Citrus Research and Development Foundation, Inc.

Grower Field Trial Methods for the Evaluation of Bactericides Against Huanglongbing (Citrus Greening)

Michael E. Rogers

Compiled by:
Stephanie Slinski Ph.D.
CRDF Project Manager

Updated April 16, 2016
See http://bit.ly/1PQEPGT for updates

700 Experiment Station Rd. • Lake Alfred, FL 33850 • Phone: (863) 956-5894

Acknowledgements

The following people provided editing assistance, comments and reviews:
James Syvertsen, Brandon Page and Harold Browning,
Citrus Research and Development Foundation;
Mike Irey, United States Sugar Corporation;
Megan Dewdney, Evan Johnson and James Graham, UF/IFAS, CREC

Table of Contents

Introduction

This manual has been developed by the Citrus Research and Development Foundation to assist growers in collecting meaningful data on the effects of bactericide use on grove health and fruit yield. Bactericides have recently become available to growers, but with limited prior field trials and use of these materials on citrus, information on best use and effectiveness is minimal. Growers have expressed an interest in evaluating treatments to determine the benefit of their investment and to help develop recommendations for future seasons. To this end, CRDF has compiled this document to aid growers in the development and implementation of field trials. These trial designs can be implemented on any size block, and the only requirement is to leave a portion of the block or an adjacent block untreated for a side-by-side comparison. This comparison will provide information on the effectiveness of the specific use pattern of the bactericides applied to the block to help to decide if the treatment is justified and economically viable.

Field Trial Development

Planning

The first step in setting up a field trial is to outline a treatment plan. Changes in the treatment plan may be made as the season progresses and as more information becomes available, but a general idea of use pattern and application timing should be developed at the initiation of the trial. Suggested use patterns for the bactericides can be found at http://www.crec.ifas.ufl.edu/extension/greening/management.shtml. Use patterns should be compliant with the current legal use. It's best to evaluate one type of practice, in this case bactericides, rather than to combine multiple practices such as new nutritional product applications, into a single trial. Since this is a bactericide evaluation all other practices and treatments should be applied uniformly across the treated and untreated portions of the trial.

The next step is to determine how much time can be dedicated to evaluating a field trial. If no time can be dedicated to a trial, simply leaving an untreated control block is enough to have a comparison of yield. The untreated control is maintained similarly to the treatment in terms of grove maintenance, but no bactericide is applied. The untreated control must be adjacent to or within a treatment block. A block with replants, and various levels of disease severity may be used as long as the untreated control and treatment blocks are similar in these factors. A single field trial will provide some data, but having several sets of this type of field trial will provide greater resolution of any treatment effect.

If more time is available, data on tree health and fruit drop may be gathered from individual evaluation trees. The amount of time required to gather these data depends of the number of blocks with evaluation trees. Data collection could take from a couple of hours for one trial to a couple of days for many trials.

Block Selection

The next step is to pick test blocks. Citrus is inherently variable, and within a block, individual tree performance varies, due to the trees themselves as well as the block environment. Block to block variation also is large. Teasing out the effects of bactericides has to take into consideration how to separate the bactericide effects from background "noise". By choosing similar, adjacent treatment and control blocks, the variation due to other factors can be minimized.

The untreated control and treatment trees must be similar in:

- Average size
- Average age
- Average disease severity/Disease index (page 6)
- Same scion variety
- Same rootstock
- Soil type, elevation, irrigation and moisture status
- Same horticultural care (pest control, fertilizer, etc.)

Trial Length

The recovery of a tree from the symptoms of HLB depends on the severity of decline of an individual tree. A tree in severe decline will not recover as rapidly as a tree in mild decline. The regeneration of phloem cells and the recovery of the root system and the canopy is a slow process. Little improvement in tree health may be observed after six months of treatment; more significant responses may be measured after one season or more. For this reason, extending a field trial into a second season is recommended. When continuing the trial for a second year, the same treatment and untreated control blocks should be used and it is recommended that the use pattern remain similar.

