importation of plants for planting, where the risks may be too high, approval for entry is generally denied. Also, in some countries without post-entry quarantine stations, propagative material not known to be free from disease is prohibited entry.

Citrus propagative material that is granted entry may be subjected to one of two approaches while in quarantine, as follows:

The classical approach involves containment and propagation of imported plant material, followed by observation and indexing, using biological or laboratory tests to detect the presence of pathogens. Infected material is destroyed or subjected to a therapeutic procedure. This approach has been used for many years with success in areas with low disease pressure.

The tissue culture approach involves culturing imported plant budwood *in vitro*, recovering plants through shoot-tip micrografting (STG) *in vitro*, and testing for the presence of pathogens by indexing and by direct laboratory testing.

The therapied and indexed material is released from quarantine **only when no pathogens are detected**. Even though other therapy techniques are available (e.g., nucellar embryony and thermotherapy), STG has proven effective against all types of citrus pathogens without inducing juvenility or other adverse effects in the citrus variety, and today is recognized as the standard therapeutic procedure.

The tissue culture approach is more conservative than the classical approach, as therapy is applied under all circumstances to ensure that the risk from potential diseases is minimized. In some cases, this approach has been incorporated into regulations that previously gave discretion to the quarantine facility at the point of entry as to which approach to use.

As stated above, it is a requirement in many citrus-producing areas that citrus propagative material comes from clean sources. However, in some areas, there are no legal restrictions on the movement of citrus propagative material within a country or state. This can result in the spread of pests that may be catastrophic to the citrus industry. In such situations, all material moved within the same country, state, or region is advised to be sanitized/therapied to remove pathogens and be included in a certification program. Where no certification program exists, obtaining propagative material from a reliable source of pathogen-tested material outside the country (either a gene bank or a certification program) carries less risk than obtaining material from within the country.

1.3 Producing clean plants and verifying that plant material is disease-free

Protocols for producing clean plants and verifying their disease-free status usually involve therapies to rid the plant of pathogens and various methods of testing for pathogens. These protocols are generally carried out under controlled conditions and may be conducted before moving plant material, following the introduction of new plant material, or after a post-entry quarantine period.

Current pathogen-elimination techniques rely mainly on thermotherapy or STG (see NAPPO treatment protocols for thermotherapy (TP 01: 2015) and STG (TP 02: 2015) for details). STG has become the most common technique because it is effective in eliminating all citrus pathogens.

Detection of graft-transmissible pathogens is a fundamental component of a program for the safe introduction of new citrus varieties or the sanitation of existing varieties. Pathogen detection is based primarily upon biological indexing with citrus indicator plants, complemented by laboratory tests. The specific tests required will be determined by various factors but are ultimately approved by regulatory authorities.

Laboratory tests commonly used for the identification of graft-transmissible pathogens include culturing on media, microscopy, serological tests, such as enzyme-linked immunosorbent assay (ELISA), and nucleic-acid based tests such as polymerase chain reaction (PCR), being PCR and DNA sequencing the main detection method. More recently, high throughput sequencing (HTS) paired with in silico bioinformatic analysis have also been developed and are under evaluation for their potential use for citrus testing in quarantine programs (see also below) (Dang *et al*., 2023).

Laboratory tests may offer advantages over biological indexing: robust assays can offer higher specificity, sensitivity, and accuracy; they are more rapid; they can be automated for large-scale testing to enable the processing of large numbers of samples in a short time; and require fewer human and physical resources, so are generally less expensive. However, the disadvantages of laboratory testing are: requirements for a well-designed facility equipped with specialized instruments could be costly; the need for well-trained and proficient staff; validated standard operating protocols (SOPs); and the need for a robust quality control system. Limitations in testing can also occur if the staff do not have prior knowledge of what pathogen(s) they are trying to detect or if the antisera or PCR primers needed are unavailable. Also, in some cases, laboratory testing may not be accepted by the regulatory authorities of some countries/states/regions. If this is the case, these limitations can be avoided by performing biological indexing on specific indicator hosts (Navarro *et al*., 1984).

Hence, biological indexing in greenhouse studies and pathogen detection in laboratory tests complement but do not replace each other. For a complete index, if a biological test is available for a pathogen, a laboratory test may be used as an adjunct but should not replace the biological test. In cases where laboratory tests are not accepted by the regulatory authorities, the "index of record" is the biological index. In contrast, for re-testing a large number of pathogen-tested germplasm accessions, laboratory tests are often the only feasible method. In this case, a large-scale laboratory test such as HTS, together with specific oligonucleotide e-probes also known as next-generation sequencing (NGS), may be a feasible method. HTS methods have now been evaluated for routine virus and/or viroid detection in grapevine and fruit trees with promising results (Al Rwahnih *et al*., 2015; Soltani *et al*., 2021), and are under investigation in citrus (Dang *et al*., 2023), suggesting their potential future contribution to phytosanitary certification processes for citrus (Candresse *et al*., 2014).

1.4 Facilities

Quarantine and sanitation programs for citrus have very specific facility requirements (Gumpf 1999). Ideally, facilities should be located in an area with climate suitable for growing citrus, but not adjacent to areas with commercial citrus production. This is not always feasible. It is therefore important that quarantine facilities are designed, constructed, and operated in a manner that minimizes the risk of pathogens escaping from the quarantine material held within them. Measures should be taken to minimize other phytosanitary risks, such as contamination of other plants in the facility, entry/access by insects and other pests, and spread of fungal, bacterial, and viral pathogens within the facility. Facilities must be designed to maintain environmental conditions suitable for indexing. In some cases, regulatory agencies will have specific requirements for facility design (USDA-APHIS-PPQ, 2010).

Access to facilities should be prohibited except for those who are authorized and trained in proper phytosanitary conduct. Facilities should always be kept locked, ideally behind a fence with a locked gate. Citrus canker decontamination spray stations should be installed at the entrance of these facilities, where possible.

Facilities generally belong to federal, or state agencies involved in plant protection, but these agencies may have agreements with outside entities, such as a research institution, to conduct the actual sanitation program. In any case, the facilities themselves as well as processes and records must be accessible and available for inspection by regulatory authorities.

Facilities should include/have greenhouses, screenhouses (only if the disease and pest pressure and risk allow it), a laboratory for tissue culture and pathogen testing, office space for personnel, and a reception and meeting area for visitors and educational programs.

1.4.1 Greenhouses

Greenhouses are used to produce indicator plants and rootstocks, for biological indexing, as well as for maintenance of the tree collection of positive controls used in bio-indexing and laboratory tests. Greenhouses must have at least two chambers, one maintained at cool temperatures and another at a higher temperature, for the detection of graft-transmissible pathogens. The rooms must have independent environmental controls. The environmental control system is the most critical component of the greenhouse, as it is vital for maintaining required temperatures. A third room of intermediate temperature is often recommended, and, if available, can be used to produce indicator plants and used for propagation. Greenhouses must be constructed so they protect plants from insects and should have double-doors with vestibules and positive pressure airflow and/or air curtains. Air intake and exhaust must also be safeguarded (e.g., screened) to exclude insects.

1.4.2 Screenhouses

Screenhouses are used for protected maintenance of plants or for positive control plants of lower risk, and for endemic and non-vectored diseases. They may be constructed of metal (preferred), or wooden frames screened with nylon or stainless-steel insect-excluding materials. In the past, the standard construction utilized “aphid-excluding” screens, but current practice uses “thrips-excluding” screens or 95% shade cloth. The roof should be at least three meters from the ground (preferably four to five meters). Screenhouses for foundation plant material must have a vestibule with double doors and positive pressure airflow and/or air curtains. If airborne pathogens such as the bacteria that causes citrus canker, or other insect, disease or environmental risk factors are present in the area, the use of screenhouses should be avoided in citrus quarantine programs. A yellow sticky trap should be placed in the vestibule with double doors. Yellow sticky traps should also be placed inside the facility, one for every 100 m^2.

1.4.3 Laboratory

Laboratories are used for testing and therapy. Separate areas for therapy and tissue culture are highly desirable. The design and construction of the laboratory depends on the extent of its use. If limited laboratory tests are conducted (e.g., ELISA, culturing, microscopy), a small laboratory with enough space to accommodate equipment for tissue preparation, testing, and for sample storage may be sufficient. If a larger range of laboratory tests are carried out, or a large number of samples are handled, a larger laboratory will be needed to accommodate additional equipment, cold storage, and personnel. If molecular tests are performed, additional areas need to be established to separate functions such as sample processing, nucleic acid extraction, master mix preparation, reaction set up, thermocycler(s) room and, if required, result visualization/gel electrophoresis and staining areas.

1.4.4 Personnel and visitor areas

Personnel should have access to appropriate office space to store and access records and literature, use computers to prepare indexing experiments, reports, and educational materials, as well as print or prepare labels, work on plant inventories etc. It is not uncommon for government inspectors, industry stakeholders, international visitors, or others to request or need to visit a citrus quarantine facility either for official business (e.g., inspection for a permit or shipment) or for educational purposes (e.g., tour the facility for a regulation, or to learn about a new pest of quarantine importance). Such human traffic should be controlled through a reception area where visitors are registered in a logbook and given instructions on the rules guiding facility visits (e.g., do not open any doors or you are not allowed in the facility if you had been in contact with any citrus plants prior to the visit). After a supervised visit or tour is completed, visitors should be hosted in a meeting room, away from any plant materials to complete the business, meeting, or educational event.

