CALIFORNIA CITRUS NURSERY BOARD

PROJECT PLAN

Fiscal Year: 2016 **Duration of Project:** 3 Years*

**Note: Year 4 may be required to complete screenhouse experiment.*

This project is: Continuing, year 3 of 3

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Project Title: Implementation and streamlining of the newly developed high throughput diagnostic system for citrus nurseries registration.

Executive Summary

The Citrus nurseries depend on their disease-tested scion and seed sources for the propagation of high quality nursery trees. In California, the Citrus Clonal Protection Program (CCPP) has been responsible for the development and execution of the registration program of nursery owned citrus source trees for many years. The CCPP has been constantly trying to improve the testing of source trees and upgrade its diagnostic platform given the available technologies. The registration program began with biological indexing, it was later enhanced with ELISA and Imprint Hybridization, and today we are moving towards the universal, molecular, real time, high throughput detection of citrus regulated pathogens. In doing so, the CCPP is providing high quality services to the industry under the auspices and commitment to the CCNB mission in a timely and economical manner and thus fulfilling its mission under the new mandatory "Citrus Nursery Stock Pest Cleanliness Program" program.

The development and validation of the universal/multiplex detection methods has been completed at the laboratory level with the CCNB funded project "The Future of the Cooperative Registration Program of Nursery Owned Citrus Source Trees". Now it is time for the implementation of the developed methods in a large scale at the industry level. There are a few basic questions that need to be resolved for the successful, economical, practical, and timely implementation of the new diagnostic methods.

1. Streamlining and minimizing the chance for false results of the viroids, RNA viruses, and DNA pathogens developed methods.
2. Optimizing the time and frequency of sampling in regard to time of the year, temperature, and tree phenological stage.
3. Building a cohesive efficiently working system of sample collection, tracking, and processing, record keeping, data management, and results reporting.

In this proposal, we intend to incorporate the three high throughput detection assays into a routine workflow, starting from optimum time for sample collection, processing, multiplex detection of RNA and DNA pathogens, tracking each sample from start to finish, documenting and timely reporting of the results. We believe that optimizing and fine-tuning the system as a whole it will reduce time and cost as well as offering flexibility for the future direction of a certification program for citrus nursery stock.

Keywords: Disease-tested propagating material, pathogen detection, multiplex Reverse transcription real time quantitative polymerase chain reaction (RT-qPCR), qPCR, Time course, Laboratory Information Management System

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Benefit to the Industry

Citrus is a perennial crop susceptible to many graft transmissible disease agents including at least nine arthropod transmitted pathogens (Roistacher, 1991), threatening citrus production worldwide (Bar-Joseph et al., 1989; Bovè, 2006). This makes co-infection of a citrus tree by two or more pathogens common. The worst citrus diseases include stem pitting of *Citrus tristeza virus* (CTV) and Huanglongbing (HLB), also known as citrus greening or yellow shoot disease (Bar-Joseph et al., 1989; Timmer et al., 2000; Bovè, 2006). Therefore, efficient high throughput multiplex qPCR based diagnostic tests are needed for these and other citrus pathogens to maintain a vigorous citrus industry.

Since a number of these important citrus pathogens are spread by propagation, arthropod vectors and inadvertent import and dissemination of infected plants, citrus disease management and clean stock programs require economical, reliable, high throughput pathogen detection systems in order to maintain a productive, competitive, and sustainable industry.

To this end, developing a complete workflow and optimum use of resources for reliable pathogen detection is key for the industry. Each component of the existing testing program needs to be optimized, fine-tuned and coordinated for efficiency, cost, and time reduction so it can set the platform for a future citrus nursery certification program. Concerns expressed among citrus industry members for the cost, sample collection and testing frequency, practical business consequences of false results, and timely reporting of testing results are justified. The proposed timeline experiment for optimum time of sample collection, deep sequencing of citrus pathogens for "hidden" genetic variation that may affect the accuracy of qPCR, and the incorporation of a Laboratory Information Management System (LIMS) for accurate sample tracking and documentation, and timely results reporting will address many of these concerns.