Field Trial Set-up

The following is a guide for the selection of blocks and three trial designs that can easily be implemented by growers. Design 1a is the comparison of adjacent blocks with no specific data collection other than block yield. By adding evaluation trees, which will be monitored during the season (1b), additional useful data can be collected. Design 2 is an alternative method for setting up an untreated control within the treatment block. Combinations and/or replications of any of the presented designs may be used in different blocks to add power to the evaluation. For example, implementing trial design 1b in one block along with several replications of design 1a in other blocks will provide better resolution of any treatment effect.

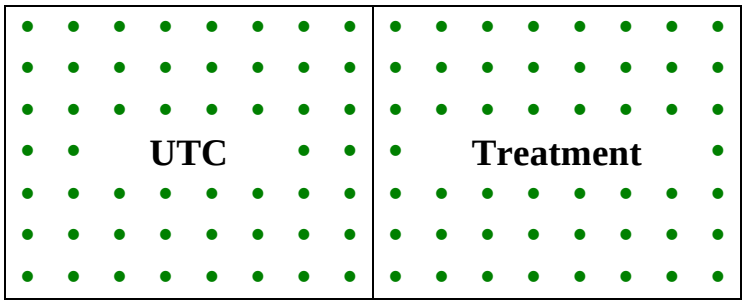
This approach can be used to compare a group of blocks treated with the same seasonal bactericidal use plan with another set of blocks treated with a different seasonal bactericide use plan. The methods presented here can also be used for assessing other products, varieties, or management practices. Please contact UF/IFAS Citrus Extension Agents or CRDF project managers (cpd@citrusrdf.org) with any questions on field trial design and implementation.

Trial Designs

1a. Simple partial-block or adjacent-block design

- One block split in half or two similar adjacent blocks.
- Half treated and half an untreated control (UTC)
- Block yields at end of season to compare block performance

This design provides some information on treatment effects when evaluating blocks. By evaluating two or more pairs of blocks, using the same treatment material and timing, a more meaningful comparison of treatment effects can be made.



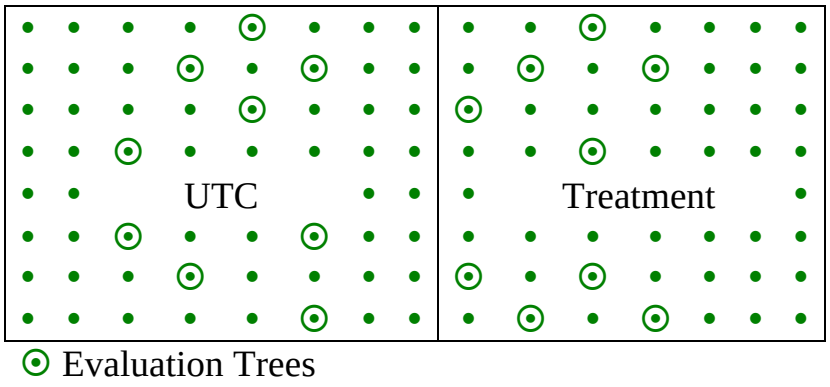
1b. Partial-block or adjacent-block design with evaluation trees

Evaluation trees are individual trees chosen for evaluation using the methods described later (page 6). Even though the block being used for the trial may contain trees of various ages, disease status and other variables, evaluation trees chosen within the block for data collection should be uniform (rather than merely similar) in size, age and disease severity (DI page 6) as well as similar in the other factors described earlier under block selection. The greater the uniformity, the easier it will be to determine significant treatment effects. Twenty to twenty-five evaluation trees for the untreated control and for the treatment should be selected and labeled using flagging and mapped on grove maps for later relocation. Growers should include the maximum number of trees that they feel that they can evaluate in a reasonable amount of time, because with greater numbers of evaluation trees, greater resolution of treatment effects can be obtained.