1.4.5 Plant materials

After quarantine release, plants are maintained under protection in a germplasm bank (gene bank – see below), foundation block, and/or increase block, as potted trees growing in a sterilized substrate. This has some obvious advantages for disease prevention, fertilization, frost protection, and the ability to manipulate and move the trees, among others. Growing trees in pots allows for varying the number of trees maintained per variety or genotype. However, maintaining trees in soil is in some cases the only way to provide large quantities of budwood. Trees maintained under screens or other protective structures, whether in pots or in the ground, are generally not suitable as sources of fruit for characterization and evaluation of fruit quality. Observation of any fruit produced inside protective structures helps to ascertain the trueness to type of genotypes and to detect possible misidentified accessions and possible chimeras. These are important considerations when releasing budwood for certification.

1.5 Germplasm bank

A germplasm bank is a collection of the widest possible range of genotypes, maintained regardless of their commercial use or potential. A germplasm bank may support scientific research and supply specific genotypes to industry via a certification or clean plant system. Maintenance of a germplasm bank is a resource-intensive activity that does not generate revenue; therefore, they are usually maintained by a governmental or an academic institution and, consequently, are subject to competition for resources from other programs and projects. Some have a program for long-term preservation under cryogenic or tissue culture conditions. These programs are important for long-term preservation of valuable genetic material and diversity, although they are restricted to only few facilities.

Germplasm banks should include access with double-doors, footbath station, air curtains, aphid-proof screens, and sticky traps for insect vectors. A germplasm bank, especially one that supports a certification or clean plant system, should be maintained in structures to protect against pests. When sufficient resources are available, a field-planted collection is an option to serve in characterization and evaluation and as a source of seeds and tissue for research. In some cases, resources are insufficient for protective structures, and the only trees present in a germplasm bank may be field planted. This is not desirable because field planted trees are vulnerable to pests and abiotic stresses, such as high temperatures and moisture stress. The current standard advises the protection of germplasm bank material inside a structure.

Many germplasm banks established in the twentieth century and earlier include genotypes of unknown phytosanitary status. Currently, only sanitized genotypes are maintained, and the addition of new genotypes or the release of genotypes from the bank to a certification or clean plant system requires that a sanitation program be in place. Sanitation of propagative material can occur before the material is added to the germplasm bank, while the material is within the bank, or before the material is released to a certification or clean plant system. The latter option is mostly associated with accessions acquired prior to the adoption of current phytosanitary standards; current practice is to have clean material in the germplasm bank. Therefore, diagnostic testing of accessions prior to public release is recommended to detect HLB.

Documentation of material maintained in a germplasm bank is crucial. At a minimum, origin data and unique identifiers for genotype and individual trees as well as management information (e.g., propagation, location) are necessary. As much additional characterization and evaluation data as possible should be obtained and recorded. Each tree should have a registration number on a permanent label.

1.6 Foundation block

The basis for all further propagation in a certification or clean plant system is the foundation block. The scope of a foundation block is narrower than that of a germplasm bank. A foundation block directly supports an industry and is comprised of commercial varieties and varieties with commercial potential. Varieties not used in commercial production should be maintained in a germplasm bank and should meet a high phytosanitary standard – see below.

Foundation blocks should include access with double-doors, footbath station, air curtains, aphid-proof screens, and sticky traps for insect vectors. Material maintained in a foundation block must meet a high phytosanitary standard; usually this equates to the absence of all known graft-transmissible pathogens, a status met after passing through an introduction or a sanitation program (e.g., therapy and indexing). Foundation block plantings are periodically re-tested for pathogens as required by phytosanitary regulations. The most important diseases from a re-testing standpoint are endemic diseases that are naturally spread by their vectors. Material in a foundation block that meets a high phytosanitary standard can be maintained indefinitely, although practical matters (e.g., tree size or quality of vegetative growth after several years growing in a pot) may limit its life.

Depending upon regulations and circumstances, foundation blocks may be maintained by governmental or academic institutions and/or by private nurseries. In the past, foundation blocks have included both protected foundation blocks of initial (mother) material and field-planted or protected foundation blocks located at nurseries. The current practice is to protect all blocks of the program. Field-planted foundation blocks are being phased out. A field-planting of trees propagated from foundation material is sometimes established by the certification or clean plant system or by researchers or industry representatives to observe, evaluate, and document fruit production and tree growth characteristics. Vegetative material, other than seed, from field-grown trees should not be used for propagation.

Documentation associated with a foundation block includes some of the same information as that used to manage a germplasm bank, such as origin data, phytosanitary data, and management data. Generally, each foundation block tree is supplied with a unique identifier that allows tracing of all buds distributed to the industry back to an individual tree in the foundation block. This is important if a disease or an abnormality is observed in trees propagated from buds originating directly or indirectly from the foundation block. The presumed source tree can be checked to determine whether it is the source of the problem or whether the problem originated after the buds left the facility. Unique identifiers are generally issued and maintained by regulatory agencies. In addition, replicates of plant material with at least two plants per variety must be kept.

1.7 Increase blocks

Because of the expense associated with maintaining and re-testing foundation block trees, typically only two to six trees from each variety are maintained. This number is not enough to supply budwood to propagate nursery trees. Therefore, material from the foundation block is used to establish budwood increase (multiplication) blocks, allowing rapid and efficient multiplication of buds. In some cases, increased blocks can be propagated from other approved, pathogen-tested source trees.

As with germplasm banks and foundation blocks, increase blocks should include access with double-doors, footbath station, air curtains, aphid-proof screens, and yellow sticky traps for insect vectors. Increase blocks generally have a defined lifetime, parameters of which are determined by the regulatory authorities. Lifetime can sometimes be extended after re-testing for specific pathogens. The phytosanitary status of increase block trees must be equivalent to germplasm bank and foundation block trees. As with germplasm bank and foundation blocks, the increase block is authorized and inspected by the regulatory authorities.

The final step in a certification or clean plant system is the production of certified nursery trees from either foundation block or increase block material. Production may be protected or may be in the field: the selection of method will depend upon the phytosanitary conditions in the area, which, in turn, determine contamination risks. The actual requirements for certification are mandated by the regulatory authorities and vary by location. The preference is to produce and maintain trees intended for sale under protection, but this is not always feasible. At a minimum, yearly diagnostics for HLB detection are required in increase blocks.

1.8 Seed source trees

The current practice is to maintain seed source trees in the field because of the difficulties in fruit and seed production using currently available technologies in protective structures. Seed source trees are tested for a relatively small number of pathogens, and the standards for testing are not consistent across governments or certification programs. Reports of seed transmission of citrus pathogens are sporadic and inconsistent, and most major graft-transmissible citrus pathogens, including the bacterium associated with HLB, have not been reported to be seed-transmitted as they are phloem limited and the citrus seed anatomy does not include phloem connections between maternal seed tissue (e.g., seed coats) and the developing embryo. Therefore, there is no pathway by which a phloem-limited pathogen can colonize the embryo, so germinated seedlings do not contain the pathogen.

The preferred practice is planting seed source trees that meet all relevant clean plant or certification criteria, protecting them against vectors, and re-testing them periodically for vector-transmitted pathogens endemic to the area. Testing should include pathogens reported to be seed transmitted and other pathogens of phytosanitary or quarantine concern for the area, to reduce the risk of seed sources serving as inoculum that can threaten a clean stock program such as foundation or increase block trees. Seed production plantings are also subject to authorization and inspection by regulatory authorities.

Completely protected production of citrus seed would be preferable; however, this is not practical because protective structures would need to be much larger to accommodate seed source trees rather than smaller bud source trees. In addition, flowering and fruiting are impeded or inconsistent under protective structures.

Tissue-cultured rootstocks are produced and used by a few commercial nurseries but are currently not routinely used. Adoption of tissue-cultured rootstocks and indicators for certification programs will undoubtedly increase in the future.

1.9 Nurseries

1.9.1 Wholesale nurseries

Wholesale nurseries producing citrus nursery stock should follow all regulations concerning citrus and general nursery regulations. Wholesale nurseries must be authorized by regulatory authorities and should be accessible for inspection. The origin and sale destination for nursery stock must be recorded. This information must be made available to regulatory authorities upon request. The actual format for documentation will be determined by nurseries and regulatory authorities depending on local conditions.

Production of citrus stock for sale should be carried out inside protective structures. A pest control program, including monitoring, control actions, and appropriate documentation, should be in place. Citrus trees should be protected until they leave the wholesale nursery. Exposure to unprotected conditions should be minimized during loading for transport off-site. Wholesale nurseries are subject to inspection. Trees should be treated for disease vectors (insects and mites) with a systemic and foliar insecticide before being transported off-site. Phytosanitary requirements of the NPPOs should be considered for these treatments. Trees must never be returned to a nursery once they are sold.

