

CALIFORNIA CITRUS NURSERY BOARD

PROJECT PLAN

Fiscal Year: 2020

Duration of Project: Ongoing

This project is: Ongoing

Project Leader: Georgios Vidalakis

Location/Department:

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Collaborators: none

Project Title: Citrus Nursery Stock Pest Cleanliness Program-Annual Research, Troubleshoot and Program Review Period.

Executive Summary

Citrus scion and seed material that has been tested and found free of graft-transmissible diseases consistently produces high quality fruit necessary for the continued productivity and growth of the California citrus industry. Diseases now present in California could cause serious problems to the citrus industry are spread through propagations using infected nursery stock. To counteract this problem a disease testing program to systematically eliminate diseased registered budwood and seed source trees owned by California nurseries is necessary. The sustainability of the California citrus nursery industry is based on its ability to provide disease tested, high quality citrus trees and propagative material.

Historically the scion source trees owned by the California citrus nurseries have been tested annually for tristeza, and every five years for psorosis and viroids. Since 2010 the nursery source trees are also tested for Huanglongbing in the CRB Jerry Dimitman laboratory or the CDFA laboratory at Sacramento. The seed trees have been tested every six years for psorosis. Comprehensive protocols for citrus tissue processing, the universal RT-qPCR detection of citrus viroids and the multiplex RT-qPCR detection of tristeza, psorosis, and leaf blotch have been developed with the support of the CCNB and have been approved by the CDFA (NO. QC 1354 and NO. QC 1388) for use in the Citrus Nursery Stock Pest Cleanliness Program.

The CCPP has been receiving approximately 1,500-2,000 scion and 300-600 seed sources samples in each registration program cycle. In the last two cycles, CCPP received for testing 1129 scion and 390 seed (2017-18) and 1477 scion and 361 seed (2018-19) and samples from nursery tree sources.

This year, we have requested CDFA to collect whenever possible a double sample from suitable nursery trees. The extra sample will be processed using the newly developed instrument budwood tissue extractor (BTE). BTE was developed to serve the exact need of processing budwood tissue of primarily healthy budwood trees sources from nurseries and the CCPP. BTE allows for the sample processing towards RNA extraction in a single instrument within minutes eliminating the need for the lengthy, labor and laboratory instrument intensive steps of bark removal, chopping, and freeze-drying.

As it was discussed in the September 22, 2014 and September 01, 2015 webinars organized by the CDFA and CCNB R&C Protocol Committee the CCPP typically receives nursery samples between the months of October and February. Samples are processed and tested as they arrive and test results are reported back to CDFA by the month of May. Between the months of June and September, the CCPP researchers are evaluating the completed testing program and perform research in order to troubleshoot and fine-tune the tissue processing and testing protocols. In the same period (i.e. June to September) the CCPP Citrus Research and Diagnostic Laboratory is getting prepared for the next cycle of testing

beginning in October. This proposal requests support for the period of June to September to prepare for the following citrus nursery testing cycle.

In March 2018, the CCPP held a three day workshop for the CDFA diagnostic laboratory personnel in order to proceed with technology transfer of the laboratory diagnostic tests for viral pathogens in the Citrus Nursery Stock Pest Cleanliness Program. For the past two years the CDFA lab received CCPP virus and viroid controls and they will also test a subset of citrus nursery samples in parallel to the CCPP laboratory. This way we will collect data points for the capacity of the CDFA lab to run tests for viral pathogens. Depending on the results, in the future we will run the experiment in reverse. CDFA will take the lead on nursery samples and CCPP will run controls and a subset of citrus nursery samples in parallel. When the CDFA lab is ready, we will troubleshoot any remaining issues, if any, and if results are satisfying CDFA will be solely responsible for all the citrus nursery testing i.e. one sampling visit for HLB and viral tests, one laboratory, one cost, and this project will become obsolete freeing CCNB resources for additional research.

Benefit to the Industry

The use of the highest quality propagating materials is most important if California is to continue to compete in the world markets with a quality product. This program insures that citrus nurseries will have a supply of clean propagation budwood and seed so that they can produce the highest quality nursery stock able to be moved in California, national and international markets. This program insures the production of quality nursery stock by eliminating the dissemination of bud-transmissible diseases that are already present in California (Calavan et al. 1978, Wallace 1978, Timmer et al. 2000).

Objectives

This program is initiated to perform annual evaluations, troubleshoot, and fine-tune the testing of the budwood source trees owned by California nurseries for:

1. *Citrus tristeza closterovirus* (CTV)-tristeza disease
2. *Citrus psorosis ophiovirus* (CPsV) and psorosis like diseases i.e. *Citrus leaf blotch virus* (CLBV)
3. *Candidatus Liberibacter asiaticus* (CLAs)-Huanglongbing disease
4. Citrus viroids-Seven characterized detected by the Apsca and non-Apsca tests

This year the Citrus Clonal Protection Program (CCPP) will be responsible for the laboratory testing of the CTV, CPsV, CLBV, and Citrus viroids. The CDFA laboratory at Sacramento will be performing the qPCR-CLAs testing while testing its capacity to perform virus and viroid testing. The overall goal is in 2-3 years for the CDFA to be solely responsible for all the laboratory tests of the Citrus Nursery Stock Pest Cleanliness Program and terminate this project.