- One block split in half or two similar adjacent blocks.
- Half treated and half an untreated control (UTC)
- Evaluation trees for evaluation of:
    - Disease index
    - Yield
    - Fruit drop
    - Further types of evaluation (see Evaluation Methods, page 6)

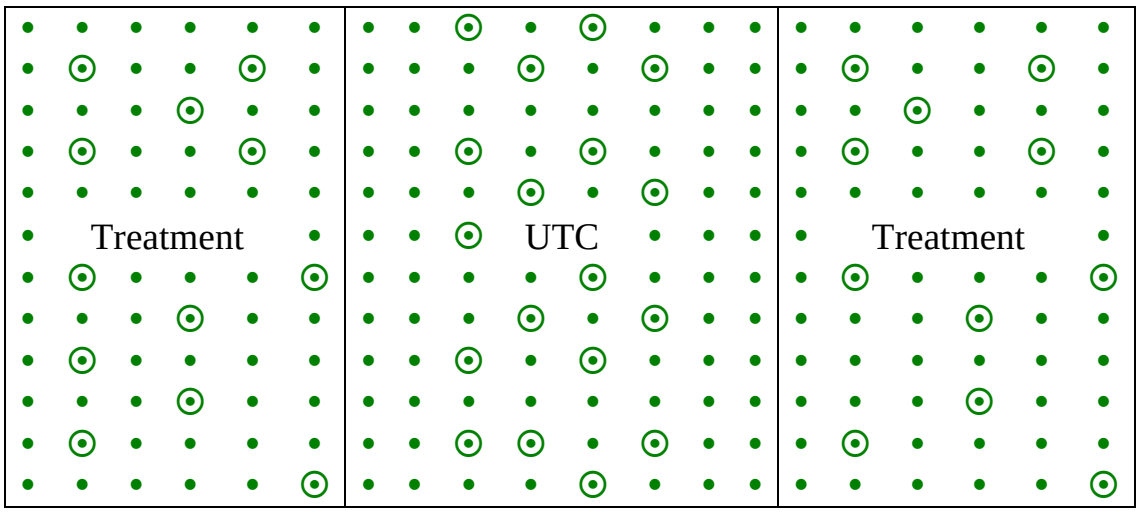
The additional data collection will increase the power of the trial and is more likely to show effects of the use of season-long bactericides compared to similar blocks not treated with bactericides.

Evaluation trees are set up in the untreated control and treatment blocks. Evaluation trees may be scattered throughout the block or chosen from a few rows. Avoid placing evaluation trees along the edge of the block because the trees on the edge are more variable than in the internal trees. Evaluation trees should not be selected from the row immediately adjacent to the treatment block to avoid drift from bactericide applications.



2. Plot design with central control rows inside of the treated block

- One block
- Four or more untreated control rows through the center (eight shown below).
- Evaluation trees are selected in the rows adjacent to the untreated control.
- All evaluation trees (in treatment and UTC) are similar, as described above.
- Harvest untreated control rows separately to collect yield data.
- A simpler version of this design can be implemented similarly to design 1 by eliminating the evaluation trees and collecting yield data from the UTC and treatment rows separately.



Evaluation methods

The most important methods suggested for evaluating the effect of treatments are the following, listed in order of importance.

- **Yield**
- **Disease index (DI)**
- **Pre-Harvest fruit drop counts**
- **Leaf sampling for PCR**

Yield Data Collection

Comparing with prior years block yields is the most basic method for yield evaluation. To determine the most meaningful treatment effects, treatment and control plots should be repeated two or more times in different sets of blocks. This could be accomplished by setting up field trial design 1a two or more times using the same bactericide seasonal use pattern. Alternatively, evaluation trees could be harvested individually and the individual tree yields could be compared to the untreated control.

Disease Index (DI)

A good method to evaluate changes in tree health is by a numerical score based on the observable amount of HLB decline, also known as **disease or decline indexing (DI)**. This provides a method to quantify visible changes in tree health over time. Several methods for rating trees have been developed, and while no method eliminates subjectivity, the method described by Gottwald et al. (1989), minimizes the amount of subjectivity. DI rating should occur at the initiation of a field trial before or at the time treatments are applied and every six months thereafter. Evaluation trees are selected based on a similar initial DI rating. Getting a DI rating is rapid and should require no more than 10-20 seconds per one side of the tree for one person. For consistency, the same person(s) should rate trees each time. If a team is doing the rating, they should work together and all team members rate in both untreated and treated plots.

