HLB scouting and interpretation of PCR results

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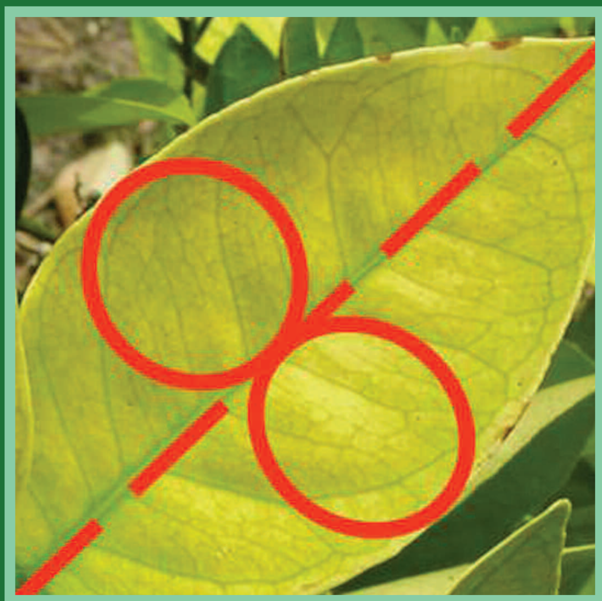
Picture 1. Leaves showing the blotchy mottle symptom of HLB/greening.

Since the initial finding of Huanglongbing (HLB) or citrus greening disease in the South Florida area in August 2005, the disease has quickly spread to 32 Florida counties, including all citrus-producing counties. The disease was also discovered in July in Louisiana, and the Asian citrus psyllid vector is present in Texas and California. Although the disease has become widespread in Florida, it is still critical to scout for the disease on a regular basis and, if feasible, remove infected trees since there is no cure or proven management strategy at this time. To accomplish this, growers must have no doubt about the symptoms of the disease they are scouting for, periodically verify samples by PCR analysis for accuracy, and understand how to interpret PCR results and be aware of its limitations.

SCOUTING

When developing an HLB scouting program, growers should consider their ultimate objectives. These objectives will determine the most effective way to measure the performance of a scouting program. HLB scouting can be broken down into three distinct types based on scouting objectives: 1) identifying new geographical areas of infection, 2) locating newly infected trees in known HLB blocks, and 3) identifying symptomatic trees for removal.

All three of these objectives require trained scouts (either in-house or contracted) who know what to observe when scouting for HLB. The greatest challenge in scouting for HLB is recognizing definitive symptoms and distinguishing them from other disorders. The definitive symptom of the disease is blotchy mottle (asymmetrical chlorosis) on leaves (Picture 1). An easy way to be certain the symptoms are blotchy or asymmetrical is to draw two circles opposite one another on either side of the mid-vein (the "pen test," Picture 2). The circles help to focus your attention and it is easy to determine if the areas within the circles are similar (= not blotchy) or different (≠ blotchy or asymmetrical → may be HLB). Also important to remember is that HLB symptoms can appear anywhere in the canopy and may be more or less visible depending on lighting and viewing angle. For these reasons, "windshield scouting" will not be sufficient; trees need to be looked at carefully and from different angles.



Picture 2. A leaf with asymmetrical HLB/greening symptoms (left) and a leaf with symmetrical nutrient deficiency symptoms (right) demonstrating the "pen test."

HLB symptoms should not be confused with nutrient deficiency symptoms. Nutrient deficiencies have a symmetrical symptom pattern. It is very common to find several different nutrient deficiency symptoms on an HLB infected tree, for recent studies show that HLB infected trees are deficient in a number of nutrients. However, nutrient deficiency symptoms alone are not diagnostic of HLB. Nutrient deficiency symptoms may catch your eye, and they are a good indication that something is wrong with the tree, but a careful check for blotchy mottled leaves is essential before selecting samples for PCR or diagnosing HLB. Remedial foliar applications of minor elements may be useful in eliminating nutrient deficiency symptoms to make scouting for blotchy mottle symptoms easier. However, there is currently no data to indicate that this strategy will slow disease development or maintain tree productivity, but a healthy tree is always better off than one which is deficient in any resource.

Other symptoms associated with HLB infection which are commonly seen are yellow veins, lopsided or misshapen fruit and corky veins. Yellow veins can also be caused by a girdled/ broken branch, and foot rot or root rot. Corky veins are a symptom of boron deficiency, which may or may not be caused by HLB. For these reasons, it is the authors' opinion that these symptoms are best used as indicators that something is wrong with the tree, but the tree should be more closely inspected for the definitive blotchy mottle symptom. Misshapen fruit in citrus are not exclusively caused by HLB infection, but in Florida, where stubborn disease is not present, they are generally considered good symptoms for HLB diagnosis. If a tree has leaves with yellow or corky veins and misshapen fruit, and blotchy mottled leaves cannot be found, the peduncles of the misshapen fruit can be sampled for PCR and in the authors' experience are more reliable than yellow and corky veins.

Once a suspect leaf has been found, make sure that other problems (girdling, broken branch, foot rot, etc.) can be ruled out. If the symptoms are not associated with other problems and you have previously found HLB trees in the area which have been confirmed by PCR analysis, then you may feel confident enough to diagnose the tree on visual symptoms alone. If no other HLB trees have been found in the area, or symptomatic leaves have not previously tested positive by PCR analysis, then a sample should be submitted for PCR testing.