1.9.2 Retail nurseries

Retail nurseries should obtain plants for sale to the public from wholesale nurseries. Citrus trees for sale in at-risk areas (where HLB or its vector are present) should be maintained inside a protective structure. Trees should be monitored and treated for disease vectors, especially in areas where HLB or its vectors are present and in unprotected situations. Regulations may limit the time citrus trees can remain in retail nurseries and the number of trees allowed at any one time, especially if protective structures are unavailable. Alternate hosts of HLB and its vector should be subject to the same standards as citrus if they are present at the retail nursery, as once they leave the nursery, they may come into contact with citrus.

2.0 Regional Management for the Asian Citrus Psyllid (ACP)

2.1 Background

The regional management of ACP requires a multi-faceted approach encompassing social, economic, operational, epidemiological, and biological aspects. ACP is a significant pest affecting citrus crops, and effective management strategies involve controlling its populations to mitigate economic and ecological impacts. Recent research (Bassanezi *et al*., 2013) showed that in Brazil, regional (area-wide) management of ACP was much more effective at significantly reducing populations compared to management on an orchard-by-orchard basis. In Florida and Texas (USA), similar observations were made, where coordinated sprays provided effective and longer residual control than orchard-based spray application programs (Graham *et al*., 2020), critical components of a systems approach included cooperation of growers, coordinated control actions, and interactions with plant-protection authorities, researchers, and government In Mexico (Martinez-Carrillo *et al.* 2019).

The most effective management of ACP usually occurs when a group of growers in a defined region or area agree to work in a coordinated manner. The nomenclature used to designate this concept varies. The most utilized terms are "area-wide management" and "regional management," although other terms are used. Area-wide management is the "management of the total pest population within a delimited area" (Hendrichs *et al*., 2007). These coordinated programs aim to reduce ACP populations, mitigate the risk of disease introduction, and protect citrus crops.

In Mexico, these programs, called Phytosanitary Epidemiological Management Areas (AMEFIs, because of their Spanish acronym), operate in 22 citrus-producing states, where coordinated actions take place to reduce ACP populations and avoid HLB re-infestations in orchards, thereby decreasing the inoculum load among and within areas through monitoring, chemical and biological control activities. AMEFIs are designed to implement grower-coordinated measures in strategically defined citrus areas. AMEFIs create blocks of 1,000 hectares or more and use a rotation schedule of pesticides and, when possible, liberation of biological control agents in an integrated management approach that also includes vector monitoring and controlling disease outbreaks (SENASICA 2021). Mexico, also has a national resistance monitoring program where the effectiveness of pesticide field applications is evaluated while reviewing changes in adult response, characterized as three times the 95% lethal dose (García-Méndez *et al*., 2016; Villanueva *et al*., 2019; Osorio *et al*, 2019).

In the United States, areas like AMEFIs are called Citrus Health Management Areas (CHMAs) in Florida (Singerman and Page 2016, UF/IFAS 2023), citrus pests and disease management areas in Texas (Sétamou, 2020), and Psyllid Management Areas (PMA's) in California (Milne *et al*., 2018). These areas and designations have been devised specifically to coordinate efforts; however, coordinated efforts can also be implemented in existing designated areas (such as Pest Management Districts), if appropriate. In California, coordinated treatments generally occur three times per year and are applied to target vectors that survived the winter and during periods of citrus flush. The California Department of Food and Agriculture (CDFA) may augment coordinated grower treatments by treating residential properties near commercial citrus if growers meet the requirements to qualify for an additional residential buffer surrounding the PMA. The AMEFIs also supported the reduction of vector populations in residential areas near commercial citrus growing areas. In these areas, where applications of conventional insecticides were not always feasible, the usage of oils, soaps, and by-products was promoted, as well as the release of biological control agents.

2.2 Components of regional management

2.2.1 Organization

A working group dedicated to HLB management should be created in each state or region, comprised of representatives from relevant agencies, institutions, stakeholder groups and organizations (e.g., regulatory agencies, local government, and citrus industry [citrus growers and processing associations], beekeepers, certified nursery associations, and citrus research institutions) (SENASICA 2021). These working groups are instrumental in the implementation of area-wide management strategies and play a crucial role in ensuring compliance with regulations related to HLB (Singerman and Page, 2016; Stelinski *et al.,* 2022; UF/IFAS 2023).

In Mexico, the AMEFIs are responsible for analyzing data on implemented phytosanitary actions and providing recommendations for HLB and other citrus pest management, recommending periods of regional pesticide application, action thresholds to control ACP, and pesticide rotation schedules. The group can also participate in training for stakeholders, growers, and other technical staff belonging to state, federal, or extension organizations and growers. The AMEFIs are made up of technical staff from the NPPO and local government, growers, beekeepers, and processing associations, as well as the AMEFI HLB program coordinators, a technician from the Growers' State Committee and researchers from citrus research institutes in the region.

In Texas, a Citrus Pest and Disease Management Corporation (CPDMC) was created in 2013 to specifically address ACP and HLB related issues, but later the scope was expanded to address all invasive citrus pests. In California, Assembly Bill 281 and the California Food and Agricultural Code established the California Citrus Pest and Disease Prevention Committee (CPDPC). The CPDPC is composed of 17 members representing the fruit and nursery industries, regional representatives, and the public, and works closely with the state government to evaluate and guide pest management activities.

A dedicated HLB technical group plays a vital role in providing recommendations for management and addressing technical issues, such as the number, size, and location of regional management areas; prioritization of areas prone to HLB endemic outbreaks; periods of regional pesticide application; action thresholds to control ACP; and pesticide rotation schedules. The group can also participate in training stakeholders, growers, and other technical staff belonging to state, federal, or extension organizations and growers. In Texas the CPDMC is composed of growers, scientists, extension agents and regulatory personnel, but is led by growers.

HLB is not only a technical problem, but also must be considered from the economic, social, environmental, and commercial points of view and, as such, regional management needs the involvement of all stakeholders. Collaborative efforts among stakeholders increase the likelihood of achieving goals and reducing individual costs. The roles of some of the authorities and personnel involved in the implementation and operation of ACP regional management are discussed below.

Role of the phytosanitary authorities
The phytosanitary (and regulatory) authorities at both the state and the federal level play a crucial role in leading the establishment of working and technical groups and promoting the implementation of regional management areas. They are responsible for developing protocols, outlining strategies, defining management components and responsibilities, and following up on regional control activities. These activities may include biweekly monitoring, scheduling pesticide applications, and conducting targeted control measures in specific focus areas. The involvement of phytosanitary authorities is essential for the successful implementation and supervision of regional management activities.

Role of researchers
To enhance the effectiveness of regional management, it is essential to continually optimize all of its components. The management strategy should consider the significance of each factor, including social, economic, operational, epidemiological, and biological aspects. It is up to the identified subject matter experts to identify all the factors, establish priorities for research, and conduct appropriate research so that results are quickly applicable in the field. Biological and epidemiological aspects need to be quantified and validated experimentally (not only in case studies). These aspects include:

1. Understanding the impact of abiotic factors such as wind (intensity and direction), humidity and temperature on the flight and spread of ACP (Martini *et al*., 2018).
2. Evaluating the efficacy of systemic and contact insecticides applied to control ACP as well as how insecticide resistance is evolving (Qureshi *et al.,* 2014a).
3. Investigating the influence of bud phenology on the biology and population dynamics of ACP and transmission of HLB (Sétamou and Alabi, 2018).
4. Examining the influence of environmental conditions and root stock selection on the intensity and seasonality of outbreaks and the susceptibility of different varieties.
5. Managing other pests present in management areas.

By addressing these aspects through research, the regional management approach to HLB can be further refined and optimized.

In relation to the social aspects of regional management, it is crucial to identify the causes behind the lack of cooperation. This is particularly important for neighboring growers and their participation in implementing control actions. Without cooperation among growers – the principal beneficiaries of the process – regional management will not be effective.

To address this challenge, it is essential to investigate and understand the factors that hinder grower cooperation. This could involve analyzing issues such as competing interests, lack of awareness or understanding, economic constraints, conflicting schedules, or limited or outdated communication channels. By identifying the specific barriers to cooperation, strategies can be developed to address and overcome them.

Mamani (2013) noted the main reason for lack of collaboration is a lack of willingness to participate in a coordinated fashion. The distrust of leaders and authorities considerably increases the vulnerability to HLB for growers in two locations in Veracruz, Mexico (Aguilar-Roman *et al*., 2020). Therefore, processes to improve local leadership and increase social participation must be implemented to improve the results of phytosanitary campaigns. Sociologists, psychologists, and anthropologists may be brought in to resolve these issues using participatory extension methods appropriate to the context. Mexican government programs employ a Phytosanitary Technical Facilitator (TFF because of its acronym in Spanish) to assist with promoting collaboration.

Providing education, training, and incentives can also play a role in motivating growers to actively participate in control actions.

Role of extension personnel

The role of extension personnel is to provide information to stakeholders and work with them to implement the regional management program, develop and implement training workshops focusing on identification, control, and suppression of HLB and its vector, and provide community outreach on crop integrated management. It is critical for extension personnel to provide feedback on the success of previous years' coordinated ACP and HLB mitigation efforts during stakeholder meetings. The extension service and other available resources should be brought together to enhance current public outreach efforts on pest and disease prevention and management, as well as to educate on the importance of protecting agricultural resources. This requires collaborating with research institutions, industry associations, government agencies, and other stakeholders.