Reference: Gottwald, T. R., Aubert, B., and Xue-Yuan, Z. 1989. Preliminary analysis of citrus greening (Huanglungbing) epidemics in the People's Republic of China and French Reunion Island. Phytopathology 79:687-693.

Pre-Harvest Fruit Drop

This method is used to evaluate differences in pre-harvest fruit drop due to season-long treatment programs with bactericides. Starting at about 2 months prior to fruit maturity, all fruit on the ground is removed beneath all evaluation trees by raking. Fruit drop counts are made approximately every two weeks to one month thereafter through harvest by raking out, counting and removing fruit from beneath each evaluation tree. Looking at differences in the numbers of fruit subject to pre-harvest drop will provide some information, but calculating percent of fruit dropped will be much more valuable. To calculate percent fruit drop, on-tree fruit will need to be counted at harvest in each evaluation tree. The percentage of fruit drop is calculated by dividing the total number of total fruit dropped by harvest (fd) by the total fruit dropped plus fruit on the tree at harvest (fh) (fd/(fd+fh)). The fruit on the ground is removed after each count is completed.

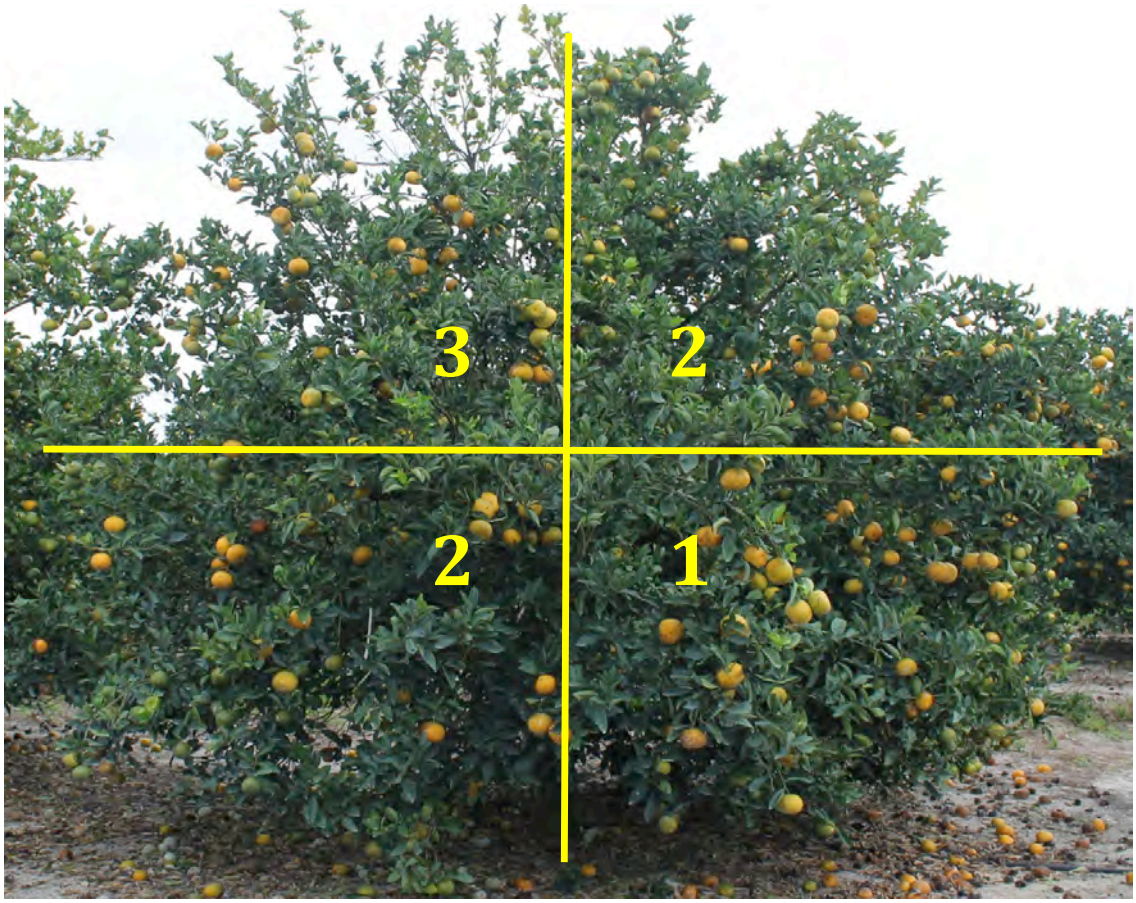
PCR Analysis

PCR is the only method available to quantify the amount of bacteria in a tree. However, this evaluation method is highly variable and results may not correlate well with the relative health of the tree. For a better understanding of PCR, see this document: http://citrusrdf.org/wp-content/uploads/2012/10/What-is-PCR.pdf

How to Conduct Disease Index (DI) Rating

Facing the tree, divide the first side of the tree into quadrants by imagining a vertical and a horizontal line at mid-canopy height. Score each quadrant individually for visible symptoms on the 0-5 scale (described on the following page) and combine the scores. Rate each quadrant on the opposite side of the tree from 0 to 5 in the same way, and combine the scores from each side of the tree for a total DI score of 0-40. A DI of 25 is a moderately declined tree, CRDF often uses this as a cut-off DI for the selection of trees for field trials.

The following is an example of the scores given to four quadrants of a diseased tree:



Disease Index (DI) Rating Values

0 = No foliar disease symptoms visible in the quadrant.