Workplans and Methods

The California registration program for the mother scion and seed tree sources is in a transition phase since November 2, 2009 when the Senate Bill 140 "Citrus Nursery Stock Pest Cleanliness Program" was chaptered.

Prior to May 17, 2010, the registration program (active since 1962) included annual mandatory testing for the *Citrus tristeza virus* (CTV) and voluntary testing for the psorosis (*Citrus psorosis virus*, CPsV) and psorosis-like diseases and citrus viroid diseases every five years. The mother seed tree sources were tested for the psorosis and psorosis-like diseases every six years also on a voluntary basis.

On May 17, 2010, regulations for a mandatory Citrus Nursery Stock Pest Cleanliness Program were filed as an emergency action, based on the authority conveyed in Food and Agricultural Code, Sections 6940-6945. The new registration program includes the use of enclosed structures and the testing for Tristeza, Huanglongbing, Psorosis, and Citrus Viroids.

On May 21, 2012, a comprehensive protocol for the high throughput nucleic acid extraction and purification for citrus tissues and the RT-qPCR universal detection of citrus viroids was submitted to the CDFA by the CCPP research team. The protocol development was supported by a CCNB research grant.

On September 10, 2012, CDFA issued the "NO. QC 1354, Permit For PCR Protocol For Viroid Testing In Citrus Nursery Stock Pest Cleanliness Program", thus moving forward with the mandatory Citrus Nursery Stock Pest Cleanliness Program.

On January 14, 2014, CDFA issued the “NO. QC 1388, Permit For PCR Protocol For Virus Testing In Citrus Nursery Stock Pest Cleanliness Program”.

On March 6-9, 2018, CCPP held a workshop-hands on training for the CDFA diagnostic laboratory personnel for technology transfer of virus and viroid testing for the Citrus Nursery Stock Pest Cleanliness Program.

Today, the CDFA approved test for tristeza, Huanglongbing, citrus viroids, psorosis, psorosis-like diseases, and leaf blotch is PCR. The testing is performed by the CCPP personnel and CDFA approved laboratories. Budwood is collected at the nurseries by CDFA Biologists. Budwood is harvested from the four quadrants (NSEW) of all nursery owned registered trees during fall or spring when pathogen titers are expected to be high. As it was discussed in the September 22, 2014 and September 01, 2015 webinars organized by the CDFA and CCNB R&C Protocol Committee the sampled budwood is labeled by CDFA field staff and is submitted for testing between the months of October and February. Samples are processed and tested as they arrive at the CCPP Citrus Research and Diagnostic Laboratory (CRDL) and test results are reported back to CDFA by the month of May. Between the months of June and September the CCPP researchers are evaluating the completed testing program and perform research in order to troubleshoot and fine-tune the tissue processing and testing protocols. In the same period (i.e. June to September) the CCPP CRDL is getting prepared for the next cycle of testing beginning in October.

Briefly, budwood from nursery source trees is processed (bark removal, chopping, and freeze-drying) in the CCPP CRDL and stored at -80°C. The frozen citrus tissue is used for the extraction of total nucleic acids (DNA and RNA) using the newly developed semi-automated protocol “Cryo-station, Geno Grinder 2010, and MagMAX Express-96 -Nucleic Acid Extraction and Purification Protocol for Citrus Tissue”. Quality of the nucleic acid extracts is assessed by spectrophotometry.

The nucleic acids are used as template for RT-qPCR reactions that provide detection of different citrus graft-transmissible pathogens.

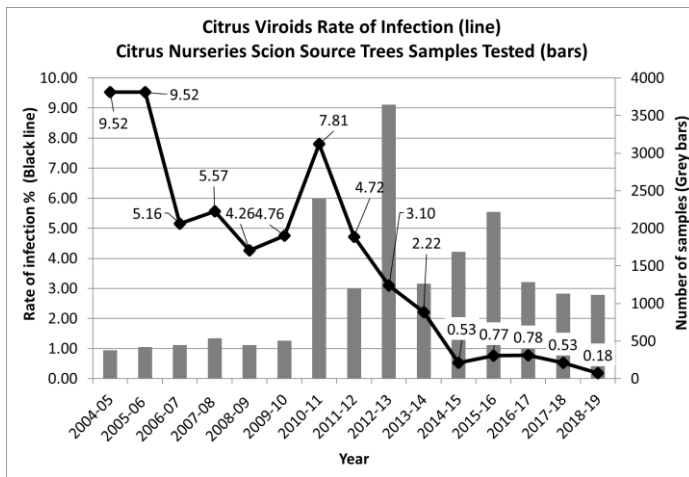
This year, we have requested CDFA to collect whenever possible a double sample from suitable nursery trees. The extra sample will be processed using the newly engineered and developed instrument budwood tissue extractor (BTE). BTE was developed by Technology Evolving Solutions (TES) in collaboration with the CCPP to serve the exact need of processing budwood tissue of primarily healthy budwood trees sources from nurseries and the CCPP. BTE allows for the sample processing towards RNA extraction in a single instrument within minutes eliminating the need for the lengthy, labor and laboratory instrument intensive steps of bark removal, chopping, and freeze-drying.

