HS1122

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An Iodine-Based Starch Test to Assist in Selecting Leaves for HLB Testing [1]

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Diagnosing huanglongbing (HLB or citrus greening disease) can be difficult under field conditions when relying solely on visual symptoms. The best diagnostic symptom of HLB is the blotchy mottle pattern on leaves (Figure 1A). However, it can be difficult to distinguish blotchy mottle caused by HLB infection from similar symptoms caused by girdling of the branches and other physiological disorders or diseases. For example, deficiencies of micronutrients such as zinc, manganese and iron (Figure 1B-D) can be mistaken for HLB. Currently, the only definitive test for HLB is polymerase chain reaction analysis, or PCR, analysis, a DNA-based test. PCR analysis, however, is time consuming and expensive, and is not suitable for large numbers of samples. Thus, a rapid, simple field diagnostic test that could be used to pre-screen samples intended for PCR analysis would be beneficial.

Anatomical studies conducted in the 1960s, found "massive accumulation" of starch in leaf samples collected from HLB-infected sweet orange trees. More recent studies have quantified starch accumulation in HLB-infected leaves at six times more than healthy leaves. Starch readily reacts with iodine, resulting in a very dark grey to black stain. Recently, a number of researchers from Vietnam and Japan have been working to adapt this starch/iodine reaction into a diagnostic tool for HLB, and they report up to 90% agreement between PCR analysis and starch tests with iodine. IFAS has not performed a similar correlation analysis, although studies are ongoing. An IFAS-developed version of this test, how to perform it, the required materials, its potential benefits, its limitations, and how to interpret the results is presented here.

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*All photos by Pedro Gonzalez unless otherwise noted.

Fig**ure 1.** Citrus leaves showing HLB symptoms (A), manganese (B), zinc (C) and iron (D) deficiency symptoms. Photo credits: Tim Spann (A) and Tom Obreza (B, C, and D), University of Florida.

Selecting Leaves for Testing

Choosing leaves to test is a crucial step in the diagnostic or confirmation process. Just as submitting hundreds of leaves for PCR testing would not be efficient, it is not efficient to use this test on leaves whose symptoms can easily be determined to be caused by something other than HLB. We recommend the following criteria be used in selecting leaves:

1. Select leaves expressing strong symptoms and avoid those with physical damage or symptoms clearly related to some other problem, e.g. nutrient deficiency. Use of the IFAS Citrus Greening Field ID Pocket Guide or other such tool is helpful.

2. Select symptomatic leaves only from healthy, undamaged branches. Branches that are broken, girdled or are otherwise physically damaged may cause starch to accumulate in leaves even if HLB is not present.

3. Leaves that are in full sun locations are best; try to avoid heavily shaded leaves.

4. Always test at least 2-3 leaves displaying prominent symptoms of HLB.

Iodine Solution

There are a number of iodine solutions available at drugstores and pharmacies (Table 1). For this test, purchase products labeled as either "tincture of iodine" or "iodine tincture." These products contain iodine and sodium iodide dissolved in alcohol and water. Other iodine products that are labeled "iodine solution" such as Betadine® (povidone-iodine) contain surfactants and other chemicals that prevent them from reacting with starch.

Table 1. Iodine solutions commercially available at most pharmacies and drugstores

Name	Active ingredient(s)	Starch reactive
Betadine, Xenodine or generic equivalent	Povidone-Iodine 10% (PVP)	No
Iodine tincture USP	Iodine 2% (may or may not also include sodium iodide 2.4%)	Yes
Lugol's iodine*	Iodine 2%, potassium iodide 4%	Untested, but should react
*This is a dietary supplement sold to alleviate iodine deficiency in humans, and thus is expensive and impractical to use for this test.		

For use in this test, the purchased tincture of iodine should be diluted 1 to 10 with water (i.e. 1 part iodine mixed with 9 parts water). Do *not* use the iodine tincture straight; the undiluted tincture will react very strongly with even small quantities of starch, potentially leading to false positives. The diluted iodine solution should be stored in a dark tinted (e.g. brown glass) or opaque container. Clear containers can be covered with aluminum foil. The prepared solution will last for a few days when properly stored.

Performing the Test

The steps to follow to perform the iodine test are outlined below. As with leaf selection, the portion of the leaf tested is important. In cases where the entire leaf is not symptomatic, the symptomatic section is preferred for testing. This test is intended to be used in the field; however, if it is impractical to test leaves in the field, they may be sealed in a zip-top bag and stored in a cooler with ice until the test can be performed. Leaves should not be stored for more than 24 hours, and then only under refrigerated conditions.