1 = Foliar disease symptoms on <20% of the quadrant. A dense quadrant with no twig dieback and minimal blotchy mottle.

2 = Foliar disease symptoms on 20-40% of the quadrant. Dense quadrant, some twig dieback, some blotchy mottle, and possibly some tufted growth on the canopy.

3 = Foliar disease symptoms on 40–60% of the quadrant. Thinning quadrant with noticeable twig dieback and a few areas of open canopy. Blotchy mottle of leaves is common and some tufted growth is apparent.

4 = Foliar disease symptoms on 60–80% of the quadrant. Abundantly thin quadrant with obvious twig dieback. The majority of the branches have blotchy mottle and/or tufted growth.

5 = Foliar disease symptoms on >80% of the quadrant. The decline has resulted in dieback of large branches and remaining leaves are small, have blotchy mottle and may be deformed

Tufted growth: elongated, bare branches with clusters of small branches and leaves

Blotchy mottle

Photo Credits: Brandon Page

Citrus Leaf Sampling Protocol for Submission for PCR Analysis

Contributed by Mike Irey, United States Sugar Corporation

Collection of Samples

Time of Year

1. If possible, samples should be collected from **late July through March** although samples will be accepted throughout the year. *Samples collected during non-optimal times of the year may not give an accurate representation of the disease status of the trees sampled.*
2. Avoid collecting samples during leaf flushes (stop when feather flushes are approximately 2-3 inches long. Resume sampling when leaves from the most recent flush are fully expanded and beginning to harden off.

What Tissue to Sample

Asymptomatic Field Trees **(no symptoms at all on the trees):**

1. Samples should be collected from branches that are dark green and angular to slightly rounded in cross section. The bark should be green and not brown.
2. Leaves from the sampled branch/twig should be fully expanded and hardened.
3. Collect 6-8 leaves (including the leaf petiole) from around the tree.
4. Alternatively, cut 6-8 branches (at least 4 inches long) and trim them so that only the angular (to slightly rounded), dark green section of the branch/twig containing fully expanded hardened leaves is left. Leave the leaves on the twig.
5. Place the leaves/twigs into a sealable (e.g. zip lock) plastic bag and keep the sample cool and out of the sunlight.

