Citrus Research and Development Foundation, Inc.

Appendix – Plant Improvement Germplasm Evaluation Guidelines

Plant improvement germplasm evaluation guidelines

Section 1. Guidelines for proposals seeking funding to collect data on existing germplasm evaluation trials

CRDF recognizes that there are existing preliminary greenhouse and field trials as well as advanced stage field trials that may require funding for data collection. Although, these trials may not fit the criteria outlined for new trials, they may be considered for funding for data collection. Programs seeking funding should follow these guidelines when preparing a proposal to seek funding to evaluate existing trials.

Preliminary greenhouse and field trials

These trials should be embedded in core breeding proposal objectives as they occur earlier in the breeding cycle. Principal investigators seeking funding for data collection for these projects should describe the objectives, population development, targeted traits of interest, evaluation materials and methods, statistical design and analysis of ongoing experiments. PI's should submit available summary preliminary data for each cycle of evaluation on the trials.

New greenhouse experiments and preliminary (early stage) field trials should adhere to the minimum guidelines below which were adopted from a document developed by the team of plant improvement researchers from Arizona, California, Florida and Texas, nominated at the National Citrus Breeding Collaboration meeting on February 27, 2018 in Denver, Colorado.

Greenhouse experiments

Experimental design:

- Experiments must use control (non-inoculated) plants of the same variety and age as the inoculated or infected plants.
- Experiments must maintain controls and infected plants under the same conditions. Plants should be randomized.
- It is suggested to use at least 10-12 plants per treatment and variety to conduct disease/health evaluations. For additional genomics, metabolomics, or other analyses, a small but representative subsample of plants can be used.
- Plants to be used in the experiments should be as homogeneous as possible
- Use a minimum of 10 psyllids and 2 tissue pieces per plant for psyllid transmission and graft transmission of *C*Las, respectively. Number of psyllids and tissue pieces may be adjusted based on the size of experimental plants. Bud stick grafting is also a suitable method for inoculation. Provide

information on percentage of infection of psyllid colony and titer levels of plant variety used for inoculation. Use standardized PCR methods and provide Ct-values and DNA concentration of plants from which inoculation material was obtained.

- Experiments must define the source of *C*Las used and reference to any previous publications or work with the same source.
- Researchers must do one of the following:
    - Use a *C*Las strain that is maintained in the exotic pathogens collection of citrus at USDA-ARS, Beltsville, MD.
    - Use a strain that has been sequenced or extensively genotyped.
    - Maintain the strain that they use.
    - Maintain the strain that they use as -80 frozen tissue for future sequencing or other analysis.

Data collection:

- One or more measures of plant size must be recorded at beginning and end of the experiment:
    - Stem diameter (preferred). For non-grafted trees measure at 10 cm above soil level. Height above graft union for measuring stem diameter of grafted trees is preferred at 5 cm but can vary depending on factors such as site of graft inoculation. Must be defined and consistent within an experiment.
    - Plant height or, if plants were pruned, the sum of the length of all branches after regrowth. Indicate times and frequency of pruning.
    - Biomass at the end of experiment. May be separated into shoot, leaf, and root portions.
- Greenhouse conditions and management practices should be recorded in as much detail as possible, including pot size, potting medium, nutritional program, temperature ranges, light levels, and any trimming or training of plants.
- Conduct foliar disease symptom ratings at different time intervals throughout the experiment using a scale from 1 to 5, with 1 = no foliar disease symptoms, 2 = foliar symptoms on less than 25% of leaves, 3 = 25-50% of leaves with symptoms, 4 = 50-75% of leaves with symptoms, 5 = more than 75% of leaves with symptoms.
- Document type of foliar symptoms (chlorosis, blotch mottle, reduced leaf size, vein corking, etc.).
- Collect at least 3 leaves (number may vary based on plant size) at different time intervals for *C*Las detection (may have this coincide with disease ratings). Choose mature leaves randomly from different areas throughout the canopy to account for variation. Pool tissue for analysis. Use petioles/midribs for *C*Las detection.
- If *C*Las testing of roots is to be part of the experiment, fibrous roots (≤ 2mm) should be used.
- Use the "Li primers" (Li et al., 2006) for real-time PCR detection of *C*Las. New guidelines on primers may follow.
- Report DNA concentration, Ct-values, and percent of infected plants. If the Ct values are used for classification of which trees are infected and which are not infected by *C*Las, the cutoff should be clearly indicated on reports.
- Duration of experiment and time intervals for disease ratings will vary depending on the age of plants used. Duration of experiments should be a minimum of 6 months following inoculation, but preferably 12 months. Time intervals for PCR detection may vary based on resources and main purpose of the experiment.