Outreach is also necessary to encourage home gardeners to manage HLB and other citrus pests in their properties, and to help promote a better understanding on how their decisions may affect commercial producers. It is essential that homeowners understand the need to purchase citrus plants only from state-certified nurseries. They must be aware that they should not accept citrus plants from other sources or give their own citrus plants to others. By effectively communicating these guidelines through targeted outreach efforts, their understanding and compliance can be improved. Homeowners play a significant role in preventing the spread of HLB, and by promoting responsible practices, they contribute to the overall management and protection of citrus resources.

Garcia-Figuera (2021, 2022) assessed the factors that drive or prevent adoption of management practices in California and how outreach efforts could assist in providing a more effective response to an invasive disease. Their results show that perceived vulnerability to HLB, willingness to stay informed, communicate with formal and informal networks, and farm size are relevant factors for adoption of control programs.

Role of growers

To ensure effective regional management, growers are key players in the successful management of the disease and its vector and should actively engage in consultation with technical experts to evaluate collected data. Mexico has encouraged growers to adopt management strategies since the first detection of HLB. However, the number of growers carrying out actions alongside regulatory authorities is low, therefore, it is essential to implement an active training program. In Brazil, a cooperative alert system has growers post weekly data from yellow sticky traps and outbreaks to a website that publishes information every two weeks, as well as recommendations for pesticide applications in hot spots. Treatments must be done in cooperation with growers. In Florida, a "tap sampling" method that provides data on ACP and other arthropod numbers every three weeks was implemented by growers and regulatory agencies. The information is used to identify hot spots and coordinate ACP control efforts (Martinez-Carillo *et al*. 2019; Qureshi and Stansly 2007; Qureshi *et al*., 2009; Stansly *et al*., 2009a).

Growers must be convinced of the importance of actively participating in a coordinated program to achieve a reduction in ACP populations through coordinated application of insecticides and entomopathogens (Martinez-Carillo *et al*., 2019; Qureshi and Stansly 2010; Qureshi 2021; Stansly *et al*., 2009b).

In Mexico, the role of growers is fundamental in the regional management of HLB and its vector. To accomplish this, outreach and educational programs are implemented through workshops led by the Phytosanitary Technical Facilitator (TFF because of its acronym in Spanish) who uses methodologies and techniques that promote participation and raise awareness to help reduce populations of ACP, which has a direct impact on orchard production and on safeguarding the national citrus industry.

Growers from different citrus producing regions organize to apply regularly timed treatments. This is coordinated with the TFF and the staff in charge of the AMEFI. The definition of regional treatment period is based on monitoring data for ACP and other pests, climatic conditions, crop phenology, and reports of decreased susceptibility of ACP to the pesticide's active ingredients. It is important to note that the number of regional treatments may vary based on funding available to the NPPO or the growers. In this respect, regional treatments are prioritized. These treatments can be done with pesticides or entomopathogenic fungi depending on the region, time period, citrus species, and target pest.

In Texas, early adopter growers were instrumental in convincing their peers of the importance of area-wide management programs and this led to an adoption rate approaching 90% of total acreage. Hence, in most outreach meetings, representative growers always present their success stories to other growers. One of the primary obstacles in convincing growers to participate in regional management working groups and programs is the expectation of immediate results from the actions taken. This is not possible when dealing with diseases with characteristics such as those of HLB (long incubation time, seasonal and unclear symptoms [especially for early infection], difficulties in controlling the vector, etc.).

As such, strategies should be employed to incentivize grower participation. The CDFA may conduct additional synchronized insecticide applications in residential buffer areas around orchards participating in coordinated area-wide applications. This synchronized strategy maximizes ACP suppression benefits for participating growers. Within a given PMA, a high degree of voluntary grower participation in the area-wide applications (90%) is required to qualify for the residential buffer treatments.

Experiences in Florida have demonstrated that the removal of infected trees during the initial stages of HLB epidemic at a regional or grove/orchard level has shown increased effectiveness. As the infection becomes more widespread, growers are reluctant to remove large numbers of trees. Consequently, they have adopted a less aggressive management strategy that includes various approaches such as enhanced plant nutrition, ACP control, and improved root health, among others (Atta *et al.*, 2023; Chinyukwi *et al.*, 2024; Hallman *et al.*, 2022; Hallman *et al.*, 2023; Kadyampakeni *et al.*, 2023; Kwakye *et al.*, 2023).

2.2.2 Social participation in the program

Social participation in the context of plant health and regional control programs is fostered through organized activities that facilitate understanding and implementation of program strategies and objectives. These activities aim to gather first-hand information and evaluate the impacts of specific strategies at different locations, enabling effective monitoring and assessment.

To achieve effective regional control of ACP, and consequently of HLB in Mexico, it is crucial to strengthen communities and foster coordinated actions among growers, the Plant Health Auxiliary Bodies (OASV, because of its Spanish acronym) and other key interested parties in citrus production. For regional control to be successful, it is important to encourage growers to participate in actions, through social organization and unity, that allow the creation of a working network to meet the AMEFI's objectives.

It is crucial that extension agents in all affected areas raise awareness of program objectives with the community and with growers. It is important for growers to understand that their participation is not an imposed obligation but a necessary and valuable contribution. They should also understand that their active participation in surveillance, monitoring, and pest control will bring direct benefits to their standard of living, making them responsible for the protection of their patrimony and for national citrus production.

For successful implementation, extension personnel should possess a combination of field experience, organizational skills, communication expertise as well as technical knowledge of the disease, its vector, and the regional control program. This will allow them to design strategic, and participatory methodologies and strategies to encourage social participation and community organization. Moreover, their input is also important in gathering and analyzing data on the social characteristics of citrus production areas, to help decide on the establishment of regional control programs and operational campaigns against HLB.

2.2.3 Operational activities

Program coordination

The coordination of the area-wide management program should be led by a regulatory authority (state, regional or national) or by industry stakeholders closely engaged with research and extension personnel. In Texas, the success of the program hinged on the fact that it was a grower led effort. Although the scientific community and regulatory agencies worked in tandem to develop the program, it was constantly adjusted to meet growers' needs and these efforts were led by the Texas Citrus Pest and Disease Management Corporation (TCPDMC), a grower group. The establishment of area-wide management areas such as CHMAs, PMAs and AMEFIs is crucial for developing an effective program. Establishing a coordinating body for each management area is important to ensure timely follow-up and program continuity. For example, California uses grower liaisons to disseminate outreach information and coordinate treatments using approved pesticides (Citrus Pest Disease and Prevention Division (CPDPD) Action plan 2022)

Training

Training workshops for growers and technicians, focusing on symptom recognition, vector identification, approved pesticides for ACP management, best practices in agrochemical use and management, biological control agents, and operation of ACP and HLB area-wide management bodies (e.g., AMEFIs, CPDMAs, CHMAs, and PMAs) are routinely offered. It is also important to conduct training sessions for the trainers themselves and facilitate coordination meetings among researchers, extension personnel, and growers. California's grower liaisons are required, as part of their tasks, to coordinate seminars in addition to well-timed pesticide applications (CPDPD action plan 2022)

Communication and outreach

One of the most important aspects of a regional management program is building an outreach and communication strategy that focuses on developing training materials on management of HLB and ACP to inform producers, growers, nursery owners/workers, and the public. In California, the state government contracts with a media outreach coordinator that promotes awareness of citrus pests and management activities to elected officials, the citrus industry, and the public. Social media and websites are essential for delivering information on HLB and ACP to the public. Extension publications together with trade journals and scientific literature provide important stakeholder information. By emphasizing public and stakeholder education, early detection of ACP and HLB can be achieved, ultimately contributing to effective management.

Websites maintained by working groups will assist in data management and information dissemination. For example, pesticide treatment schedules as well as recommended pesticides may be published on a dedicated webpage, or an outreach channel chosen by each country's phytosanitary authority.

2.2.4 Prioritization of areas for regional control

The regulatory authorities should prioritize the establishment of area-wide management programs for ACP and HLB where regional conditions are conducive to HLB outbreaks. The following biological and epidemiological criteria should be considered when assessing such conditions: (1) host abundance; (2) host susceptibility; (3) significant geographical barriers and distance between sites (as sources of infection); (4) inoculum load; (5) climatic factors; and (6) number of sites with previous and current positive disease detections and distance between sites (as sources of infection). These criteria will inform decisions regarding the size and shape of the control area as well as the number of participating growers.

In California, PMAs are designed to optimize communication between regional Citrus Pest and Disease Prevention Program Grower Liaisons, local volunteer Team Leaders, and the group of growers with commercial citrus groves located within each PMA. PMA boundaries are established to limit either the number of growers (25 – 30, in North Central areas), or the area under production (<2,000 acres, in Southern areas). This is not the case in Mexico, where, due to the orchard size per capita, the AMEFIs include hundreds of growers at a time, which makes it more challenging to implement.

In addition to the aforementioned criteria, the determination of the number and location of regional control areas considers practicalities such as the availability of infrastructure, and human and economic resources in the citrus-producing states. The desired level of suppression is also important. By applying epidemiological approaches, rational criteria based on the principles of prevention and protection are used to establish regional areas for ACP control in Mexico.

