Citrus Research and Development Foundation, Inc.

Field Trial Tree Evaluation Methods

These field evaluation protocols have been developed by CRDF project managers to evaluate CRDF field trials. These evaluation methods will provide valid data on the impact of various treatments on tree health, as well as the impact of treatments on bacterial infection and HLB disease. Methods are intended to be straightforward and easily adaptable to different locations and treatments.

Tree canopy decline index: The most basic field trial evaluation includes a visual decline index (DI) rating before and after treatment and twice a year thereafter. DI ratings can be done on 12 treated trees and 12 control trees in about 1 hour. This rating plus yield data will provide sufficient information to compare the treated trees with the untreated control trees. It is important to include untreated control trees or standard practices or treatments in a field trial to serve as a basis for comparison of results.

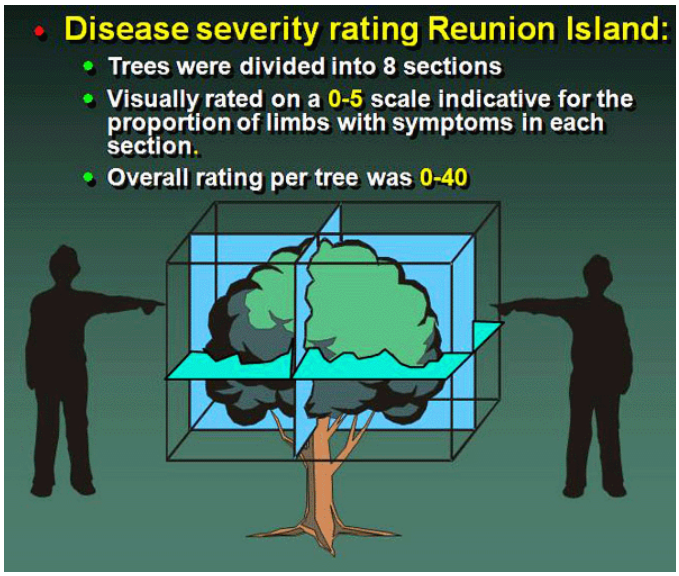


Illustration from Gottwald *et al.* (1989)

Tree canopy decline index (DI) score: Each canopy hemisphere is divided into four equal quadrants by two imaginary perpendicular planes (vertical and horizontal at mid canopy height) passing through the axis of the tree trunk. The resulting eight sections (4 on each side of the canopy) are scored individually on a 0-5 scale indicative of the proportion of limbs expressing HLB disease symptoms within each section (0 = no limbs, 5 = all limbs). The sum of the eight scores for each tree results in a severity rating of 0-40 for each tree on each survey date. For example, trees with a DI score of 32, have an average DI of 4 x 8 sections = 80% declined with symptoms.

Pre-treatment tree evaluation: Taking measures of tree health and disease status prior to treatments is important to help establish a baseline for the comparisons of treatments.

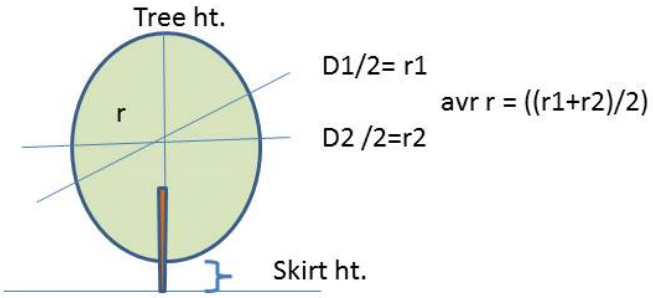
Pre-treatment tree and grove documentation should include tree age, tree spacing/density, scion, rootstock, soil type, soil pH, general moisture status, and cultural practices including irrigation scheduling, water quality (salinity, bicarbonates), fertility programs, previous leaf nutrition, pest/psyllid control, yield records and fruit quality. Good horticultural care should continue uniformly in the entire trial area. At each site, there should be at least 12 replicate trees of each treatment plus 12 similar untreated control trees. 3-4 replicates of these 24 tree sets are desirable. Tree evaluations should focus on the middle 10 trees (excluding end buffer trees) of each of the treated and untreated trees for a total of 20 measurement trees in each replicate per site. Each of the 20 trees in each group should be assigned a unique treatment and replicate number. Treatment and control trees could be in rows of trees or in blocks with buffer trees on the borders.

Photographs of each of the 20 measurement trees at a standard distance, direction and size, are taken just prior to the treatment for comparison with later photos.

PCR analysis. A description of PCR can be found at http://citrusrdf.org/wpcontent/uploads/2012/10/What-is-PCR.pdf. This document includes the contact information for the two laboratories in Florida that provide this service to growers. Instructions for PCR sampling and submission can be found at http://www.flcitrusmutual.com/content/docs/issues/canker/sg_samplingform.pdf. Briefly, 6-8 mature leaves with petioles attached are selected from around the canopy, placed into a zip-lock bag and labeled with the tree unique ID code. Sealed sample bags should be kept on ice and out of the sunlight and immediately transported to the laboratory.