Early-stage field trials

Experimental design

- Experimental design should be completely randomized or randomized blocked. Design will depend on trial objectives, number of rows available, row length, and tree spacing. Should be balanced across treatments/genotypes as far as numbers of individuals and reps in the test.
- As much as possible, all trees to be compared in a particular trial should come from the same nursery and be planted at the same time.
- Variability of soil and irrigation conditions should be taken into account in the experimental design (blocking).
- A minimum of 6 replicates should be used. Depending on the trial objectives, replicates can be 3 trees, or more; a minimum of 3 trees is preferred. The relative balance of number of replications and number of trees per replication may vary according to the particular situation.
- Plant border trees at end of rows, and in adjacent rows on each side, when possible.

Data collection

Data to be collected from a field trial may vary by trial objectives, conditions, and resources available. The following metrics should be used:

- Tree size. Measure at one or more time intervals before the completion of the trial.
    - Trunk diameter for scion and rootstock. Measure at 5 cm above and below graft union. Be consistent and return to the same spot on the trunk every year. Measure in two perpendicular directions and use average. Alternatively, trunk circumference can be measured, and trunk diameter calculated using the formula [circumference/π]. Report trunk cross-sectional area (TCSA) using the formula [π x (diameter/2)^2].
    - Tree height to top of canopy (do not include height of vigorous shoots that extend significantly past the top of the canopy).
    - Canopy diameter (parallel and perpendicular to the row).
    - If hedging and/or topping are done to the block, this needs to be clearly noted, and may significantly change the value of subsequent canopy size measurements.
- Once tree height and diameter are measured, calculate canopy area and/or volume. Measure canopy diameter parallel and perpendicular to row.
- Calculate standard canopy volume according to the formula: [(diameter parallel to row x diameter perpendicular to row) x height]/4, modified from Wutscher and Hill (1995).
- Determine leaf macro and micronutrient concentrations annually during July-August from 12 mature, 4 to 6-month-old spring flush leaves from each or a subset of trees depending on experimental design.
- Report percentage of dead trees periodically or at the end of a trial period. Dead trees should be excluded from further ratings and analyses, or if included, this should be noted. Inferred or hypothesized cause of tree death may be noted. In many cases, trees that die in the first year are not the result of *C*Las effects and may be excluded from HLB-associated assessments.

- If a trial is located in an HLB-endemic environment, conduct foliar disease ratings using a scale from 1 to 5, with 1 = no foliar disease symptoms, 2 = foliar symptoms on less than 25% of leaves, 3 = 25-50% of leaves with symptoms, 4 = 50-75% of leaves with symptoms, 5 = more than 75% of leaves with symptoms. Calculate disease index as described below based on tree size and age:
    - For very small trees, rate the entire canopy as one unit. The maximum score per tree will be 5.
    - For medium trees, divide canopy into two sectors and apply ratings to each sector. The maximum score per tree will be 10.
    - For larger trees, divide canopy into 4 sectors and apply ratings to each sector. The maximum score per tree will be 20. If trees are very large, divide into 8 sectors for a maximum score of 40.
    - To standardize ratings across trees sizes, divide the total score by the number of sectors used, so that all tree ratings are expressed on a 1-5 scale.
- Conduct canopy thickness and color ratings using a scale from 1-5 as described below. Apply ratings to one, two, four, or eight sectors of the canopy depending on tree size, with a maximum score of 5 for smallest trees and 40 for large trees. To standardize ratings across trees sizes, divide the total score by the number of sectors used, so that all tree ratings are expressed on a 1-5 scale. Dead trees are not to be scored for canopy thickness or canopy color, and so will not affect average values in analyses.
    - *Canopy thickness*
      1 = very thin canopy, 2 = thin canopy, 3 = medium canopy, 4 = thick canopy, 5 = very thick canopy. It is recommended to illustrate differences between ratings photographically.
    - *Canopy color*
      1 = very yellow unhealthy canopy, 2 = yellow unhealthy canopy, 3 = moderately healthy canopy, 4 = healthy green canopy, 5 = very healthy dark green canopy. It is recommended to illustrate differences between ratings photographically.
- Document foliar diseases not associated with HLB if commercially relevant (e.g., canker) particularly when evaluating different scion varieties.
- Foliar disease and health ratings should be conducted at the same time of year. In Florida and Texas, fall is recommended for scoring disease symptoms, as that is the time they will usually be most pronounced (once temperatures are dropping). Additional ratings during spring and/or summer can provide important information and are recommended, particularly when evaluating new scion varieties.
- Tree appearance may be documented photographically using a measuring pole as reference.
- PCR evaluation of trees for *C*Las:
    - Collect mature leaves from most recent flush and use petiole/midribs for CLas detection. Depending on tree size, collect one or more leaves randomly from each of the four cardinal directions.
    - Collect fibrous roots (≤ 2mm) for *C*Las detection. Depending on tree size, collect fibrous roots from a minimum of two different cardinal directions, avoiding zones of overlap between adjacent trees.
    - Conduct leaf and root sample collections annually or at the end of the evaluation period (such as the end of four years of harvest). May coincide with disease and health ratings.