2.2.5 Monitoring

Trapping

Yellow sticky traps are used to detect populations of ACP and relate these to host species. These traps serve several purposes including: (1) evaluate the effectiveness of pesticide applications and releases of biological control agents; (2) determine optimal timing for regional pesticide applications; (3) minimize unnecessary treatments; and (4) identify vector outbreaks by grove/orchard (sources of infestation). In a comparative study in Brazil and Texas, yellow sticky traps were the most sensitive method for detecting low ACP populations and comparing effectiveness of different treatments (Miranda *et al.*, 2018; Monzo *et al.* 2015). Trapping data should be collected weekly or biweekly and delivered to a centralized point for processing. Information systems should store the data and enable its analysis for decision-making at different levels (e.g., state, orchard). To render trapping more efficient, yellow sticky traps may be deployed along the border of groves/orchards where most of the ACP concentrate (Sétamou and Bartels, 2015). In California, yellow sticky traps are placed within orchards and surrounding areas to detect ACP and guide the application of pesticides.

Mexico designed and implemented a monitoring system for ACP based on a trapping system called SIMDIA (Monitoring system for *Diaphorina citri* (because of its Spanish acronym http://www.siafeson.com/simdiatecnicos/)). The system provides information on ACP levels at the national, state, AMEFI, orchard and trap levels. The SIMDIA system facilitates timely decision-populations and adjustments to scheduled regional pesticide applications, targeted interventions in areas with increasing vector populations, and informs other management strategies.

In Mexico, direct visual orchard/grove monitoring is conducted biweekly on four young shoots per tree, each located at a different cardinal point (N, S, E, W) at inspector's height. The number of ACP nymphs and adults is quantified, and the infested shoot size is determined. Data is useful to understand ACP population fluctuations, which cause primary infection in orchards as well as to determine the appropriate timing for control actions. The data can also help mitigate the risk of ACP spread to areas where the disease is not present, as well as avoid secondary infections in areas with presence of HLB.

Tapping

Tap sampling ("tapping") is an efficient method to monitor moderate to high ACP populations (Qureshi and Stansly 2007; Qureshi *et al*., 2014a; Monzo *et al*., 2015). In the USA, it is recommended for routine monitoring by using 100 taps per block of any reasonable size, taken in groups of 10 blocks per location in 10 different locations, with five taps on the periphery and five in the interior of the block (Monzo *et al.,* 2015). This approach provides population numbers with approximately 25% accuracy down to about one ACP per 10 taps. Inspection of 10 young shoots per location, to determine percent infestation and flush density is also recommended (Sétamou *et al.,* 2008). Very low ACP populations are better detected using yellow sticky traps or vacuum sampling methods instead of tap sampling.

Tap sampling was adapted in Florida by the Citrus Health Response Program (CHRP) with the goal of monitoring 6,000 "multi-blocks" every three weeks. Fifty tap samples are taken, 10 in each of the four cardinal extremes of the block and 10 in the center. This information is uploaded to the CHMA management area website where it is available to the multi-block owner and whoever else he or she designates. The data are mapped and made available to members of participating CHMA and others to chart surveillance progress and ACP population densities in groves/orchards.

In Texas, a combination of trapping, tap sampling and visual observation is used in 200 sentinel groves every two weeks for ACP monitoring and determining infestation levels.

In Mexico, tap sampling is used (after an outbreak is confirmed through direct monitoring) to delimit outbreaks of ACP and to monitor localized pesticide applications. Outbreaks could be delimited to a group of trees or areas such as orchard borders. Tapping consists of three quick successive taps on a branch with shoots or with young leaves at approximately 1-1.5 meters height. Displaced insects will fall on a white sheet of paper where counts are made. Data is digitally recorded to allow for decision-making on needed treatments.

To determine the effect of regional pesticide applications on ACP load in Mexico, a sample of up to 100 adults is collected at each AMEFI, one week before each regional application, followed by another sample one month after the application. Monitoring only applies to AMEFIs where HLB and the vector are present. This activity is more relevant in areas where the disease is endemic and allows the establishment of regional action thresholds to mitigate the spread of the disease to low-incidence areas.

Visual Observation
APHIS PPQ has conducted visual observations of nymphal densities since 2010 to monitor the impact of biological control efforts against ACP (Flores and Ciomperlik, 2017).

Detection of HLB outbreaks and determination of infection level
To detect presence of the HLB bacterium, plants and plant tissue with symptoms and possible vectors are collected for laboratory diagnosis. Scouting for HLB is considered valuable if the incidence is low enough to justify removal of symptomatic trees. Incidence of more than 3 - 4% per year is considered too high by growers. In Texas, growers continue to actively remove infected trees during grove establishment (from planting to 3 yrs-old). However, HLB-affected trees are seldom removed in bearing groves. HLB scouting for detection and removal purposes needs to be undertaken frequently - at least four times a year. Scouts must be trained to recognize symptomatic trees and have the equipment necessary to inspect tall trees. California uses an HLB risk-based survey for early detection of HLB in residential areas. The risk is determined using several factors such as data on international travel, ACP density, *C*Las positive ACP samples, potential ACP source, transportation corridors, packinghouses, farmer's market, military installations and sovereign land, organic citrus, and weather suitability. Using these factors, total risk is determined for each square mile grid, resulting in a recommended sampling density (CPDPD Action Plan 2022).

If the objective is to evaluate the effectiveness of management programs or provide information on positive HLB detection rates, it may be valuable to dedicate time to estimating HLB incidence. One straightforward method in California is to count the number of symptomatic trees among the 50 or 100 trees in some or all the blocks being monitored for ACP.

It is important to recognize that inconclusive or suspect samples (e.g., those with any measurable detection by real-time polymerase chain reaction (qPCR), that exceed Ct thresholds) are likely infected with Liberibacter but may not meet the regulatory standards for conclusive diagnosis. Such cases should be closely monitored as part of ongoing surveillance. In California, properties that had inconclusive plant samples are resampled six months after the laboratory identification is confirmed (CPDPD Action Plan 2022).

2.2.6 Rational use of insecticides

For applications both in an entire region or in individual outbreak sites, priority must be given to insecticides registered by the regulatory authority against ACP in citrus production. The AMEFI protocol used in Mexico (https://www.gob.mx/senasica/documentos/estrategia-operativa-plagas-reglamentadas-de-los-citricos-69755), recommends pesticide rotation to manage insect resistance and reduce emergence of secondary pests, such as the citrus blackfly (*Aleurocanthus woglumi*).

California's protocol for ACP is to apply a foliar contact spray for immediate effect and a systemic treatment for longer lasting protection (https://ipm.ucanr.edu/agriculture/citrus/asian-citrus-psyllid/).

For resistance management and good pesticide stewardship, growers should not rely on a single class of insecticides for ACP control. A rotation of Insect Resistance Action Committee (IRAC)-mode of action products needs to be used in season-long ACP control programs.

Chemical control of ACP will still provide benefits in terms of increased yield even if incidence of HLB approaches 100% (Chen *et al.,* 2022). However, a threshold approach during the growing season may be more cost-effective than monthly sprays. The choice of insecticide will depend on other pests present at the time of application. Back-to-back pesticide applications of the same class (mode of action) are not recommended. The choice of chemical depends on several parameters, including what has already been used during the season (to avoid exceeding the maximum allowable amount per season), the pre-harvest interval (because dormant sprays are also applied at harvest), and costs deemed reasonable by the grower. The selection of citrus commodities to be exported must consider the requirements of the country of destination. In general, the chemicals used should be of low environmental risk, and harmless to humans and animals (Cortéz *et al*., 2013).

ACP is not endemic in most areas of California. The state applies a foliar and systemic insecticide treatment on all residential hosts within 250 meters of an HLB detection, as well as within 50-400 meters of an ACP detection outside of the generally infested areas. Chemical control is targeted and serves to reduce or eliminate ACP populations. Furthermore, in addition to growing season applications, in regions where ACP is established, the University of California Citrus Pest Management Guidelines also recommends a fall and a winter spray for orchards (UCANR 2022).

Coordinated insecticide application

When growers are organized into regional or area-wide management areas (such as AMEFIs, CPDMAs, CHMAs, or PMAs) insecticide applications can be efficiently coordinated. A coordinator from each management area can communicate with growers on precise application timing. All growers would be urged to spray their orchards within a two-week time frame.

Pest outbreaks often occur when growers fail to respond promptly during a vegetative flush cycle or when orchards are near residential zones with abundant citrus trees, from where infected ACP adults can move into groves (Sétamou *et al*., 2022). In these situations, targeted low volume or reduced volume spraying or a number of applications can be used.

Some growers have adopted the orchard border treatment program, specifically between major flush cycles. Orchard border treatments prevent ACP incursions into the orchard (Sétamou and Alabi, 2018). Its success depends on a good monitoring program to detect adult ACP before a new outbreak. To further prevent ACP incursions into orchards participating in coordinated treatments in California, CDFA may also apply insecticides to citrus growing in 250-meter to 800-meter buffer areas around these orchards. CDFA's treatments in residential buffer areas are coordinated with orchard's treatments to drive ACP populations further away from the orchards.