Symptomatic Field Trees

1. Samples should be collected from the symptomatic areas/branches of the trees.
2. Samples should consist of short sections (4-6 inches or greater) of symptomatic branches with the attached leaves. If fruit are present on the branches, the fruit can either be left on or they can be trimmed off. If the fruit are trimmed off, please leave the fruit stem on the sample (i.e. trim the fruit off as close to the button as possible leaving the stem on the branch).
3. If a variety of symptoms are present, the preferred samples (in order of preference) would be:
    a. Branches with mottled leaves
    b. Branches that contain shoots that are almost entirely yellow
    c. Branches that have leaves with yellow veins
    d. Branches with leaves that have either green islands on a yellow background or yellow islands on a green background
    e. Branches with nutrient deficiencies that have a “rabbit ear” appearance (small, upright leaves)
    f. Branches with leaves that show chlorosis and “vein corking”
    g. Branches with zinc and/or iron deficiencies that are not related to blight or other known causes


Vein Corking

4. Place the leaves/twigs into a sealable (e.g. ziplock) plastic bag and keep the sample cool and out of the sunlight.

Sample Handling and Shipping

1. Each sample should be in an individual sealable plastic bag (zip lock).
2. A completed sample submission form should be included in the bag (or stapled) with each sample
3. Samples should be double bagged.
    a. When multiple samples are being submitted, multiple samples can be placed in one or more larger sealable bags, however each individual sample needs to be bagged individually and have its own individual completed sample submission form attached.
    b. If a large number of samples are being submitted from a nursery (i.e. a nursery submission from an increase block) it is permissible to use one form to cover each bench/block.
4. Samples should be kept cool and out of direct sunlight until shipping (i.e. cooler with ice, cooler or box w/o ice kept in the shade, etc.).
5. If at all possible, the samples should be shipped or hand delivered the same day that they are collected. If same day shipment/delivery is not possible, samples should be shipped/delivered the following day provided the samples are kept cool (but not frozen).
6. **Samples should be shipped by overnight mail or courier and must not be shipped or held over the weekend.**

PCR Sample Submission

Two laboratories in Florida accept leaf and psyllid samples for PCR analysis. Both labs use similar methods and provide similar quality results. Both labs are funded by CRDF and samples are analyzed at no cost to growers.

Southern Gardens Diagnostic Laboratory

United States Sugar Corporation
Technical Operations
Attention: Mike Irey
111 Ponce de Leon Ave
Clewiston, FL 33440
863-902-2249
msirey@ussugar.com

Instruction for sample submission at:
www.flcitrusmutual.com/content/docs/issues/canker/sg_samplingform.pdf

UF/IFAS SWFREC HLB Laboratory

SWFREC HLB Lab
2685 State Road 29 North
Immokalee, Florida 34142
239-658-3400
ssteems@ufl.edu

Information and sample submission form at:
http://www.imok.ufl.edu/programs/plant-path/hlb-lab/

Submission forms for both laboratories can be found online and also at the end of this document.

Additional Evaluation Methods

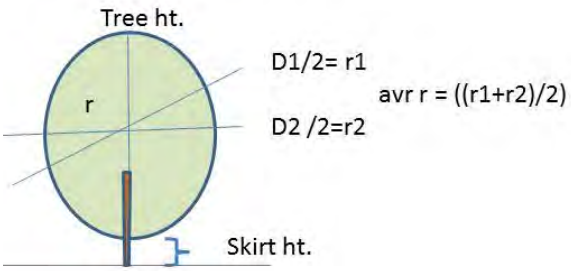
These methods may be used to add more information and thus power to the evaluation of bactericide treatments to improve citrus health.

Trunk diameter

Two perpendicular trunk diameters (D) are measured with calipers and averaged to get D on each of the trees before/at treatment and annually thereafter. Diameters are measured at exactly 8 to 12 inches above the ground (or above the graft union) depending on tree size. It is important to be consistent so you can return to the same spot on the trunk next year. You can also use a tape measure to measure trunk circumference (C) as trunk diameter can come from C/p; C=pD. The trunk cross sectional area (CSA) can be calculated geometrically from the trunk radius (r=D/2) using the formula pr^2 (where p = 3.14).

Canopy volume

Canopy volumes (in cubic feet or cubic meters) is calculated using a geometric prolate spheroid formula: [(4 / 3) (p) (tree height/2) (average canopy radius)^2]. p=3.14, D = average diameter, r = radius, and ht. = height.