- Use the "Li primers" (Li et al., 2006) for real-time PCR detection of *C*Las. New guidelines may follow.
- Once trees reach maturity, collect fruit yield and fruit quality data each season. Conduct yield and fruit quality assessment at dates that are standard harvest times for that cultivar, or harvest times that are proposed for new cultivars. Report date of assessment.
    - Yield - assess directly by weighing fruits per replicate or indirectly by counting number of fruits per tree. Report as fruit weight per experimental unit. Alternatively, yield can be measured as boxes of fruit per tree.
    - Fruit weight – determine from random subsample of fruits from each tree, or group, depending on what is practical.
    - Fruit size - determine from subsample of fruits from each tree, or group, depending on what is practical for the situation. Measure the horizontal or vertical diameter (as appropriate) of the subsample of fruit collected for determination of fruit weight.
    - Fruit quality – depending on the type of fruit and trial purpose, determine percent juice, brix, acid, brix/acid ratio, external color, and juice color from subsample of fruits according to standard laboratory methods.
    - Sampling time will vary based on scion variety maturity and other factors. Select time that is most appropriate for the scion variety under evaluation.
    - If appropriate, assess percentage of visually abnormal putatively greening-affected fruit per tree.
    - If appropriate, assess fruit drop pre-harvest. Report as percent drop from fruit number data.

Existing Pre-commercial or large-scale field trials

PI's seeking funding to collect data on existing field trials should describe the field trials using the following guidelines.

1. What are the objectives of the trial?
   Describe the criteria used to select each nominated candidate rootstock or scion variety. Include a brief description of pedigree, population development, and experimental design and phenotyping methods used to characterize populations at each screening stage. Submit summary data from preliminary trials on each candidate rootstock or scion.
2. Provide information on ownership of the genotypes and technology associated with the candidates selected including but not limited to transfer agreements or constructs containing proprietary technology in the case of engineered genotypes.
3. Provide a timeline for the experiment describing establishment date. Provide all planting dates in cases where some genotypes were planted later. What was the source of plant material for the field trial?
4. Provide information on the site characteristics, field layout and a field map of the trial. The field map should provide sufficient detail to identify blocks, treatments (rootstock or scion) and site characteristics (ponds, ditches, slope, windbreaks, etc.).
5. Describe the field trial in detail including the number of candidate genotypes, commercial standards, number of sites, experimental unit (number of trees), observational unit (number of trees), replications and statistical design.

6. What are the horticultural practices used to maintain the trial? Please identify all treatments applied to the trial. If there are unbalanced, treatments explain how they are managed for statistical variation.
7. Describe in detail the traits of interest and data collection and analysis methods.
8. Submit summary data collected in the pre-commercial trial.
9. Identify candidates that have been submitted for disease indexing.

The scientific advisory board or ad-hoc reviewers, CRDF Committees and Board will review the information provided above to identify trials or portions of trials that merit funding for data collection will review the information provided above. As with new pre-commercial trials, CRDF will engage the citrus industry for comment on the relevance of the trials to industry needs to inform decisions on funding.

Section 2. Proposal guidelines for new Pre-commercial citrus field trials:

Proposals developed to solicit funding to conduct new large-scale pre-commercial variety field trials must describe the following.

Description of genotypes nominated for pre-commercial citrus field trials

1. Describe the criteria used to select each nominated candidate rootstock or scion variety including:
    a. A brief description of parental pedigree, population development, experimental design and phenotyping methods used to characterize populations at each screening stage.
    b. Provide information on ownership of the genotypes and technology associated with the candidates selected including but not limited to transfer agreements or constructs containing proprietary technology in the case of engineered genotypes.
    c. Submit summary data on each candidate rootstock or scion showing performance for all the traits of interest measured in preliminary trials. PI's should include a coded name for the candidates in a separate column. For example US2018R. To protect intellectual property these codes will be used when presenting information in public meetings where pedigrees and other confidential information **will not be disclosed.**
    d. Indicate whether candidates have been submitted for disease indexing.
    e. What is the source of plant material for the field trial?
    f. Synchronized planting of the pre-commercial field trial is vital for direct comparisons. How much disease free plant material is available for the field trial?
    g. Information provided in a – e above will be reviewed by a scientific panel and CRDF Board and Committees.