ACP control programs are typically part of a multi-pest control approach. Although ACP-specific sprays are used, most sprays are tank mixes that target other pests present at the time of application. When the production goal is fresh fruit for commercial use, growers should exercise extreme caution in the use of broad-spectrum insecticides, which pose the risk of emerging secondary pests or chemical residues remaining on the fruit. The success of the ACP area-wide management program in Texas partially hinged on using judicious pesticide tank mixes targeting pests present in the groves at the time of pesticide applications, thereby reducing the number of applications during the year.

Trees should not be treated with broad-spectrum chemicals during the flushing cycle because they will affect natural enemies and pollinators. However, sprays during flush cycles have provided significant reductions in ACP populations and may be useful in regions where biological control is not strong/active (Qureshi, unpublished). Growers should actively monitor both adult and immature ACP populations during the flushing cycle and apply sprays if increasing populations persist over a three-week period. Only registered products should be used.

Research has shown that ACP populations move between two habitats (Setamou, *et al*., 2022) resulting in reinfestation of commercial orchards. When feasible and in cooperation with regulatory authorities (federal, state, or local government entities) and commercial growers, a residential buffer treatment may be applied to protect commercial orchards. The residential buffer treatment will push populations of ACP further away from commercial orchards thus minimizing reintroduction. California may implement treatments around commercial citrus production for growers that meet the requirements to receive additional residential buffer surrounding the PMA (CPDPD action plan 2022).

2.2.7 Biological control

The use of biological control is an important strategy to reduce populations of ACP. Biological control helps decrease environmental damage due to the use of agrochemicals. Available biological control agents (BCAs) against ACP include predators, parasitoids and entomopathogenic fungi.

Some zones and sites within a management area may not be suitable for the application of chemicals such as those of organic producers that only employ Organic Materials Review Institute (OMRI) approved organic products. In the urban environment, where homeowners may have backyard citrus trees, chemical treatments may not be feasible or acceptable. In these cases, the use, conservation, mass-production, and release of BCAs is particularly helpful. Biological control is not a short-term solution but it contributes to the sustainable management of ACP populations.

In California, the CDFA rears *Tamarixia radiata* (Hymenoptera; Eulophidae) (Chen and Stansly, 2014; Chen *et al*., 2014; Michaud, 2002) in controlled laboratory facilities. These parasitoids are released in areas around HLB detections, near the California-Mexico border, along trade routes, and in residential areas to supplement chemical treatments. Mexico produces and releases millions of *Tamarixia radiata* annually.

2.2.7.1 Biological control with arthropods

Adult and immature lacewings (Neuroptera: Chrysopidae) are highly effective predators for controlling ACP because of their high feeding capacity and commercial availability. Lacewings of the genus *Chrysoperla* and *Ceraeochrysa* can regulate ACP nymph populations (Qureshi and Stansly 2009; Cortéz-Mondaca *et al*.; 2011; Pacheco-Rueda and Lomelí-Flores, 2012). Ladybeetles (Coleoptera: Coccinellidae) such as *Olla v-nigrum* are important ACP nymph predators (Qureshi and Stansly 2009; Pacheco-Rueda and Lomelí-Flores, 2012). Two other coccinellids were recently identified attacking ACP nymphs: *Exochomus insatiable* (Rodríguez-Vélez, 2018) and *Cheilomenes Immaculata* (Chávez *et al*., 2017; Chávez *et al*., 2019). Both can be commercially produced. The two-spotted ladybeetle *Adalia bipunctata* (Coleoptera: Coccinellidae) (Khan *et al*., 2016) is an efficient predator of ACP nymphs. Another parasitoid of *D. citri,* is *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae) (Bistline-East *et al*.; 2015). It was introduced to Florida from Taiwan (Hoy and Nguyen 2001), although it has not been as effective as *T. radiata*. *D. aligarhensis* is present in some regions in the United States and Mexico (Cortez-Moncada *et al*., 2010).

Other generalist predators feed on *D. citri*, such as flies from the Syrphidae family, bugs from the

Reduviidae family, and wasps from the Vespidae family, in addition to other arthropods and spiders (Kondo *et al*., 2015; Qureshy and Stansly, 2009), and a predatory mite *Amblyseius herbicolus* (Kalile *et al*., 2023).

BCAs must have certain characteristics to effectively control ACP: be host-specific; be synchronous with ACP; be able to rapidly increase their densities; complete their life cycle on ACP; have a high search rate for ACP; and aggregate in areas of high ACP density (Murdoch *et al*., 1985). Among the parasitoids, *Tamarixia radiata* has important advantages – such as its high level of specificity and parasitism on 3rd and 5th instar nymphs of ACP, and its excellent search ability for ACP nymphs – that make it an ideal candidate for use as a BCA, above all in abandoned orchards, organic orchards , or in orchards with little management (Aubert 1987; Étienne *et al*., 2001; Skelley and Hoy 2004; Chow and Sétamou 2022; Milosavlievic *et al*., 2022). *Tamarixia radiata* has been imported and released in control programs on the islands of Réunion and Taiwan (Étienne and Aubert 1980; Chien 1995; Hall 2008; Qureshi *et al*., 2009), as well as in Florida, California, Louisiana, Puerto Rico, and Texas, United States (Hoy and Nguyen 2001; Qureshi *et al*., 2014b; Kistner *et al*., 2016; Flores and Ciomperlik 2017, Chow and Sétamou 2022). Although there are no records for an official introduction, *T. radiata* is present in Mexico (Sánchez *et al*., 2015) and Ecuador (Chavez *et al*., 2017), and augmentative releases have been implemented in the United States and Mexico (Rodriguez Vélez, 2018; Chávez *et al*., 2019). México has a mass-rearing laboratory for *Tamarixia radiata* (CNRCB, 2016), and has implemented augmentative releases in most of the citrus-growing states of the country. Similar mass-rearing facilities are available in the United States (California).

In Florida, residential citrus plantings are problematic because of inconsistent or complete lack of pesticide applications, but their negative impact on ACP populations is small compared to that of abandoned and unmanaged orchards (Hall D., pers. comm.). Texas and California share the concern for ACP spreading from urban neighborhoods (where many varieties of citrus trees are found) to nearby commercial citrus-producing zones (Sétamou *et al*., 2022; Arredondo *et al*., 2013). In most cases, ACP moves from residential to commercial citrus and not vice versa, because of the abundance in type and quantity of host material (Sétamou *et al*., 2022). If left unmanaged, populations of ACP in residential areas can interfere with the effectiveness of regional or area-wide management programs such as AMEFIs. Control of ACP in urban environments must rely on BCAs; it is the most practical and acceptable method, as there are challenges to acceptance of chemical treatments because of problems with contamination and intoxication when chemicals are used in urban environments.

Parasitoid releases

Releases of *T. radiata* should be timed based on flushing patterns and infestations on host material. The number of parasitoids to be released is based on a variety of risk factors considering vector population and the capacity of mass-rearing facilities. For example, in Texas, 100–500 parasitoids per site have been released. Releases are targeted to parks and residential neighborhoods as well as areas with host material within one mile of orchards. An alternative method used by APHIS PPQ in both Texas and California uses field cages to cover citrus trees in the urban environment. These cages are used for the production and release of a large volume of parasitoids in Texas (Daniel Flores, pers. comm.). Releases made in conventional orchards have been shown to increase ACP parasitism rates in Florida (Qureshi and Stansly 2019).

In Mexico, depending on the levels of ACP nymph infestations in urban areas, 100, 200 or 400 parasitoids are released per 100 m^2; in commercial and abandoned orchards, and depending on the infestation level, 1,400 up to 7,000 parasitoids/ha are released.

Parasitoid releases in unmanaged orchards

In unmanaged orchards, releases of *T. radiata* may be made at any time of the year if eggs or nymphs of ACP are present and temperatures are between 20 and 35ºC (Sánchez-González *et al.*, 2015). The parasitoids should be released at the rate of 400 insects per hectare (Sánchez-González *et al.*, 2011b). This rate may result in a reduction of up to 92.6% of the population of third to fifth instar ACP nymphs after five months of weekly releases (Sánchez-González *et al.*, 2011a). Studies on the dissemination of *T. radiata* show that parasitoids disperse in groups, using the wind; wind direction and velocity should therefore be considered before parasitoid release (Sandoval-Jiménez *et al.*, 2013).

Parasitoid releases in urban areas

There is concern that ACP may be spreading from abandoned citrus orchards and urban areas into commercially viable orchards. Therefore, it is important to manage ACP in these areas to protect commercial orchards. The rate of parasitoid release in urban areas should be 100 parasitoids per 50–100 linear meters, depending on the extent of the infestation; or in other words, if more than 20 ACP nymphs per bud per tree are observed, then 100 parasitoids should be released every 50 meters (CNRCB 2011). Just as in the case of unmanaged orchards, releases in urban areas may be done at any time of the year if eggs or nymphs of ACP are present and temperatures are between 20 and 35ºC. The effectiveness of *T. radiata* released in urban areas can reach up to 71% (Moreno- Carrillo *et al.*, 2012).