Finally, a well-functioning, optimized, cost effective system for testing the nursery budwood and seed source plants will open the road for the establishment of a complete certification scheme of citrus nursery stock that will facilitate the marketing of disease-certified nursery products for the local, national, and international markets.

Objectives

The objectives of this proposal aim on the implementation and streamlining of a complete, reliable, high throughput diagnostic system-from sample collection to results reporting-for citrus nursery regulated pathogens**.** Objectives can be summarized as follows;

1. Streamlining and minimizing the chance for false results of the viroids, RNA viruses, and DNA pathogens developed methods.
2. Optimizing the time and frequency of sampling in regard to time of the year, temperature, and tree phenological stage.
3. Building a cohesive efficiently working Laboratory Information Management System (LIMS) for sample collection, tracking, and processing record keeping, data management, and results reporting.

Work plans & Methods

1. Streamlining and minimizing the chance for false results of the viroids, RNA viruses, and DNA pathogens developed methods.

Singleplex qPCR has been reported for the detection of different woody-plant infecting viruses (Marbot et al., 2003; Osman et al., 2007, 2008, 2012a, b, 2013b; Osman and Rowhani, 2006; Schneider et al., 2004; Varga and James, 2005). It has also been reported for various citrus infecting pathogens such as CTV (Ananthakrishnan et al., 2010; Bertolini et al., 2008; Loconsole et al., 2010; Rosa et al., 2007; Ruiz-Ruiz et al., 2007, 2009a; Saponari et al., 2008, 2013; Sieburth et al., 2009; Yokomi et al., 2010); CLBV (Ruiz-Ruiz et al., 2009b); CPsV (Loconsole et al., 2010); *Candidatus* Liberibactersp. (Kim and Wang, 2009; Manjunath et al., 2008;Morgan et al., 2012; Li et al., 2006, 2007; Schuenzel et al., 2007); citrus viroids (Saponari et al., 2013; Loconsole et al., 2013; Vidalakis and Wang 2013) and *S. citri*. In qPCR, multiplex is done by using multiple fluorescent reporter dyes with various non-overlapping wavelengths to detect several pathogens in a single reaction. Multiplex qPCR has several advantages, whereby several organisms can be identified in a single assay as reported for citrus (Osman et al., 2015; Saponari et al., 2013; Loconsole et al., 2010) as well as many other pathogens (Pallás, Más, and Sánchez-Navarro, 1998).

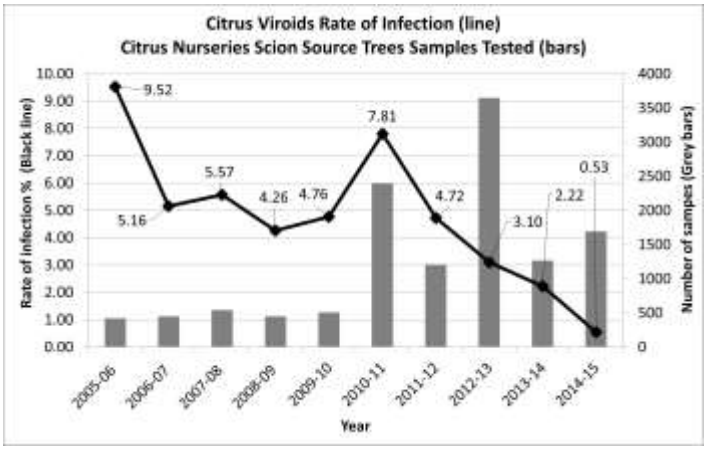
Three universal/multiplex assays have been developed (CCNB-The Future of the Cooperative Registration Program of Nursery Owned Citrus Source Trees) for the detection of citrus nurseries regulated pathogens:

a) Universal detection of citrus viroids: *Citrus exocortis viroid*, *Hop stunt viroid*, *Citrus bark cracking viroid*, *Citrus bent leaf viroid*, *Citrus dwarfing viroid*, *Citrus viroid V* (CVd-V) and CVd-VI.

b) Multiplex detection of RNA viruses: *Citrus tristeza virus*, *Citrus psorosis virus* (CPsV), and *Citrus leaf blotch virus* (CLBV) (Osman et al., 2015).

c) Multiplex detection of DNA pathogens: *Candidatus* Liberibacter sp. (asiaticus, americanus, africanus) and *Spiroplasma citri*.