Final candidate list determination

The pre-commercial variety field trials serve as a powerful source of data for the citrus industry, and therefore it is important to engage the industry at large for input on the strength of the candidates and data summaries provided by the breeding programs. CRDF committees and Board will invite the principal investigators, collaborators, and industry stakeholders to evaluate the list of candidates and the associated summary data. Data presented at this public meeting will not provide confidential

information such as pedigrees. Summary data of coded lines will be shared publically along with ranking from the scientific panel, CRDF committees and Board, breeding program ranking and finally industry stakeholder ranking. At this meeting, the final list of candidates will be selected and approved for the pre-commercial field trial. Principal investigators MUST submit any/all revisions to the approved list of candidates for CRDF committees and Board consideration and approval before implementation.

General Pre-commercial field trial design requirements

The field trial design minimum requirements:

1. Number of sites: Dependent on the state geography and the targeted market. In Florida, at least 3 locations in the Central Ridge, Southeast, Southwest regions for most varieties.
2. Strategic site selection: To evaluate genetic, genetic x environmental and environmental effects on candidate variety performance.
3. At each site, the trial should be mapped to reduce variation and confounding factors.
    a. Account for topography, soil type changes, unbalanced windbreaks, drainage, ditches, ponds, etc.
    b. Multi-location trials should be set up given 3a so that genotypes can be compared for adaptation and regional performance.
4. Statistical design and analysis.
    a. A randomized complete block design (RCBD) or Latin square design is preferred. If more factor levels are necessary, the statistical design must be clearly described for review and approval.
    b. The trial should contain necessary standard (check) rootstocks or scions appropriate to the region for comparison to candidate varieties.
    c. Number of replications: Minimum of 5 replications.
    d. Experimental unit (plot) to which a treatment is applied: Minimum 8 trees. Treatments defined as the candidate varieties evaluated against industry standards.
    e. The observational unit or sampling unit (e.g., trees, branches, leaves) from which data is collected must be clearly defined in trials where the experimental unit has more than the 8 minimum trees required in b, above.
    f. The same observation trees will be measured in each data collection cycle. Due to the high variability of individual trees, a minimum of 8 trees is required for yield data collection. Death of observation trees should be noted in trial data summary reports each calendar year and if possible similar trees should be selected at random within the experimental unit (plot) for evaluation.
    g. The trial should incorporate appropriate rows (2) of buffer around the trial and within the trial. Buffers between plots minimize the competition effect of removing genotypes due to poor performance.
    h. The objective of the advanced field trial is evaluating candidate varieties for commercial relevance. Therefore total yield, fruit and juice quality traits must be measured every season after cropping. Early production is an important goal for new plantings due to higher production costs and shorter life expectancy for trees/groves. This situation has made it imperative that growers make an income as soon as possible so the field trials must be harvested each year until the trial is retired.

i. Trial tree spacing and grove design should be specified taking into account tree size data collected from preliminary field trials if it is available.
j. Horticultural practices: The trial should be managed using appropriate best management commercial practices for plant fertilization irrigation, pest management, pruning, hedging or topping. Grower cooperators will communicate horticultural practices annually. The incidence of wind, freezes, drought or flood or other phenomena that impact the trial should be recorded and reported. Pre-commercialization trials should not serve as a location for overlaying cultural practice treatments or to evaluate any other variable treatments which would interfere with the primary purpose of the trials.

5. Trial establishment : Tree survival should be monitored after planting, and a protocol defined if replanting or removing genotypes is necessary.
6. Location characteristics: Soil type, pH, organic matter content, irrigation water quality, incidents of drought, freezes, hurricanes or other phenomena which affect the field trial should be noted and evaluated. Trials that are affected negatively should be retired if the validity of the data collected is questionable.

