OPERATIONAL DIAGNOSTICS OF HLB (HUANGLONGBING) IN THE USA

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ABSTRACT

The rapid and accurate detection and identification of the species of Candidatus Liberibacter that cause Huanglongbing (HLB, citrus greening) is essential to a successful response and recovery system. Ideally a diagnostic method would be available for use in the field and which could detect infection even prior to visible symptoms. Since reliable and economical field-based diagnostic methods aren’t available it is important that a careful field sampling and laboratory diagnostic system be developed and utilized. This talk will focus on the operational diagnostic system used in the U.S. to survey for and diagnose the disease, including sample collection, use of a laboratory network, molecular diagnostic assays, communication of diagnostic results, and select agent considerations.

DIAGNÓSTICO DEL HLB (HUANGLONGBING)

Palabras clave: Enfermedades de los Citrus, Psílido, huanglongbing

RESUMEN

La rápida y adecuada detección e identificación de las especies de Candidatus Liberibacter que causan Huanglongbing (HLB, Enverdecimiento de los cítricos) es esencial para una respuesta exitosa y un sistema de recuperación. Idealmente un método de diagnóstico debería estar disponible para usarse en el campo y el cual pudiera detectar la infección antes que los síntomas fueran visibles. Aunque no exista un método de diagnóstico económico y de campo, es importante que se desarrolle y utilice un sistema de muestreo cuidadoso en campo y diagnóstico de laboratorio. Esta presentación se enfocará en el sistema operacional de diagnóstico usado en los Estados Unidos de Norteamérica para una revisión del diagnóstico de la enfermedad, incluyendo la toma de muestras, el uso de una red de laboratorios, los ensayos de diagnóstico molecular, la comunicación de los resultados del diagnóstico y consideraciones del agente seleccionado.

Sample collection

Plant

Visual observation of citrus plants in the field for symptoms of HLB is the first step in a survey for the disease. It is best to select six to eight twigs, each approximately 4-8 inches long, of the current year’s growth from the upper canopy of a symptomatic tree. Each sample of the twigs with symptomatic leaves attached should be kept separate from other samples and double bagged in resealable plastic bags. The samples should be kept at cool temperatures and shipped by overnight courier to the diagnostic laboratory in a sturdy shipping container that will keep them cool.

Although fruit symptoms are relatively characteristic for HLB, the molecular testing of fruit usually is not reliable because of the low numbers of bacteria in the fruit. Therefore, fruit samples alone should not be submitted for identification. Leaves or leaves on twigs should always be sent along with fruit
samples. Those fruit samples should be placed in a paper bag and then double bagged in resealable plastic bags and cooled.

For each sample a USDA/APHIS/PPQ Form 391 is required which includes details of location, host, date, collector, number of plants, plant part, and other information. When possible, the tree as well as symptomatic plant parts should be photographed.

**Psyllid**

Testing of Asian citrus psyllids (ACP) for the presence of the HLB bacterium is problematic because of low titers of the pathogen in the psyllids. However, psyllids are collected as part of some surveys. In those cases the first step is the identification of the insect. For psyllids collected during US domestic surveys the identification is done by a PPQ identifier for the area or a state entomologist. If they determine that it is likely an Asian or African citrus psyllid and it is from a state where it is not known to occur, the psyllids are placed in 95% alcohol and shipped by overnight courier to the U.S. Department of Agriculture, Agricultural Research Service (USDA/ARS) Systematic Entomology Laboratory (SEL), with whom PPQ works closely for insect identifications. If SEL confirms that it is an ACP, the sample will be tested by PPQ for presence of the HLB pathogen by personnel in Beltsville Maryland using a molecular diagnostic that is currently under development.

**Diagnostics – laboratory network**

In the U.S. a network of laboratories is involved in the diagnostics of HLB. In most cases the initial screening and diagnosis of survey samples are conducted by Plant Protection and Quarantine (PPQ) approved laboratories at the state, federal, or university level. Final identification is conducted by the PPQ Molecular Diagnostic Laboratory (MDL) in Beltsville Maryland because federal action or money will be part of the response to a positive detection.

**Within the regulated area**

In Florida most of the initial screening and diagnostic testing was conducted by the Florida Department of Agriculture, Department of Plant Industry (DPI). When a suspect positive was found in a county not previously known to have HLB, the sample was sent to the PPQ lab in Beltsville for confirmatory molecular testing.

Currently, if the sample is from a regulated area, i.e. any of the counties in Florida known to have HLB, the state laboratory can make the final determination. For example, the Southern Gardens Diagnostic Lab, is a partnership between US Sugar Corp, Southern Garden Citrus and the University of Florida (IFAS, CREC). This lab provides free PCR based testing of leaf and fruit samples symptomatic of greening. Paperwork submission form is required and the samples are prioritized. Nursery and foundation budwood are given highest priority, then grove samples, and finally research samples.