Canopy dimensions are sighted using a pre-measured marked pole. Tree height is the distance from the ground surface to canopy top ignoring any escaped branches. An average canopy radius is calculated from ½ of the average diameter width.

Canopy density

Canopy density is qualitatively estimated by visually classifying overall tree canopy density into 3 classes: **Healthy** = thick green canopy and few visible woody branches. **Moderate** = some yellow leaves, some leaf loss, woody branches visible, a few fruit dropped. **Declined** = some dieback, visible leaf loss, obvious fruit drop and an open declined canopy.

Nutrient analysis

Twelve mature, six month-old spring flush leaves from each of the trees are sampled during July-August for nutrition analyses and submitted for routine analysis of major and minor elements.

Data Analysis

Once the data collection is complete, there are some simple descriptive statistics that can be used to compare results. Instructions for this analysis are beyond the scope of this document, however this YouTube video (https://www.youtube.com/watch?v=siqx4PbqJ6s) demonstrates an easy method for comparing data using Microsoft Excel®. Complete this procedure with all of the treatment and the untreated control data, if there is no overlap in the upper and lower bound value range, there is a treatment effect.

Appendix A

Huanglongbing (Greening) Sample Submission Form

US Sugar Corporation

(► Required Information)

Submission Information

►Date Sample Collected:_____ ►Date Sent:_____

►Submitter name:_____ Affiliation:_____

►Address:_____

_____

►City:_____ ►State:_____ ►Zipcode:_____

Email address:_____

Phone Number:_____Fax Number:_____

►Results To Be Returned By: Mail ☐ Email ☐ Fax ☐

Grove/Nursery/Sample Information

►Grove/Nursery Name:_____

►Address/Location Where Specimen Was Collected:_____

_____

►City:_____ ►County:_____

Block/Row/Tree Designation *(must provide enough information to be able to locate the specific tree sampled)* :_____

Latitude:_____ Longitude:_____

Section/Township/Range:_____ Sample Id (local id):_____

Host Plant Name/Variety:_____

Tree Age ☐ Tree size ☐ Symptomatic ☐

Additional Comments:_____

_____

_____

Do not write in this area

Date Received:_____ Lab Id:_____

Shipping/Delivery Address: United States Sugar Corporation, Technical Operations
111 Ponce de Leon Ave, Clewiston, FL 33440, 863-902-2249 (Mike Irey)

HLB Lab Sample Submission Form

Ship Samples To: HLB Lab, UF-IFAS-SWFREC, 2685 SR 29 N, Immokalee, FL 34142

Client:

This is the person submitting samples and the lab's contact for correspondance

*Name: _____

Association (Company): _____

Address: _____

City: _____ State: _____ Zip: _____

Phone: _____ [ ]Office [ ]Other: _____

_____ [ ]Cell [ ]Other: _____

Fax: _____

*E-Mail Address: _____

*Preferred Method of Contact for Receiving Results:

[ ] E-Mail [ ] Fax [ ] Mail

Sample Location:

UNIVERSITY OF FLORIDA
Institute of Food and Agricultural Sciences

This is the grove or nursery where samples were taken

[ ] Check **here** for Residential Samples

[ ] Check **here** if **Sample Location** is the *same* as **Client Address**
(please still provide County and Grove Name, if applicable)

*Grove Name: _____

Individual fields within a Grove or Company are not to be listed for the Grove Name.
Please list fields, if applicable, in the comments section at the bottom of the page.

Address: _____

City: _____ State: _____ Zip: _____

*County: _____

Date Samples Sent to Lab: _____

Items marked with an asterisk (*) are required information

Sample Information must include Sample ID and/or unique identifying Block/Row/Tree information

Lab Use Only **Lab ID**	Sample ID	Date Sampled	Variety	Rootstock	Block	Row	Tree	Township	Range	Section	Latitude	Longitude	Age (yrs)	Size (ft)

Comments: _____ Page ____ of ____

_____ Date Received by Lab: _____

Appendix B