These three assays will be streamlined for a rapid, high throughput, low cost qPCR based diagnostic protocol. Multiplex qPCR detection will be performed using the QuantStudio™ 12K high throughput detection instrument.



The benefits to the registration program from the use of just one of the newly developed methods i.e. universal detection of citrus viroids (Vidalakis and Wang 2013) since 2001-11 (see figure) are already visible. The testing capacity of the registration program has increased dramatically (grey bars). As a result a trend of reduced viroid infection is developing (black line). In other words, during the years of biological indexing (2005-2010) the number of samples

tested was limited. As a result the program was always behind the curve and the viroid infection was maintained stable. From the 2010 forward and with the number of samples tested dramatically increased the program is getting ahead of the curve and the viroid infection has moved downwards (0.5% in 2014-15 testing cycle). If the trend continues and the combination with best management nursery practices the frequency or individual tree testing (i.e. sample pooling) can be modified and eventually the cost for viroids testing can be reduced. When all three high throughput methods are harmonized and streamlined the benefits to the industry will be maximized.

a) Universal detection of citrus viroids: Citrus exocortis viroid, Hop stunt viroid, Citrus bark cracking viroid, Citrus bent leaf viroid, Citrus dwarfing viroid, Citrus viroid V (CVd-V) and CVd-VI.

The currently CDFA approved citrus viroid detection protocol is based on the use of SYBR Green qPCR technology. PCR reactions using SYBR green require an extra step (i.e. melting curve) before the results are finalized. In addition, SYBR Green is not specifically binding to the target viroid but rather to any DNA present in the PCR reaction.

Today there are available chemistries that improve the qPCR detection of targets. Minor groove binding (MGB) probes labeled with a reporter fluorophore and a non-fluorescent Black Hole Quencher® dye (BHQ) (e.g. TaqMan) are increasingly becoming the preferred probes for qPCR detection due to their elevated specificity. This real-time qPCR technology allows the use of multiple fluorescent reporter dyes with various non-overlapping wavelengths for the multiplex detection of several pathogens in a single reaction. After processing over 9,500 citrus nursery samples with SYBR green reactions in the past five years we felt that it was important to evaluate the TaqMan technology for the multiplex detection of citrus viroids for the improvement of the workflow and results reporting.

A one-step multiplex real-time quantitative polymerase chain reaction (multiplex-qPCR) based on TaqMan chemistry for the simultaneous detection of three non-Apsca viroids infecting citrus trees was developed during 2015 (report on preparation). This year we will focus on the development of the multiplex-qPCR for the simultaneous detection of the four Apscaviroids.

All GenBank sequence of the seven distinct viroid species representing four genera of the Pospiviroidae family will be separately piled up for the design of primers and MGB probes for the multiplex citrus viroid detection.

Table 1. All GenBank sequences of the seven distinct citrus viroid species representing four genera of the Pospiviroidae family have been piled up for assay design.

Target	Total GenBank Accessions	TaqMan Probe Fluorophore
Apscaviroids		
Citrus bent leaf viroid (CBLVd)	60	VIC
Citrus dwarfing viroid (CDVd)	193	FAM
Citrus viroid V (CVd-V)	25	TET
Citrus viroid VI (CVd-IV)	23	FAM
Non-Apscaviroids		
Citrus exocortis viroid (CEVd)	270	FAM
Citrus bark cracking viroid (CBCVd)	2	VIC
Hop stunt viroid (HSVd syn. CVd-II)	70	TET

The assay's analytical specificity and sensitivity will be investigated using reference isolates, obtained from different geographical regions and different hosts available at the CCPP disease bank. In addition, the diagnostic sensitivity and the diagnostic specificity will be evaluated to assess the assay's accuracy. Finally, the new assays will be validate using “real life” samples collected from the nursery owned scion and seed CDFA registered sources.