2.2.7.2 Biological control by entomopathogenic fungi

Entomopathogenic fungi may be considered a more environmentally friendly alternative for control of ACP in commercial citrus orchards (Maluta *et al.,* 2022). Entomopathogens may be used in a rotation with pesticides when humidity and temperature conditions are favorable. Research and laboratory testing must continue to determine which species and strains of entomopathogenic fungi are appropriate for different citrus-producing regions that have ACP. The choice of strain will depend on the results of validation tests (Sánchez *et al.,* 2015).

In Mexico, the strategy in the AMEFIs includes the use of strains of *Cordyceps javanica (*prior *I. fumosorosea) (*candidate races *CNRCB-CHE 303, 305 y 307,* prior *Pf15, Pf17 y Pf21)* and *Metarhizium anisopliae* (CHE-CNRCB 224, prior Ma59) (Mellín-Rosas *et al.,* 2009; Ayala *et al.*, 2015) for the control of ACP. These strains are kept in the Entomopathogenic Fungi Collection of the Plant Health General Directorate of SENASICA.

ACP area-wide management programs in Texas and Florida rely on insecticide sprays in the dormant winter season and before major flush cycles (Stansly *et al.*, 2009b; Chow *et al.*, 2013; Wright 2015; Saldarriaga Ausique *et al.*, 2017; Sétamou, 2020). However, populations of ACP in Florida are becoming less susceptible to some insecticides (Kanga *et al.*, 2016; Chen and Stelinski 2017), and the use of entomopathogenic fungi has less adverse effects on human health and the environment (Chow *et al.*, 2013). In the United States, ACP is susceptible to several entomopathogenic fungi. *Cordyceps javanica* is showing positive results in south Texas; in one study, 94% of ACP adults and nymphs were killed within four days of infection (Chow *et al.*, 2013). In Florida, *C. javanica* alone or mixed with white oil suppressed ACP adult populations by 61-83% up to 14 days after treatment (Avery *et al.*, 2021).

Application of entomopathogenic fungi

In commercial orchards that meet the conditions for temperature (22–28°C) and relative humidity

(>80%), applications of entomopathogenic fungi may be made throughout the entire orchard (Zimmermann 2008). In Mexico, these conditions occur from November to February; however, the specific timing and effectiveness of the entomopathogenic fungi application should be assessed before field implementation and will depend on several factors including the presence of ACP, microorganism management from the laboratory to the field and proper application equipment. In general terms, applications of entomopathogenic fungi to control ACP are made at a concentration of 1×10^7 conidia/ml. The amount applied per hectare will depend on the amount of water required to cover the area. If a residual population of ACP is detected after application, the material should be reapplied 10 days after the first application, but only in areas that meet the environmental conditions. Applications should be made in the evening hours (after 16.00 hours) as the fungus will survive better under the conditions of temperature and relative humidity at that time.

The equipment used for the application of the entomopathogenic fungi should allow for all the foliage of an ACP infested tree to be sprayed to increase the probability of infection, while guaranteeing the dose of 1×10^7 conidia/ml. The equipment should be free from residues of fungicides, insecticides, fertilizers, and herbicides. Preparations of fungi should be applied on the same day they are made.

2.2.8 Inspection and abatement warrant for Huanglongbing eradication

Removing HLB infected trees using an inspection and abatement warrant for refusal properties is a crucial step in California's ability to protect the health of citrus in surrounding residential areas, and prevent the spread of HLB into commercial citrus groves. Established HLB delimitation areas by the CDFA require the removal of all HLB positive trees confirmed by qPCR testing. According to the CDFA's established Emergency Proclamation, they are authorized to take such actions pursuant to Food and Agricultural Code, section 5763, as may be necessary to eradicate HLB. If the resident does not schedule the removal, an abatement warrant is issued by CDFA staff and local law enforcement to gain access to the property and remove the infected trees.

3.0 Additional Tools for Management of Huanglongbing and its Vector

The following information is adapted from the Executive Summary of the Technical Working Group report, area-wide control of Asian citrus psyllid (*Diaphorina citri*) (USDA 2009).

3.1 Cultural management

The following tactics are recommended for area-wide control programs:

- Removal of symptomatic and/or qPCR positive trees to reduce inoculum.
- Destruction of abandoned orchards.
- Encouragement of urban dwellers to replace ACP host plants with non-host plants in their gardens or, failing that, to control ACP. Caution should be exercised when choosing a new plant to avoid attracting other significant pests.
- Management of untreated citrus and other hosts such as orange jasmine (*Murraya paniculata*) and box orange (*Severinia buxifolia*) through mass releases of *T. radiata* (especially in urban areas).
- Flush management such that reproduction by ACP is limited to twice per year. This would greatly reduce psyllid populations.
- Planting of new citrus blocks to reduce the relative proportion of edge trees with

regard to the enclosed area.
- Adjusting production practices such as fertilization and watering to ensure a vigorous root system of trees.
- Monitor and determine protection of pruned trees due to its vigorous vegetative shoot, which will become a reproduction site for ACP out of season.

3.2 Outreach, education, coordination, and extension

Important elements include:
- Appropriate information should be developed and disseminated by the NPPO, state, regional, local and industry groups to the general public and stakeholders: agricultural news media, home gardeners (backyard trees), tribal governments, packers and shippers, migrant farm laborers, people who might move fruit or plants from one place to another, farmers' market personnel, floral market personnel, and ethnic grocery stores personnel.
- Extension specialists should disseminate the appropriate information through established mechanisms in each state or area.
- The provision of information to all commercial growers, packers, urban growers, and so forth on the importance and timing of the area-wide control program will be essential.
- Recruiting urban area residents and stakeholders in the area-wide program to report any psyllids will provide information to extension personnel and regulatory officials on the presence of ACP in new areas,
- Involve urban area residents and stakeholders, especially near commercial groves, to encourage participation in biological control programs.

3.3 Residential property and commercial orchard surveys

California employs residential risk-based surveys and commercial orchard commodity surveys for early detection of HLB. CDFA utilizes the following strategies to conduct residential surveys:

- The HLB risk-based model is used to determine high, medium, and low HLB introduction risk grids (each covering one square mile = 2.5899 square km) across the state. Input factors to the model include historical detections of ACP and HLB, census travel data for international introduction, citrus transportation corridors, location of citrus related plant nurseries, big box stores, packing houses, farmer markets, military installations, Native American lands, and organic citrus orchards.
- CDFA assigns staff year-round to survey selected grids with high HLB risk of introduction. In addition, survey is also conducted for grids near commercial citrus orchards or lower-risk areas seldom surveyed to account for model bias.
- Each assigned grid has a specific target for properties to survey, ranging from 5 to 200 properties depending on risk level and density of residential trees within the grid. By surveying enough properties within the grid, the likelihood of a CDFA surveyor identifying an HLB-infected tree is significantly increased. This approach allows for a more comprehensive and thorough search, enhancing the chances of early detection.
- Samples are collected from trees showing symptoms of HLB for *C*Las testing. ACPs are also collected for *C*Las testing. The survey results serve as inputs to the risk-based model, informing future survey efforts.

CDFA employs the following strategies to conduct surveys on commercial orchards for early HLB detection:

- The objective is to survey all commercial orchards throughout the state within a five-year period. To ensure comprehensive coverage, orchards are randomly selected each year to maximize the geographical coverage.
- In general, for each orchard, CDFA inspects all corner trees and all trees every five rows. However, for orchards larger than 100 acres, staff inspect all trees every ten rows. This systemic approach enables thorough inspection while accounting for orchard size. During inspections, symptomatic plant samples are collected for further analysis.
- In areas not known to be infested with ACP, all corner trees within each orchard are tapped to check for the presence of ACP. This additional step helps monitor the potential spread of ACP and their association with HLB.
- All ACP and plant samples collected are analyzed for the presence of *C*Las. This analysis provides valuable information regarding the presence and spread of HLB in commercial orchards.

If *C*Las is detected during residential surveys or commercial orchard surveys, intensive surveys will be initiated. These surveys involve inspection of all citrus host trees within a 250-meter radius of the detection site(s).

4.0 Regulatory Actions

In addition to insecticide application and biological control, regulatory actions can be part of the toolbox for area-wide management. NPPOs may consider establishing and enforcing regulations related to:

- Detection and identification methodology for HLB and ACP
- Certification of disease-free propagative material
- Removal of plants contaminated with HLB
- Movement of HLB-free propagative material
- Movement of fruit free from other plant material
- Restrict movement of unprocessed fruit from areas with ACP into uninfested areas
- Establishment of quarantine areas
- Training and outreach campaigns

In California, the CDFA employs a pest prevention approach that includes quarantines and regulatory enforcement to manage ACP and HLB. The regulatory component, starting with exclusion to prevent the introduction of pests, complements the detection, eradication, biocontrol, and outreach. The subsections below detail California's regulatory approach.

CDFA regulates the intrastate movement of ACP and HLB host material pursuant to Title 3 of the California Code of Regulations (CCR) section 3435, Asian Citrus Psyllid State Interior Quarantine and section 3439, Huanglongbing Disease State Interior Quarantines. In addition, APHIS PPQ regulates the interstate movement of ACP and HLB host material pursuant to the Code of Federal Regulations (CFR), title 7, section 301.76, for Citrus Greening and ACP. These state and federal regulations establish the quarantine areas, hosts and possible carriers of the pest, and the prohibitions or conditions which enable movement of hosts within or from the quarantine area.