**Outside the regulated area**

For most other citrus producing states the Centralized National Survey Screening Laboratory in Gastonia NC processes survey samples. As with the state labs, if the Gastonia lab detects HLB in a new host or from a state or county where the disease was not recorded previously, the samples will be forwarded to the PPQ MDL for confirmatory molecular testing.

**Diagnostics – confirmatory testing by PPQ**

**In plants:**

The detection and identification of any of the three species of *Candidatus Liberibacter* (Ca. L.) requires the use of molecular assays because plant symptoms are not diagnostic and the bacterium cannot be cultured on sterile media. A number of methods have been used to detect and identify HLB including biological assays, the presence of fluorescent substances, examination using light or electron microscopy.
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microscopy and ELISA tests. None of these tests can consistently and reliably detect Ca. L. spp. Detection is difficult because of low concentration of the pathogen and uneven distribution in the plant. Various molecular tests have been developed that use specific primers and therefore more specifically target and detect the pathogen. However these “conventional PCR assays” are time consuming.

Li et al. (2006) developed a quantitative realtime PCR assay for detecting and differentiating the three Ca. L. species that cause HLB including Ca L. asiaticus (Las), Ca. L. africanus (Laf) and Ca. L. americanus (Lam). The assay uses TaqMan primer-probe sets based on the 16S r-DNA that are specific to the three different Ca.L. species. Included in the assay is an internal control based on plant cytochrome oxidase which allows the diagnostician to assess the quality of the DNA extracts. The realtime assay is reproducible for HLB-infected plants in the greenhouse. Additionally the test is rapid, and clearly differentiates the three HLB pathogen species. APHIS/PPQ conducts the realtime PCR test using the Cepheid SmartCycler platform. With a portable SmartCycler the test could be performed in the field in less than one hour. Although not developed for other automated systems, the assay could be adapted for equipment such as an ABI.

When plant samples are received by PPQ for confirmatory testing, the following steps are taken. The sample is given a unique laboratory number. Date of receipt and sample condition are noted. That information, as well as the data from the Form 391, are entered into NIS'computer database. Plant samples are maintained in a secure location because of the importance of the samples in the regulatory process and because HLB pathogens are considered select agents (SA) in the US. See separate discussion of the implications and additional precautions and reported needed for diagnosis of select agents.

The diagnostic procedure validated by the APHIS/PPQ Center for Plant Health Science and Technology (CPHST), National Plant Germplasm Biotechnology Laboratory (NPGBL) in Beltsville Maryland is utilized for final confirmatory testing. Plant samples are opened, observed and handled in a secure room in a biosafety cabinet. Four twigs are selected and the third through fifth leaves from the top of each twig are removed. The midribs with petioles are then removed from 12 selected leaves and cut into smaller pieces. Leaf midribs contain the highest titer of the HLB bacterium. DNA is extracted from these pieces using a modified protocol for the Qiagen DNeasy Plant Mini kit.

In the conventional assays three primer sets are used (Jagoueix et al 1996, Hocquellet et al., 1999, and Teixeira et al., 2005) to differentiate Lam from Las and Laf. The realtime PCR assays use the three different forward primers developed by Li et al. (2006) and differentiate the three different species. Results of both conventional and realtime assays are used for federal confirmations.

In psyllids:
Dr. WenBin Li of the CPHST, NPGBL is in the process of developing and validating a protocol similar to that used in plants for detection and identification of HLB in the psyllid.

Communication
Once confirmatory diagnostic assays have been conducted, the results are reported to the PPQ National Identification Services (NIS) National Domestic Diagnostics Coordinator in the Riverdale, Maryland headquarters. He then coordinates the reporting through the Emergency and Domestic Program Staff (EDP) at headquarters with the results going to the two regional program specialists and then to the individual states from which the samples were taken. Both PPQ state regulatory officials and State regulatory officials are notified so that a coordinated response can be organized, as needed. Diagnostic laboratories that submitted the initial samples are then notified by the regional HLB coordinator.
Select Agent Considerations

*Ca L. asiaticus* and *Ca. L. africanus* are listed as select agents in the U.S., as published in 7 CFR Part 331 (March 2005), because they are considered a severe threat to plant health. Diagnostic laboratories can receive plant material for diagnostic purposes without being a registered SA entity. However, the laboratory must notify the USDA SA Program within 24 hours of detecting HLB in a sample. Additionally, APHIS/CDC Form 4 must be completed and sent to the SA Program within 7 calendar days indicating the final disposition of the sample. Select agents must either be transferred to a registered SA entity or be destroyed.

Literature cited