1. Using a sharp, clean razor blade, cut a section from the selected leaf that includes the symptomatic tissue (Figure 2). Do not cut through the mid-vein, rather cut sections from the leaf blade on either side of the mid-vein.
2. Immerse the cut section(s) of leaf in the prepared iodine solution for 1.5 - 2 minutes.
3. Remove the sections and rinse with clear water.
4. Examine the cut edge of the section(s) for dark staining using a hand lens or magnifying glass.

Figure 2. A citrus leaf with the vein corking symptom of HLB properly sectioned for the iodine test. The arrow indicates the symptomatic section to be used for testing.

Figures 3-5 show a series of leaf sections, including healthy leaves, HLB-positive leaves and nutrient-deficient leaves, after iodine staining. Healthy leaves may show some starch staining; however, it is generally confined to a few cell layers at the upper side of the leaf and does not show the same intensity of staining as an HLB-positive leaf (compare Figures 3B and 4). HLB-positive leaves stain a very intense dark grey to black throughout the entire cut surface (Figure 4). Nutrient-deficient leaves generally stain similar to a healthy green leaf (Figure 5).

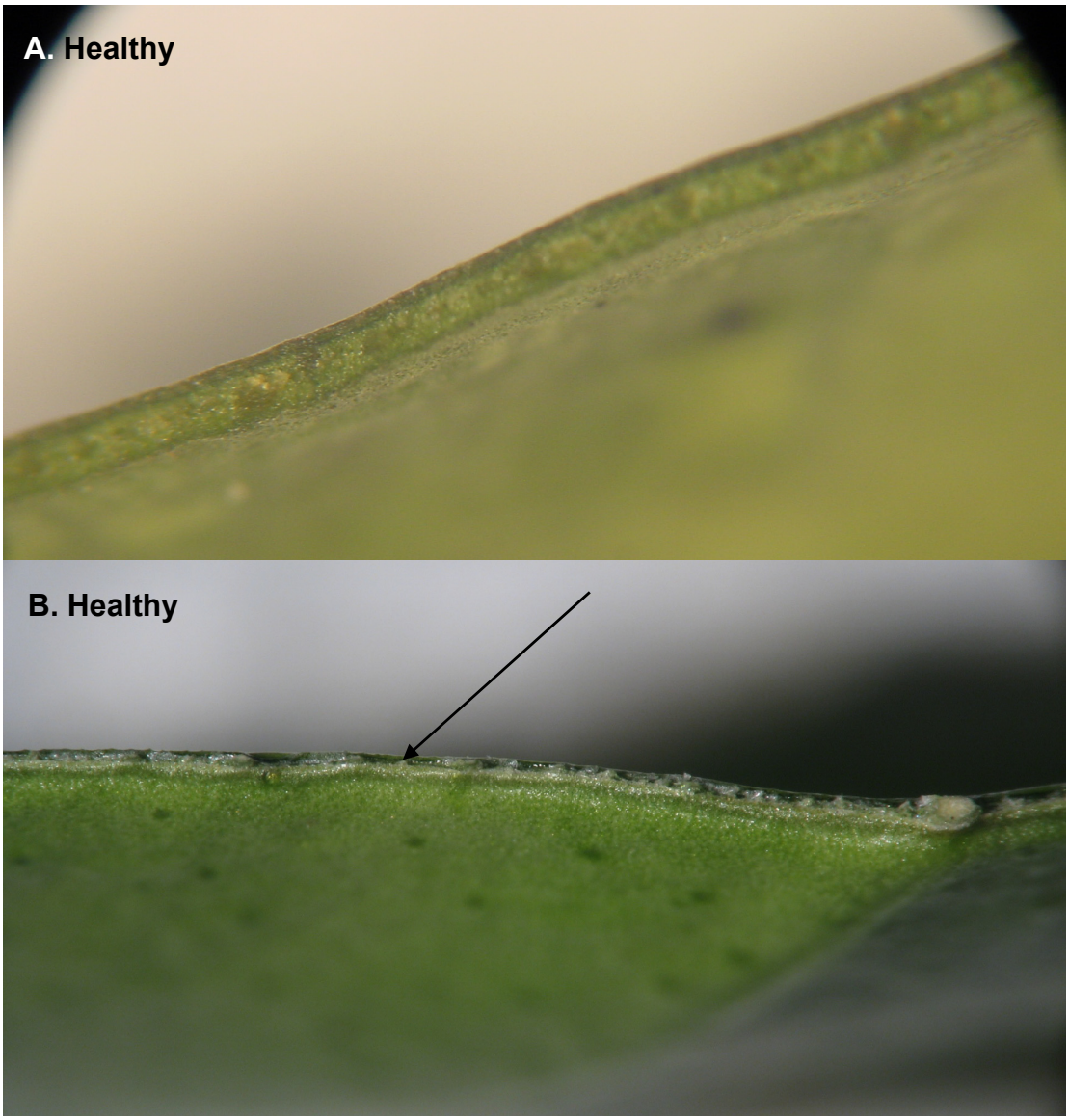

Figure 3. A healthy citrus leaf will show no (A) or very little (B) staining after immersion in the iodine solution for 2 minutes. Note how the staining that does occur in a healthy leaf (B) is limited to a couple of cell layers along the leaf surface (arrow).

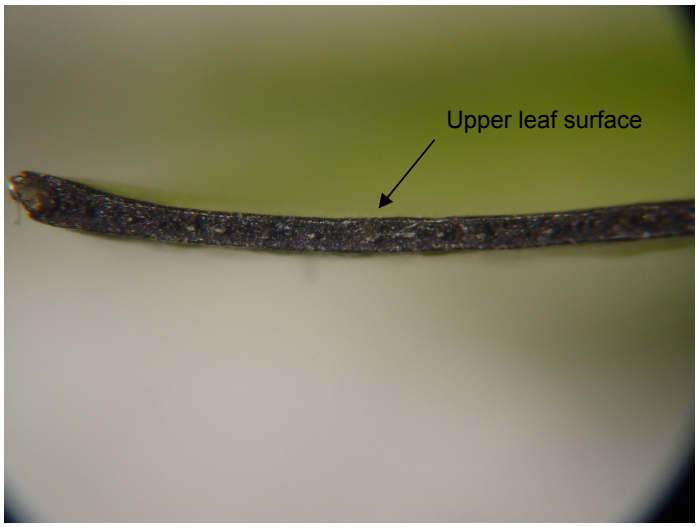

Figure 4. A leaf that showed strong blotchy mottle symptoms of HLB infection stains very dark grey to black along cut surfaces when immersed in iodine solution for 2 minutes. Note that all cell layers of the leaf are stained unlike in a healthy leaf (see Figure 3B).