In addition to requirements regarding ACP and HLB, all citrus nursery stock produced and/or sold in California must meet the requirements found in 3 CCR section 3701, Citrus Nursery Stock Pest Cleanliness Program (NSPCP). All source trees for citrus nursery stock propagative materials are registered with the Citrus NSPCP and must meet testing and maintenance requirements.

4.1 ACP regional quarantine

CDFA regulates ACP through a regional quarantine based on pest risk criteria. Using these, CDFA may modify the regional quarantine zones when survey results indicate the presence of an ACP or HLB infestation. In addition to the regional quarantine zone, ACP regulation also includes regulated articles and commodities, restrictions on movement of articles and commodities, and exemptions. All equipment used to harvest, prune, process, or transport any hosts of ACP and HLB must be cleaned and/or treated to eliminate all life stages of ACP prior to movement out of the HLB quarantine area.

Compliance agreements are signed with all regulated establishments to ensure quarantine requirements are understood and followed. Enforcement activities include conducting nursery inspections, monitoring pesticide treatments, checking treatment and sales records, and inspecting citrus fruit growers, packers, transporters, and fruit sellers.

4.2 HLB quarantine

Pursuant to 3 CCR section 3439, CDFA establishes a quarantine area with a five-mile radius from each HLB positive tree detection. HLB host nursery stock, propagative plant parts (except seed extracted from fruit), and fruit are prohibited from moving out of the quarantine area unless they meet the requirements outlined in 7 CFR section 301.76. Following each confirmed HLB detection, CDFA issues a hold notice for all host material from the property where HLB was found, and the positive tree is treated or removed. All host material is considered compromised and placed on hold to prevent further spread of HLB.

4.3 Compliance agreements

Compliance agreements issued by CDFA convey the quarantine restrictions and requirements to affected businesses located within a regulated area. They are issued to all citrus growers, harvesters, transporters, packers, fruit sellers, and production and wholesale nurseries. Under a signed compliance agreement, regulated establishments are permitted to move host material while adhering to the terms of the agreement and with general CDFA oversight. While signed agreements are self-executing, CDFA conducts periodic inspections to ensure compliance.

4.4 Safeguarding requirements

All bulk citrus transporters/haulers are required to completely safeguard citrus fruit while in transit within or from a bulk citrus regional quarantine zone. Safeguarding of fruit can be accomplished in any manner that prevents the fruit from exposure to ACP and prevents loss of fruit, leaves, stems, branches, or plant debris while in transit. Safeguards must be in place and remain in place until the vehicle reaches its destination for offloading.

4.5 Special permits

Under 3 CCR section 3154, special permits may be issued to allow movement of regulated articles and commodities which would otherwise be prohibited. This may occur when there is a specific demonstrated need and the terms and conditions of the permit adequately mitigate the risk of pest spread. These permits called Quarantine Commodity (QC) permits, may be issued to individuals, businesses, researchers, or to CDFA program staff. QC permits have been issued for movement of nursery stock and propagative material, bulk citrus fruit, mandarins with attached stems and leaves, leaves for consumption, green waste, and for the removal of HLB suspect trees for research.

Additionally, special federal and state permits are issued to support research related to ACP and HLB. State permits are required for intrastate movement and use of ACP, HLB and their hosts. Federal permits are issued to researchers for interstate movement of regulated organisms. For example, a federal permit was issued for the movement of *T. radiata* with its ACP host into Florida to establish a colony. A portion of that colony was subsequently moved interstate under another federal permit into California to the University of California (UC) Riverside's Contained Research Facility. Once it was determined that the ACP/*T. radiata* colony was not contaminated by other organisms, the federal permit was modified to allow experimental releases of *T. radiata* in California. This activity now takes place under the terms of a state permit. State plant pest permits are also issued to researchers to maintain ACP-infested nursery stock to determine the efficacy of conventional and organic pesticides in California.

5.0 Detection and Identification Methodology for HLB and ACP

Detection of *C*Las in citrus trees can be challenging due to the long latency of the disease, variation in symptom expression, low titer, uneven distribution within the tree, and difficulties in sampling large dense trees for symptomatic leaves. It is therefore essential to adopt effective sampling and early detection methods that have been rigorously validated to provide high specificity, sensitivity, and reproducibility in detecting *C*Las. Early detection leads to quick removal of HLB-infected trees, which is critical to reducing inoculum in the field and slowing the spread of the bacterium. The CDFA Plant Pest Diagnostics Center (PPDC) plant pathology laboratory performs HTS using a USDA validated qPCR test for the detection of *C*Las.

5.1 Sample collection, tracking, and handling

*C*Las is phloem-limited. Therefore, phloem-rich tissues are targeted during collection. General sampling consists of collecting 20 leaves (preferably symptomatic) with intact petioles and 6-8 peduncles from each tree. Peduncle tissue was recently reported as a reliable tissue to test for *C*Las (Hajeri *et al*., 2023). However, when collecting from higher risk trees - such as remaining trees on HLB positive find sites, trees on adjacent sites, and qPCR inconclusive trees - more intensive sampling is done by partitioning the tree into four sections and sampling peduncles and roots (Braswell *et al*., 2020) in addition to leaf sampling. Intensive sampling collects more subsamples increasing the likelihood of CLas detection especially in trees with low titer infections.

For tracking, samples are checked, sorted, assigned unique laboratory accession numbers, and batched into groups of 92 samples for HTS. The barcode labels associated with the samples are scanned using a mobile PDR Batch App to extract pertinent sample metadata from the CDFA Pest and Damage Record (PDR) database and populate an Excel master spreadsheet maintained in the laboratory by the lead pathologist. Samples are handled sequentially, assigned to individual technicians, and stored in designated cold rooms until tests are completed. Final test determinations are recorded in the CDFA PDR database accessible to state and county officials.

Samples are prepared and tested in batches of 92 samples. The samples are prepared and chopped inside a class II Biosafety hood. Supplies used such as gloves, razor blades, etc. are changed for each sample batch. DNA is extracted from the petiole, midrib, peduncle, and root tissue in a separate room equipped with robotic liquid handlers programmed for high-throughput DNA extraction. qPCR is performed in a designated room isolated from the rest of the laboratory. A unidirectional workflow and clear separation between tissue preparation, DNA extraction, and qPCR is strictly adhered to to prevent contamination.

5.2 Laboratory tests

Currently, qPCR is the standard regulatory screening method used for detection of *C*Las in both plants and ACP. It is a quick test that can be completed in an hour, has high specificity, sensitivity, and reproducibility, and can be scaled-up for testing thousands of samples per month. Three USDA validated HLB TaqMan qPCR protocols are used for screening plants and ACP and for confirming all *C*Las positive and inconclusive samples. qPCR assays with the Ribonucleotide Reductase (RNR) gene, multiplexed with the plant cytochrome oxidase (COX) gene internal positive control, and optimized for the ABI QuantStudio 5 or 7 and the ABI 7500 Fast Real-time PCR System, are used to screen plant DNA for the presence of *C*Las. The RNR real-time PCR assay targets a conserved partial sequence of the RNR gene present in five copies per *C*Las genome (Zheng *et al*., 2016). RNR qPCR Root assay is similar to the RNR assay described above but includes extra treatments to remove PCR inhibitors commonly found in roots. A HLBas 16S qPCR assay, with 16S rDNA-based primers and a probe specific to *C*Las, multiplexed with the psyllid glycoprotein (WG) gene-based probe-primer set as a positive internal control, and optimized for the ABI QuantStudio 5 or 7 and the ABI 7500 Fast Real-time PCR System, is used to screen ACP adult and nymph DNA for the presence of *C*Las. The HLBas 16S assay targets a specific partial sequence of the 16S rDNA present in three copies per *C*Las genome.

Mexico uses a qPCR-based protocol developed by the National Center for Plant Health (CNRF because of its acronym in Spanish) for the diagnosis of *C*Las in plant and insect material. Primers are specific for *C*Las. Positive internal controls for plant material include probes based on the COX gene and the 16S ribosomal RNA gene. For ACP, primers are based on the psyllid glycoprotein gene and 16S rDNA.

5.3 Laboratory accreditation

The CDFA Plant Pest Diagnostics Laboratory in Sacramento and the Citrus Research Board's Jerry Dimitman Laboratory in Riverside are the only laboratories in California with USDA accreditation to test ACP and plant samples for the HLB bacterium. CDFA's collaboration with the Jerry Dimitman Laboratory supports the department's HLB testing program and helps to protect the multibillion-dollar CA citrus industry. Both laboratories must adhere to USDA work instructions and communication protocols and maintain strict accountability for the security and integrity of all samples tested - from sample receipt to sample destruction.

To maintain accreditation, laboratory personnel must complete a three-day hands-on training at the USDA APHIS PPQ Plant Pathogen Confirmatory Diagnostics Laboratory (PPCDL) in Laurel, MD, in HLB molecular diagnostics. Laboratory personnel must also pass the annual HLB Proficiency test administered by USDA to renew their qualified standing. All laboratory equipment must be calibrated yearly and undergo annual preventative maintenance. The laboratory must provide clear separation between the different stages of testing and adhere to strict cleanliness guidelines and workflow to prevent contamination of samples and equipment.

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