The utility of the multiplex qPCR will be demonstrated with the comparison of multiplex and singleplex qPCR assays detecting single, double and multiple infection of viroids. These Multiplex qPCR assays could effectively replace three currently available molecular detection systems (i.e. conventional RT-PCR, multiplex PCR and singleplex qPCR). In addition, the multiplex qPCR test has a lower risk of contamination compared to conventional PCR detection and enables the reliable high throughput testing of large number of samples. The increased sensitivity and flexibility achieved with multiplex qPCR permits the simultaneous identification of a broad spectrum of the targeted viroids and their diverse strains by combining proper primers and (or) qPCR probes. Multiplex qPCR detection of citrus viroids would be economically beneficial for the citrus industry.

b) Multiplex detection of RNA viruses: Citrus tristeza virus, Citrus psorosis virus (CPsV), and Citrus leaf blotch virus (CLBV).

This part of objective 1 was completed in 2015. A peer reviewed article was published in the *Journal of Virological Methods* "Development and validation of a multiplex RT-qPCR assay for the rapid detection of Citrus tristeza virus, Citrus psorosis virus, and Citrus leaf blotch virus" and CDFA approved the use of this method for virus testing in the citrus nursery stock pest cleanliness program (Permit no. QC 1388).

c) Multiplex detection of DNA pathogens: Candidatus Liberibacter sp. (asiaticus, americanus, africanus) and Spiroplasma citri.

A single real-time multiplex quantitative polymerase chain reaction (qPCR) assay using two different fluorescently labeled minor groove binding qPCR probes for the simultaneous detection of *Candidatus* liberibacter spp. and *S. citri* was developed in 2015 (report in preparation). The qPCR assays have been designed to detect all *C.* liberibacter spp. (asiaticus, americanus, and africanus) and multiple California isolates of *S. citri*.

This year we will continue with the validation (specificity and sensitivity) of the assay using multiple pathogen isolates from diverse geographical regions with emphasis to mix infections of *C.* Liberibacter spp. + *S. citri* currently developed at the USDA-ARS Exotic Pathogens of Citrus Collection in Beltsville, MD. In addition, the method will be used to test "real life" samples collected from the nursery owned scion and seed CDFA registered sources.

These 2016 experiments will finalize the peer review technical publication currently under preparation that will be used to support our request to CDFA for the approval for use for testing in the citrus nursery stock pest cleanliness program.

The second part of objective 1 is to make sure that the utilized methods do not produce false results. We propose to continue with the high throughput sequencing of citrus pathogens, as it was initiated in "The Future of the Cooperative Registration Program of Nursery Owned Citrus Source Trees", in order to collect data on the intra-population variability of regulated pathogens and identify any shortfalls of the designed primers and probes thus reducing the risk of false negatives.

This year we will focus on *S. citri* since there is limited genomic information available. Unfortunately, the full genome sequence of *S. citri* has not been determined and the available genomic information is limited (the majority of available sequences at GenBank are in 16S rRNA and spiralin genes). This limited genomic information severely restricts our ability to design more efficient and specific diagnostic assays that would prevent potential diagnostic cross reactions between variable isolates and *S. citri* and potentially other pathogenic bacteria such as *C.* liberibacter spp.

The isolates will be subjected to the next generation high throughput genomic sequencing (NGS) using Illumina to examine the intra-population variability. Both sequencing and

bioinformatics analysis will be outsourced to Genome Quest that will be able to analyze the sequences quickly and economically using highly sophisticated bioinformatics tools. The sequences will be piled up with corresponding sequences from the GenBank and in house generated sequences, and subsequently singleplex qPCR assays for these pathogens will be updated to be able to detect all sequence variants of these pathogens.