Trunk diameter. Two perpendicular trunk diameters (D) are measured with calipers and averaged to get D on each of the trees before 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/π; $C=\pi D$. The trunk cross sectional area (CSA) can be calculated geometrically from the trunk radius ($r=D/2$) using the formula πr^2 (where $\pi = 3.14$).

Canopy volume. Canopy volumes (in cubic feet or cubic meters) is calculated using a geometric prolate spheroid formula: $[(4 / 3) (\pi) (\text{tree height}/2) (\text{average canopy radius})^2]$. π=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. If there is no skirt height, then subtract about 10% from the canopy volume for the flat bottom. Estimated volume corrections are made for raised skirt heights. An average canopy radius is calculated from ½ of the average diameter width.

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

Fruit drop. All fruit on the ground is removed prior to treatment. Fruit drop counts are made approximately every two weeks after treatment. Percentage fruit drop is calculated by dividing the number of total fruit dropped (fd) by the total fruit dropped plus fruit harvested (fh) (fd/(fd+fh)). The fruit on the ground is removed after each count is completed.

Post-treatment tree evaluations:

Post-treatment tree evaluations may begin as soon as one week after treatments to evaluate any incidental short-term tree injury from treatments.

Photographs of the trees at a standard distance, direction and size, can be taken one week after treatment, 3-6 months later and/or annually thereafter.

Decline Index. Depending on tree condition, DI is evaluated every six to twelve months after treatment.

Leaves for PCR test. Leaves may be sampled as soon as 45 days after treatment, 3-6 months later and/or annually (winter season will produce the most reliable results).

Trunk diameter. Trunk diameters or a trunk circumference are measured annually in December.

Canopy volume. Canopy volumes may be evaluated twice per year.

Canopy density may be rated twice per year and rated as **Healthy, Moderate** or **Declined** as defined above.

Fruit drop. Fruit drop counts may be made after treatment until harvest.

Leaves for nutrient analysis. 12 mature, 6 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 (watersag.com).

Fruit quality analysis. At the first harvest after treatment, 50-fruit samples may be sampled from each of the trees and put into labeled net bags for fruit quality analyses and be delivered to a testing lab. There are currently no laboratories in Florida offering this service to growers, but a buyer or processor may be able to assist with arranging for analysis.

Canker evaluation may occur in August through a foliar survey for characterizing canker lesions, as well as evaluating any fruit for presence of canker lesions. Disease indexing for canker is performed by dividing the tree visually into eight sectors (similar to the Gottwald et al. (1989) method). Each sector is evaluated for the percent of leaves with lesions and percent of fruit with lesions (two separate values) and given a score of 0-4 (0 = no lesions, 1 = 25%, 2 = 50%, 3 = 75%, 4 = 100% of leaves with lesions). The summation of the eight scores for each tree will result in a severity rating of 0-32 for each tree.

Data interpretation and analysis: Comparisons can be made between the treated trees and control trees. Tree photographs, DI ratings, trunk diameter, fruit drop and PCR data can be used as covariates in data analyses and collectively illustrate the effects of treatments

Reference for visual decline index methods:

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.

The following is an example of a CRDF field trial protocol; this is not necessarily a template for a grower trial, but is an example of a comprehensive field trial.

Title of Project: Evaluation of five bactericides against HLB

Investigator(s):

Situation Statement: This is a study to provide a side-by-side comparison of five bactericides products as a therapy for HLB on young trees. The impact of treatments on psyllids, tree health, foliar nutrition, disease rating, and HLB status will be evaluated. The trial will be large enough and replicated sufficiently to allow statistical evaluation. Materials will be applied to young trees that are in early stages of decline from HLB.

Objectives of the Project:

Determine ability of candidate materials to suppress CLas populations in trees (2-4 years old) already infected and showing symptoms of HLB

Six treatment plots of ten trees each will be established based on a map of the initial PCR ratings. Each treatment will be replicated four times such that there will be 24 plots of 240 total trees.. Treatment plots will include:

1. Material 1
2. Material 2
3. Material 3
4. Material 4
5. Material 5
6. Water control

All products will be foliar-applied by conventional air blast spray every 60 days at approximately 150 gallons/acre.

Proposed Start and End Date:

PCR sampling, block set-up and pre-application evaluations will occur in late 2015. Treatments will begin in early February 2016. Treatment applications will occur every 60 days for one year (six total applications) with the option to continue for a total of two years.

Narrative:

This study will test the hypothesis that these five products will mitigate the effects of HLB on tree health and yield of infected trees. After two years, we will have valid information on the impact of these treatments on tree health, disease rating, HLB status, foliar nutrition, yield and fruit quality.

