EXPERIENCES ON HLB (HUANGLONGBING) SYMPTOMS DETECTION IN FLORIDA

H.D. Gómez

USDA-APHIS-PPQ-Citrus Health Response Program,
Plantation, FL 33313
E-mail: Hilda.Gomez@usda.gov

Key words: Psyllids. Huanglongbing, citrus disease greening

ABSTRACT
Throughout the history of citrus production in Florida one of the diseases with a major impact has been the Asian form of citrus greening (HLB), which was first detected in 2005. As a response to this introduction, new survey and detection methods had to be developed utilizing field observations directed not only to corroborate symptoms already reported in the literature, but to describe any variations in symptomology observed on the different citrus varieties and their relatives growing in the area. Field and lab symptom studies were performed on Citrus aurantiifolia (Key/Mexican lime), C. aurantiifolia (Persian lime), C. aurantium (sour orange), C. jambhiri (rough lemon), C. limettioides (Palestine sweet lime), C. limon (lemon), C. limonia (Rangpur lime), C. maxima (pummelo), C. macrophylla (alemow), C. x paradisi (grapefruit), C. reticulata (mandarin-tangerine), C. reticulata X C. x paradisi (tangelo), C. sinensis (sweet orange), and Poncirus trifoliata (trifoliate orange) plants. The blotchy mottle symptom observed on the study plants support those described in the literature as the most important diagnostic symptom. Based on the results, training activities and materials were developed to compliment survey activities and sample collection protocols used to delimit and monitor the disease spread.

EXPERIENCIAS EN LA DETECCIÓN DE SÍNTOMAS DEL ENVERDECIMIENTO (HLB) EN LA FLORIDA

Keywords: Psilidios, huanglongbing, enfermedad de enverdecimiento de los cítricos.

RESUMEN
A través de la historia citrícola de la Florida, una de las enfermedades con mayor impacto ha sido la forma asiática del enverdecimiento de los cítricos (HLB) que fuera detectada por primera vez en este Estado en el año 2005. Ante esta nueva introducción, nuevos métodos de detección y muestreo fueron diseñados utilizando observaciones de campo dirigidas a corroborar no sólo los síntomas ya reportados en la literatura, sino también a describir cualquier variación de los mismos que pudieran desarrollarse sobre las diferentes variedades de cítricos y especies relacionadas a ellos que crecen en el área. El estudio de los síntomas, tanto en el campo como en el laboratorio, fue realizado sobre plantas de Citrus aurantiifolia (lima mejicana), C. aurantiifolia (lima Persa), C. aurantium (naranjo agrio), C. jambhiri (limón rugoso), C. limettioides (lima dulce), C. limon (limón), C. limonia (lima Rangpur), C. maxima (pummelo), C. macrophylla (alemow), C. x paradisi (toronja), C. reticulata (mandarina), C. reticulata X C. x paradisi (tangelo), C. sinensis (naranjo dulce) y Poncirus trifoliata (naranjo trifoliado). La observación del moteado marchito en todas las plantas estudiadas ratifica a este síntoma como el de mayor valor para el diagnóstico. Basado en los resultados, se desarrollaron nuevos materiales y actividades de entrenamiento para apoyar los protocolos de inspección y colección de muestras dirigidas a monitorear y delimitar la propagación de la enfermedad.
INTRODUCTION
Throughout the history of citrus production in Florida, many diseases and pests have represented serious threats. However, among these diseases, one which has had a major impact has been the Asian form of citrus greening (HLB) with its first detection in south Miami Dade County in 2005 (6, 10).

This disease, considered devastating to citrus production, is officially known as Huanglongbing (HLB) (1), in English “citrus greening”. Occasionally, it has been referred to as “yellow dragon disease” from the translation into English of the name Huanglongbing. However, it has been mentioned that in the local Chaoshan dialect, “long” refers to “young shoots” rather than “dragon” (5). For this reason, the use of the term “yellow shoot disease” that refers to the presence of yellow shoots as one of the disease symptoms has been suggested.

Citrus greening or HLB is caused by the gram-negative fastidious bacterium Candidatus Liberibacter asiaticus that inhabits the phloem of the host and is considered the worst disease to citrus caused by a vectored pathogen (11). The presence of the vector responsible for the spread of the Asiatic form of the disease, the Asian citrus psyllid (ACP) Diaphorina citri Kuwayama, was reported in Florida in 1998. The insect is now widespread in the state, and its large population is one of the factors that has led to making disease eradication impossible. There are now thirty Florida counties that have been confirmed positive for HLB and on January 11, 2008 a statewide HLB and ACP quarantine went into effect (14).