From 17-24hours to 15min or Less per sample

1. Hand Chopping [1-10 min]	2. Freeze-drying [16-24 h]	3. Tissue Pulverization at Low Temperatures [1-2 min]
a) Consumables (\$10/96well plate)	b) Freeze-dryer (\$20K)	c) -80C Freezer (\$12K)
d) Liquid Nitrogen Cryostation (\$3K)	e) Specialized Supplies (\$25/96well plate)	f) Pulverizer (\$20K)
6. Pathogen Detection [2.5-3 h]	5. Nucleic Acid Extraction and Purification [20 min]	4. Centrifugation [1-1.5 h]
i) Thermocycler (\$70K)	k) Automated Pipetting & Pipettes (\$13K)	l) Multichannel Pipette (\$1.5K)
j) Specialized Supplies (\$35/96well plate)	i) Nucleic Acid Robot (\$35K)	g) Centrifuge (\$6K)

This work plan is the result of long term research that generated comparative data between bioindexing and the RT-qPCR and resulted to the approval and implementation of laboratory tests in the Citrus Nursery Stock Pest Cleanliness Program. We hope to achieve the same outcome for the BTE instrument and the transition of the virus and viroid testing in to the CDFA laboratory.

This project has one overarching objective; Perform research, evaluations, troubleshoot, and fine-tune the budwood processing and testing protocols during the months of June and September while the program is not actively collecting, processing, and testing nursery samples in order to preserve the integrity and guarantee the growth of this valuable citrus nursery program.



For example, the benefits to the citrus nursery testing program from the use of just one reliable, constantly adjusted and evaluated diagnostic assay are clearly visible. Since 2010-11 when the RT-qPCR assay for the universal detection of citrus viroids was implemented (see figure) the testing capacity of the program has increased dramatically (grey bars). As a result a trend of reduced viroid infection has developed (black line). In other words, during the years of biological indexing (2005-2010) the number of samples tested was limited and the program was always behind the curve and the viroid infection was maintained stable (4.26%-9.52%). From the 2010 forward and with the number of samples tested increased, the program is

getting ahead of the curve and the viroid infection is moving downwards with 0.2-0.8% infection the last five years.

If the trend continues and the combination with best management nursery practices the frequency of the viroid testing can change or samples can be pooled and eventually the cost for viroids testing can be reduced. In addition, ongoing research on HLB and stubborn for the multi-detection of the associated pathogens with PCR based techniques will eventually pull together all the nursery testing under one, universal, efficient, and high throughput, protocol that will require constant evaluation and adjustments.

Project Management and Evaluation

Dr. Vidalakis and the personnel of the CCPP have a long experience in managing this program. Special attention is given to the timely and careful preparation and execution of the laboratory testing for accurate and reliable results.

Literature Review

1. Calavan C.E., Mather S.M., and McEachern E.H. 1978. Registration, certification, and indexing of citrus trees. In Reuther W., Calavan C. E., and Carman G. E. (eds.). The citrus industry Vol. IV. Crop protection. Chapter 3, pages 185-222. University of California, Division of Agricultural Sciences.
 2. Timmer, L. W., Garnsey, S. M., and Graham, J. H. (eds.). 2000. Graft-Transmissible, Systemic Diseases. In: Compendium of citrus diseases. Second Edition. Pages 51-69. APS press, St. Paul, Minnesota.
 3. Vidalakis, G., Wang, J. 2013. Molecular method for universal detection of citrus viroids, US Patent 20,130,115,591.
 4. Wallace, J. M. 1978. Virus and viruslike diseases. In Reuther W., Calavan C. E., and Carman G. E. (eds.). The citrus industry Vol. IV. Crop protection. Chapter 2, pages 67-184. University of California, Division of Agricultural Sciences.
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Budget Proposal

Project Title: Citrus Nursery Stock Pest Cleanliness Program-Annual Research, Troubleshoot and Program Review Period.

Project Leaders: Georgios Vidalakis

Proposed Fiscal Year: 2020

A. <u>PERSONNEL SERVICES:</u>	
4 months of 4 Undergraduate Students	<u>\$7,288</u>
Benefits	<u>\$ 132</u>
TOTAL PERSONNEL SERVICES	<u>\$7,420</u>
B. <u>OPERATING EXPENSES:</u>	
Laboratory Supplies (kits, sequencing, etc.)	<u>\$3,000</u>
Travel	<u>\$0</u>
C. <u>TOTAL OPERATING EXPENSES:</u>	<u>\$0</u>
D. <u>TOTAL BUDGET REQUESTED:</u>	<u>\$10,420</u>

Signatures of Requestors:  Date: 11-18-19

_____ Date: _____

Signature of Cooperator: _____ Date: _____

Department Chair: _____ Date: _____

LIAISON OFFICER _____ Date _____