Pre-treatment tree and grove evaluation will include scion, rootstock, soil type, soil pH, and good horticultural practices including irrigation, fertility program, leaf nutrition, recommended young tree psyllid and other pest control including appropriate rates of neonicotinoids (by the cooperating grower), subsequent yield (by the CC) and fruit quality. Good horticultural care should continue for the duration

of the trial. The field site will be chosen by the contracted crop consultant (CC) in conjunction with the CRDF field trial administrator.

Field Trial Set-Up:

Studies will focus on a subset of 6 measurement trees in the middle of each of the 10 tree plots; 6 trees x 4 reps = 24 trees x 6 treatments = 144 measurement trees total. The two end trees on each end of the 10 tree plots will be avoided as buffer trees. Measurements will focus on the 6 central trees (144 trees) in each 10-tree plot. Once the plots are established, all 144 measurement trees must be initially visually rated using canopy sectors for Disease Index (DI= 0 to 40, Gottwald et al., 1989, see below) to determine the visual disease status. All 144 measurement trees will receive a unique ID code that will include the treatment ID (1-12, replication number (1-4), row/tree ID. GPS locations for each tree should be noted if available. ID codes will be built into Excel data sheets for DI data entry.

Photographs:

All 144 measurement trees will be digitally photographed (CRDF) at the beginning of the experiments and every 12 months thereafter.

Fruit Analysis:

At the first harvest after treatment, 50-fruit will be pooled from various canopy positions from the 6 measurement trees (8-9 fruit from each tree) (CC). Each sample will be put into labeled net bags for fruit quality analyses and will be delivered to a testing lab after prior arrangements have been made. Total yield and remaining fruit counts will be collected from each of the 144 measurement trees at harvest. Weight of the 50-fruit quality samples divided by six, will be added to calculate total yield per measurement tree. Fruit counts will be used to calculate percentage fruit drop (total fruit drop / (total fruit drop + remaining on-tree fruit) for each of the 144 trees.

Leaves for PCR:

Six to eight mature leaves (with petioles attached) from each of the 144 measurement trees will be sampled (CRDF) from around the canopy at the beginning of treatments and in winter (Sept-Mar) thereafter. If visible blotchy mottle symptoms appear**,** sampled leaves should be mature symptomatic leaves and placed into a sealable (e.g., zip lock) plastic bag, labeled with the tree with the unique ID code (as above) for each measurement tree and GPS coordinates if available. Sealed sample bags should be kept cool and out of the sunlight (e.g., in ice chests). Samples should be immediately delivered to Southern Gardens Diagnostic Laboratory accompanied by the site information and sample form at http://www.flcitrusmutual.com/content/docs/issues/canker/sg_samplingform.pdf (see this site for leaf sampling details) to be tested for *CLas* titers by qPCR. If there are no visible symptoms, 6-8 mature leaves (with petioles attached) per measurement tree will be sampled from around the canopy (CRDF), sealed, labeled and delivered as above.

Leaves for Nutrient Analysis:

Leaves of measurement trees will be sampled annually in summer (July-August) for nutritional analyses. Two mature leaves from each of the 6 measurement trees per main plot, will be sampled (CRDF) from around the 6 canopies, pooled into 1 sample (12 leaves total from each of the 24 plots, sealed in Water's sampling bags (will be supplied), labeled with the site/treatment/plot ID code (CRDF) and immediately forwarded to Waters Ag Lab for leaf nutrition analyses. (24 total samples of 12 leaves each).

Fruit drop:

All fruit on the ground of the 144 trees will be raked out prior to treatment and fruit drop counts will be made every 2 weeks thereafter until harvest (CRDF).

Tap Sampling for ACP:

Tap samples will be conducted at 3 separate locations on each measurement tree (CRDF). Every effort will be made to ensure the tap sample locations contain new flush in varying stages of development. The number of psyllids collected from the 3 tap sample will be totaled and that number will be recorded for each measurement tree. The total number of tap sampled adult ACP via spreadsheet.

Tap sampling procedure:

1. Place the sample sheet (white paper, piece of cardboard, or plastic) 12 inches under the branch to be inspected.
2. Strike the branch three times with your hand, pvc pipe, broom handle, etc.…
3. Quickly examine the sample sheet for ACP and record the total number of ACP collected
4. Preform three tap sample on each measurement tree. The total number of ACP collected from the three tap samples will be the number recorded and reported

Visual inspection for ACP:

Visual inspection for ACP will be conducted on each of three different branches per measurement tree during each scouting period (CRDF). Branches that will be visually inspected should have new growth that has not fully developed (preferably the branch will have feathering flush). The visual inspection will focus on all life stages of ACP; adult, nymph, and eggs. The Crop Consultant will report the total number of adult ACP, ACP nymphs, and ACP eggs via a spreadsheet.

Data Interpretation and Analysis:

Data will be reported to the CRDF field trial administrator as data are collected. The CRDF field trial administrator at CREC will analyze data monthly.