With this new disease, new survey and detection methods, as well as sample collection methods, had to be designed based on the particular symptoms it induces. Field observations were completed in order to corroborate some of the symptoms already reported in the literature, however additional evaluations were needed to describe variations that develop on different citrus varieties and their relatives growing in the area. With this concept, symptoms on Citrus aurantiifolia (Key/Mexican lime), C. aurantiifolia (Persian lime), C. aurantium (sour orange), C. jambhiri (rough lemon), C. limettoides (Palestine sweet lime), C. limon (lemon), C. limonia (Rangpur lime), C. maxima (pummelo), C. macrophylla (alemow), C. x paradisi (grapefruit), C. reticulata (mandarin-tangerine), C. reticulata × C. x paradisi (tangelo), and Poncirus trifoliata (trifoliate orange) plants were observed in the field and laboratory. Following the review of the symptom studies and resulting descriptions, the Citrus Health Response Program (CHRP) adopted them into the training program for its inspectors which conduct Multiple Pest Surveys (MPS) directed to the detection of different citrus diseases.

Symptoms description.
The importance of a rapid and accurate diagnosis in the field enhances the success of any eradication program (11). However, the HLB symptoms that can be observed at a specific time in the field are the expression of an infection that occurred a year or more in the past, due to the long latency period of the disease. It has been suggested that HLB symptoms are more evident during the cooler months of the year (12). Any symptom on its own, however, cannot be used for diagnosis due to the similarities with symptoms produced by other diseases and/or cultural conditions. For this reason, it is necessary to use other methods such as polymerase chain reaction (PCR), which is currently considered the main test for disease diagnosis (8).

All citrus varieties and their hybrids, as well as some citrus relatives in the Rutaceae family, are considered hosts for the disease. It has been mentioned that in the early stages of the disease, the first observed symptom is the presence of yellow shoots occurring only in some sectors of the tree (1, 2, 3, 4, 8) (Figure 1), while the other parts of the tree remain symptomless. This irregular distribution of the symptoms on the tree corresponds with the irregular distribution of the pathogen inside the host. The presence of yellow shoots in tree sectors is more noticeable in commercial groves, allowing the detection from a distance. However, this symptom is not always present initially. In residential areas, this symptom has not been observed on Persian lime plants (C. aurantiifolia) even a year later.
Experiences on HLB symptoms detection…

after being diagnosed as positive for the disease. The lack of this symptom could lead to a failure in detecting the disease in its early stages.

Once the pathogen starts moving through the vascular system of the plant, specifically in the phloem, other symptoms become evident on the tree canopy. There is a tendency for other branches to become yellow, followed by twig necrosis or dieback. A severe leaf drop that affects the density of the foliage can also be observed. Another symptom is early fruit drop leading to enormous losses in production.

However, the most important diagnostic symptom is the presence of the blotchy mottle on leaves that crosses the veins (1, 8, 12, 13) (Figure 2). The blotchy mottle can be observed on the leaves as dark or light green patches that are not symmetrical to both sides of the mid vein. This symptom is generally more evident in the interior part of the tree canopy, and it was the only one observed in all of the studied species.

With a severe infection, the dark green areas are reduced to small circular spots that contrast with a light yellow or green background. This symptom is commonly known as "green islands" (Figure 3). It has been observed on numerous sweet orange (C. sinensis) plants in commercial groves and on a potted grapefruit (C. x paradisi) plant in a Miami nursery. It is important to know that the mottling produced by HLB can easily be confused with those produced by other diseases such as severe forms of citrus tristeza virus or Phytophthora infections, or even nutrient deficiencies.

On severely affected branches having acute leaf drop, some small leaves develop a symptom commonly known as “rabbit ears” (Figure 4). This name refers to the presence of small, pointed, erect leaves having a light green or yellow color. These leaves can also be observed at the tip of new flushes. They are evident on species like sweet orange (C. sinensis), pummelo (C. maxima) and mandarin (C. reticulata).
Another characteristic observed on HLB infected leaves is the yellowing of the main and secondary veins (12) that sometimes become enlarged, swollen, and corky (1). In sour orange (C. aurantium) and key lime (C. aurantifolia) plants, it was also possible to observe thicker and leathery leaves in advanced stages of the disease (1, 12). Accumulation of starch grains in the parenchyma cells may explain why the leaves are leathery (12).