Pre-commercial field trial evaluations

Environment

Evaluate candidate variety performance based on the following conditions if applicable: Data collected from grid soil sampling and testing. Soil samples to be collected within each block at the drip line to account for block and block × treatment interaction

1. Salinity
2. pH
3. Soil type
4. Organic matter content
5. Asian Citrus Psyllid infestation- relevant to HLB testing.

Horticultural Performance

Means of propagation: For each candidate variety, describe means and methods of propagation through seed, cuttings or tissue culture.
Tree height (m): Measured from the base of the tree to the top of uniform canopy ignoring errant vigorous branches. Pruning of vegetation as hedging, topping or skirting should be noted.
Tree skirt height (m): Measured from the base of the tree to the bottom of uniform canopy ignoring errant vigorous branches.
Canopy Diameter (cm): Two perpendicular diameters measured between trees along the row and perpendicular to the row.
Trunk diameter (cm): Two perpendicular trunk diameters measured with calipers on each of the observation trees. Diameters will be measured 10 cm above the bud union, a different criteria is used due to branching please describe the selected distance above the bud union.
Leaf nutrient content: Follow established sampling guidelines.
Bloom: Observations of bloom time and environmental conditions.
Yield and maturity date: Fruit maturity date and the total weight of fruit harvested per experimental unit.
Yield/acre: Calculation per experimental unit based on yield/experimental unit and planting density.

Juice quality: Juice weight, total soluble solids, brix/acid ratio, color rating and flavor profile measured on a random pre-determined sub-sample of fruit per experimental unit at harvest.
Fruit quality: Fruit size and color measured on a pre-determined random sub-sample of fruit per experimental unit at harvest.
Post-Harvest fruit evaluation: Where applicable in scion trials evaluate post-harvest handling traits, especially for the fresh-fruit market, such as diseases, bruising, degreening, etc.

Diseases and Pests

Huanglongbing (HLB): PCR testing annually for bacterial titer is required at least once a year during the highest stress period. Leaf tissue samples should be collected from each observational (sampling) unit.

HLB: Visual rating of disease incidence and severity (HLB Decline Index) adjusted for tree size.

Tree canopy decline index (DI) score: For each quadrant visually assess HLB symptoms on a scale of 1 to 5, with 1 = no foliar disease symptoms, 2 = foliar symptoms on less than 25% of leaves, 3 = 25-50% of leaves with symptoms, 4 = 50-75% of leaves with symptoms, 5 = more than 75% of leaves with symptoms.

1. Small trees (Year 1 and 2): Rate the entire canopy as one unit. The maximum score per tree will be 5.
2. Medium canopy trees (Canopy volume ≤ $3m^3$) divide canopy into bilaterally. Apply ratings to each sector. The maximum score per tree will be 10.
3. Mature trees, each canopy hemisphere is subdivided 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 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 summation of the eight scores for each tree will result in a severity rating of 0 - 40 for each tree on each survey date. Trees that were severely declined with initial DI scores greater than 32 (average DI 4 x 8 = 80% declined with symptoms) will not be chosen as measurement trees within each plot.

Evaluation of other pests and diseases based on incidence and severity.

1. Blight: Die-back and quick decline ratings in canopy sections developed for HLB DI ratings by tree age and size.
2. *Phytophthora nicotianae.*
3. *P. palmivora/Diaprepes* weevil complex and burrowing nematodes
4. Other citrus nematodes.
5. Citrus Tristeza Virus: Die-back and quick decline ratings the same as used for HLB disease rating
6. Post-bloom fruit drop: Count the number of buttons in canopy sections accounting for variability.
7. Citrus Canker: Percent lesions on leaves and fruit in canopy sections developed for HLB DI ratings by tree age and size.
8. Other: Incidence and severity of other pests and diseases should be recorded, and evaluation criteria developed and when necessary.

Guidelines specific to pre-commercial rootstock field trials.

Scion selection: For simplicity of trial design, data collection and interpretation, one scion clone is recommended for each replicated, multi-location rootstock trial.This leads to separate trials for each 'Valencia,' 'Hamlin', 'Mid-sweet', 'Grapefruit', 'Tangerine', 'Mandarin' etc. scion clone selected for each trial. Complex designs utilizing more than one clone for any scion type, for example, two Valencia clones, must be balanced and take into the effect of scion and scion/rootstock interaction on the validity of data collected.

Guidelines specific to pre-commercial scion field trials.

Rootstock selection: For simplicity of the trial design, data collection and interpretation, one rootstock variety and one scion variety-type (multiple scion genotypes) of similar maturity (sweet orange, grapefruit, etc.) is recommended for each replicated, multi-location trial. For example, Valencia candidate scions budded on Swingle only (Valencia1/Swingle, Valencia2/Swingle). This leads to separate trials for each 'Swingle, 'Sour orange, 'Carrizo'etc. scion/rootstock combination selected per trial. However, if the decision is made to test the candidate scions on more than one rootstock (e.g., valencia1/Swingle, Valencia2/Swingle, Valencia1/Carrizo, Valencia2/Carrizo) the following considerations should be made:

1. Each experimental unit (split-plot) should contain every scion/rootstock combination.
2. If known, from preliminary data, the effect of rootstocks on scion maturity should be taken into account.