Currently IFAS recommends that scouting for HLB should be done at least four times a year. If possible, scouting should be focused from September through March, when symptom expression is strong; however, symptoms can be found year-round and scouting should not be overlooked from April through August. Every grower's goal should be to detect the disease as early as possible when incidence is low in a given area and before psyllids have a long time to feed and transmit the pathogen. Some data indicate that for every tree with visible HLB symptoms, there is another tree which is not yet showing visible symptoms, further emphasizing the importance of finding the disease early.

In considering the three objectives of an HLB scouting program, one effective strategy if scouting resources are limited may be to concentrate scouting efforts in new areas without a history of HLB during the winter months. This assumes that new areas of infection will have a lower disease incidence and fewer symptoms; thus scouting when symptom expression is greatest will allow for maximum detection. The identification of newly symptomatic trees in areas with a history of HLB (along with tree removal) can then be done during summer and winter and the identification of trees in these areas can continue year-round until all symptomatic trees are removed. Even though scouting during the winter is most effective for all three objectives, if resources are limited, these guidelines may help focus the efforts of your HLB scouting.

PCR TESTING

PCR, short for Polymerase Chain Reaction, is a molecular tool used for detecting the DNA of an organism. Simply put, PCR is a method of replicating DNA in order to amplify it to levels which are detectable. The number of replication cycles it takes before the DNA has been amplified to detectable levels is reported as the PCR Ct value.

But what does the Ct value really mean to you? Actually, there is still some debate about this within the scientific community. Some researchers believe that if any Ct value is reached, that indicates the HLB bacterium is present since the method only replicates a specific portion of the HLB bacterium DNA. However, others believe that as the number of cycles increase, so do the chances for something to go wrong — thus a high Ct value may not be accurate. However, one thing that we all do agree on is that no Ct value, or an "undetermined" value, does not mean that the tree does not have HLB. Rather, it simply means that the leaf or leaves submitted for testing did not have the bacterium present.

The reliability of PCR with respect to detecting HLB diagnosis is greatly influenced by two key traits of this disease. First, the bacterium is unevenly distributed within the tree. This means that just because a tree may be infected, not all parts of the tree will have equal (if any) amounts of bacteria in them at a given time. Research is still ongoing to fully understand how the bacterium moves through the tree during the season and if samples should be taken from specific tissue types at different times of the year. Second, as previously mentioned, the disease symptoms change in intensity throughout the year. This may be related to the movement of the bacterium in the tree, temperature or other environmental factors.

These two factors (bacterial distribution and movement) together make it critical that scouts look for and key in on the blotchy mottle symptom. In our research we have sampled blotchy mottled leaves, leaves with yellow veins,

Seasonal effects on the reliability of PCR detection of HLB from blotchy mottle leaves

Season	Months (Central FL)	Expected Results
Spring to Early Summer	April-June	High Potential for False Negatives
Early Summer to Late Summer	June-August/September	Fairly Consistent PCR Results
Late Summer and Winter	September-March	Most Consistent PCR Results

leaves with zinc deficiency symptoms, leaves with corky veins (boron deficiency), and healthy appearing green leaves from the same tree. Consistently, the blotchy mottle leaves are the ones which test positive for HLB by PCR. This has also been found to be true in Brazil.

Experience has shown that PCR testing from spring, beginning with the initiation of the spring flush through late spring or early summer, can result in a large number of undetermined PCR results. During this time of year, trees with what appear to be HLB symptoms may test negative for the HLB bacterium, and subsequent testing of the same tree later in the season can result in a positive PCR test. This may be a dilution factor of the bacterium due to the production of the new growth, a fluctuation of the bacterial population in the tree or some other factor during the spring. Thus, samples tested during this period of the year may result in a significant increase in false negatives from symptomatic trees. This could give the impression of a decrease in the effectiveness of the scouting program. However, relying on the percentage of PCR results to judge your scouting program can be a double-edged sword. The expectation for a high percentage of samples tested to be positive without regard for the time of year may lead to scouting crews selecting and submitting only highly symptomatic samples, leaving the questionable samples in the field. This is one reason why many growers find it better to PCR confirm the presence of HLB when disease incidence is low, but once the disease is established in a block, they rely upon visual symptoms for diagnosis with only an occasional PCR confirmation to ensure scouts are maintaining their skills. A good rule of thumb is to recognize obviously positive samples and to rely on PCR to confirm questionable samples.

CONCLUSIONS ON HLB SCOUTING AND PCR

If a survey crew has demonstrated the ability to find PCR positive greening trees when scouting during the optimum time of the year, then a subsequent decrease in PCR positive samples during the spring and early summer would not necessarily be an indicator of ineffective HLB scouting. The most important goal is early detection when disease incidence is low. The next level would be the removal of the initial HLB positive trees with a subsequent reduction in symptomatic and PCR positive trees during future surveys. This demonstrates the variability and problems associated with basing the evaluation of a scouting program on the percentage of samples that result in a positive PCR test. The evaluation of HLB scouting should be based on the objectives of the individual scouting program.

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