One of the most interesting symptoms to look for during survey and detection activities is the presence of leaf and branch flushes with mineral deficiency-like symptoms that resemble those chlorotic patterns produced by zinc, iron, manganese, calcium, sulfur and/or boron deficiencies (2).

Disease detection is aided by observation of the notching caused by toxin secretion of psyllids while feeding on the leaves. This has been the case during nursery and residential property inspections where the notching is the clue inferring that the associated mottling is due to HLB and not to a nutrient deficiency in the potted plants.

The presence of small and misshapen (lopsided) fruit is a noteworthy symptom in field surveys. However, it is not exclusive to citrus greening. Misshapen fruit can be caused by other problems such as nutrient deficiency. For this reason, it is important to analyze all symptoms on the tree. On the other hand, if small and misshapen fruit is found in a packinghouse, it is likely that the disease is present in the grove where it originated. A Florida packinghouse did allow CHRP personnel to do a follow-up inspection of a grove upon finding this fruit symptom in the packinghouse. This follow-up inspection resulted in the detection of the disease.

When diseased fruit is cut longitudinally, the axis of the fruit is observed to be curved (1) (Figure 5). In addition, aborted seeds can be present (1, 8, 12, 13). In pummelo (C. maxima) and sour orange (C. aurantium) fruits, aborted seeds in the affected part of the fruit and normally developed seeds in the remaining part have been simultaneously detected. Another symptom on the fruit is the orange-brown stain of the vascular columella (1, 8) that has been observed on grapefruit (C. x paradisi) and sweet orange (C. sinensis) fruits. This fruit symptom should only be used as a diagnostic character when other reliable symptoms are present.

Reduced fruit size is one of the symptoms produced by greening that is evident in groves and is responsible for losses in the fresh fruit market but is not the only one. The juice from fruits affected by HLB disease has been referred to as bitter and with a low content of soluble acids (13), or as having a salty bitter taste (2, 12). In relation to this, the juice tested during our experience with sour oranges (C. aurantium) and pomelo (C. maxima) proved to be insipid, coinciding with the off-tasting characteristic of the fruit mentioned by Halbert (11).

Another interesting symptom on fruit affected by citrus greening is the color inversion that tends to keep the green color in the stylar end (1, 8). This characteristic was observed on pummelo (C. maxima) fruit in a Miami area commercial grove. Lastly, HLB affected fruit, especially sweet orange (C. sinensis), can also have a mottled appearance (8).
Survey strategies.
Detection, delimiting, and monitoring surveys have been recommended as part of the citrus greening guidelines by the United States Department of Agriculture (USDA) (7). Detection surveys should be performed to ascertain the presence or absence of the disease in an area where it is not known to occur. Once the disease is detected, a delimiting survey should be completed to define the affected area. And finally, once the control regulations have been established, a monitoring survey should be conducted to confirm success. These three survey types can also target the psyllid vector of greening. Sentinel Tree Programs directed to conduct surveys on a repeated basis can also be recommended for areas where the disease is not known to be present. A sentinel tree survey was adopted in Florida in June 2000 as an early warning system for new outbreaks of Asiatic Citrus Canker (ACC) (9). Sentinel trees need to be healthy and easily accessed. The varieties selected should be among the reported varieties susceptible to the concerning disease. More recently in Florida, a systematic Multiple Pest Survey Program (MPS) has been designed to detect multiple diseases including HLB, citrus canker, and other exotic pathogens such as citrus variegated chlorosis and citrus leprosis to take advantage of resource limitations for field inspections (8).

During survey activities it is important to count on specialists with knowledge on the targeted disease, as well as properly trained personnel (7). Surveyors should be trained not only on symptom recognition, but also on the biology of the pathogen and the insect vector for a better understanding of the disease. Some additional subjects recommended are citrus disease differentiation and host identification. Different materials have been developed by the CHRP Pathology Team to aid the training process, such as manuals and identification cards that could be used during field activities.

Procedures reflecting the strategies for field inspection should be established, including the sanitation measures needed to be taken to avoid contamination between trees or properties. Once in the field, surveyors should look first for any symptomatic trees (3, 4). In the case of HLB infections, a visual observation of yellowing tree canopy, blotchy mottling, and small lopsided fruit containing aborted seeds will be the best indicators for disease detection (3).