Figure 5. Leaves showing various yellowing symptoms, not typical of HLB, probably due to nutrient deficiency (A) do not stain darkly when immersed in iodine solution (B), indicating that these would not be good samples to submit for PCR analysis.

Interpreting the Results

A darkly stained leaf from an undamaged branch indicates that the leaf has an above-normal level of starch accumulation; it *does not* indicate that the leaf is HLB-positive. HLB infection is *not* the only condition that may cause excessive starch accumulation in leaves. Some cultivars of citrus (e.g. 'Murcott') have naturally high levels of starch in their leaves. Physical damage (e.g. girdling, limb breakage, insect feeding) that disrupts sugar transport in the phloem will lead to starch accumulation. Diseases other than HLB (e.g. *Phytophthora*) may also lead to starch accumulation in leaves. Similarly, the test does not appear to be useful when trees are growing in pots or greenhouses, or for small trees growing in nurseries.

The purpose of this test is to assist you in determining which leaves, with difficult-to-interpret symptoms, should be submitted for PCR analysis. For example, this test can help you to distinguish between confusing nutrient deficiency symptoms and leaves that may be HLB-positive. This test can be a useful tool to help you select the best samples for PCR analysis, thus helping to reduce the number of negative samples submitted. IFAS *does not* recommend that the results of this test be used in making decisions about the HLB status of a tree or whether to remove a tree. PCR testing remains the current, definitive test for HLB and it should be relied upon for making management decisions.

Additional Information

Hong, L.T.T. and N.T.N. Truc. 2003. Iodine reaction quick detection of huanglongbing disease. Proceedings of the 2003 Annual Workshop of JIRCAS Mekong Delta Project: 1-11. http://www.ctu.edu.vn/institutes/mdi/jircas/JIRCAS/research/workshop/pro03/B3-Fruit%203%20(Hong).pdf