Zinc or other mineral deficiencies can be easily mistaken for citrus greening symptoms. Surveyors should be able to distinguish between true mineral deficiency symptoms and deficiency-like symptoms caused by HLB. Nutrient-like deficiency symptoms will generally be observed at the end of branches showing conspicuous HLB mottling symptoms.

In groves, the number of blocks selected for survey completion depends on resources, time, and the proportion of infection you need to detect. In Florida, the host susceptibility has been a key factor in selecting blocks for MPS. In residential properties and nurseries all citrus trees must be inspected since the disease can be irregularly distributed and present in more than one host. In cases where nutrient related symptoms could be present, the observation of yellowing tree canopy, blotchy mottling, and small lopsided fruit containing aborted seeds will be the best indicators for disease detection (3).

Once a suspect tree has been detected, either on commercial sites, nurseries or backyard locations, the affected branch should be marked or taped and GPS readings should be taken to facilitate future inspections. Lastly, a map should be prepared showing the location of the infected tree within the grove or property. It is important to consider a photograph submission, if possible, to document the findings. The use of digital images in this sense will not only allow for a faster confirmation or feedback on a suspect tree or sample that has been collected, but also helps on symptoms evaluation.
from remote locations via the internet. A system for survey data collection should be installed taking into consideration that recording negative results is also important (4, 7).

Sample collection and processing.
Excessive sample collection could result in heavy workload at the reception offices and in the diagnostic laboratory. Hence, it is important to grade or screen samples prior to submission. Samples can be classified as high, medium, and low suspect according to the observed symptoms (7). A high suspect sample that will be sent to the lab for PCR diagnosis should present the typical mottling and/or nutrient deficiency-like symptoms, corky and yellow veins, and lopsided or irregular colored fruits. A medium suspect sample will be considered a sample that shows non-classic mottling symptoms with some of the other mentioned symptoms. A low suspect sample will be one containing only nutrient deficiency symptoms, naturally senescing leaves, variegation, and mottling as a result of insect injuries, fungal diseases or damages to the leaves.

Sample collection procedures should be established taking into consideration the following factors. Each sample collected should contain green twigs of six to eight inches long with approximately twenty leaves, preferably with the petiole still attached and with good recognizable symptoms. The symptomatic leaves can be accompanied with non-symptomatic leaves from the same branch. In the case of fruit collection, sample should be collected together with the peduncle, leaves, and twigs from the same tree because the amount of pathogen they can harbor is not known. It is important to avoid fruit that could decompose during the submission. Also, measures should be taken to avoid the presence of the insect vector in the sample bag.

Sanitation procedures should also be established to avoid further contaminations through shears or other tools used during the sample collection process. The use of a liquid bleach solution followed by a water rinse has been recommended as a decontaminant (7).

The samples should be placed inside a plastic bag which is then placed inside a second bag with the corresponding information. The bagged samples need to be stored in a cool location above freezing, and sent to the laboratory avoiding weekends and holidays. The receiving lab should be notified about the shipment.

Samples collected as a result of survey activities should be accompanied with information regarding the date of collection, sample number from a predetermined numbering system, collector’s name and agency, full address of the collection site, type of property (residential or commercial), coordinates of the host plant, name of the host species and cultivars, number of trees that show symptoms and any other relevant information (7). Suitable forms to contain this information should be developed. It can be suggested to establish a chain of custody form, reflecting the name of each person who has handled the sample. In order to complete the data collection process, a copy of the final diagnosis should be included.

CONCLUSION.
Citrus greening (HLB) symptoms on leaves, branches, and fruits have been observed on various citrus varieties including *Citrus aurantifolia* (Key/Mexican lime), *C. aurantifolia* (Palestine sweet lime), *C. aurantium* (sour orange), *C. jambhiri* (rough lemon), *C. limettioides* (Persian lime), *C. limon* (lemon), *C. limonia* (Rangpur lime), *C. maxima* (pummelo), *C. macrophylla* (alemow), *C. x paradisi* (grapefruit), *C. reticulata* (mandarin-tangerine), *C. reticulata X C. x paradisi* (tangelo), and *Poncirus trifoliata* (trifoliate orange) plants. It is confirmed that the blotchy mottle on leaves is the most important diagnostic symptom, since it is present in all the species involved during the field observations. The symptoms and procedures described in this manuscript can aid in the detection and management of
HLB. These symptom studies and descriptions are today the basis of the training activities for CHRP personnel in order to assist with the completion of the Multiple Pest Survey Program (MPS) in Florida.

REFERENCES