2. **Optimizing the time and frequency of sampling in regard to time of the year, temperature, and tree phenological stage.**

Heterogeneous distribution, low concentration and seasonal titer variations of pathogens in citrus are main problems for the implementation of molecular biology-based laboratory detection protocols. Detection of the citrus pathogens is influenced by factors that affect concentration as the erratic distribution within the plant (Lee et al, 2001; D'Urso et al., 2000), the type and age of tissue used for analysis, the season (Lee et al., 2001), mixed infections, and environmental factors.

A time course study will be performed for at least 24 months with regular sampling intervals to evaluate the pathogen titer in different seasons within the period of vegetative growth throughout a range of temperatures.

In spring 2015 we received from the CCPP Foundation Operations at the Lindcove Research and Extension Center 260 trees of navel orange, mandarin, satsuma, lemon and grapefruit and established them (e.g., set up of growth benched, irrigation lines, labelling etc.) at the CCPP Quarantine Screenhouse. In addition, we developed sources of inoculum for the CDFA regulated pathogens in the citrus nursery stock pest cleanliness program (e.g. CTV, CPsV, *S. citri*, and citrus viroids), and designed a replicated experiment for mix infections of these pathogens.

This year, during the winter months in order to avoid the high summer temperatures, we will execute the mix infection experiment. The trees will be tested prior to inoculation for the presence of graft-transmissible pathogens. Samples (e.g. bark, leaf petioles or newly emerged flush) will be collected at specific time intervals (e.g. 2-3 weeks) and flushing stages (e.g. feather flush, 1-2 fully expanded leaves etc.) while the temperature will be monitored 24 hours a day. The samples will be processed with the high throughput semi-automated nucleic acid extraction system approved by CDFA and tested with the newly developed singleplex and multiplex qPCR and RT-qPCR.

After the preliminary data analysis indicate a specific time period, temperature range, or phenological tree stage that is preferable for pathogen detection we can evaluate the performance of our protocols on citrus nursery regulated pathogens exotic to California. For example, if our data indicate that temperatures 25-28^{o}C are optimum for sample collection and testing of CTV, CPsV and viroids we can contact fellow scientists in Florida and request nucleic acid extracts or lyophilized tissues of trees infected with *C.* Liberibacter asiaticus and CLBV collected at that temperature range so we can test all our protocols.

3. **Building a cohesive efficiently working Laboratory Information Management System (LIMS) for sample collection, tracking, and processing record keeping, data management, and results reporting.**

LIMS is used to keep track of projects, samples, plate maps, assay information, results, and cost. Each entry is linked, making it easy to track the path of a sample from receipt, to what assays were run with it, to the results, and finally cost.

As an example, when a set of samples is submitted, it is given a unique project ID # (PID). Under the PID; information regarding the project, contact information for the researcher and PI, account #, project start date, explanation of the project etc. are entered. Individual sample is then assigned "a sample ID #" (SID), that includes; the sample name and origin, date received, species, etc. After SID is entered into the LIMS, 'permanent homes' for each sample is assigned by linking the sample to a well position on a qPCR reaction plate. qPCR is then run, where the samples will

also be linked with what qPCR assays were used to test the samples. Finally, after the completion of the qPCR, the data are exported from the qPCR thermocycler into the LIMS database. The results will match up with the samples for easy and automatic analysis. After completion of a project, the project is assigned a cost #. All this information is just a click away...no searching through an Excel file and no back tracking through stacks of paper. This system can be developed in conjunction with the CDFA database or independent pending the nurseries preference.

After careful study of the latest version of the Complete Guide to LIMS and defining our lab-work-flow along with our laboratory's current and future requirements, we identified several potential LIMS vendors (Table 2 and progress report in preparation). We also purchased an advanced Zebra label printer (GX430t) with barcode readers which will allow us to electronically log in and label all nursery samples with a unique identification number and barcode. Sample processing, storage and results will be tracked by barcode avoiding any labeling errors.

This year we will proceed with the evaluation of the different products in regard to annual cost, functionality for the current and future needs of the program, and alignment with regulatory requirements. This objective is also supported by the Citrus Research Board and the National Clean Plant Network.

Table 2. Laboratory Information Management System (LIMS) vendors and descriptions

Vendor	Description
LabLynx	WebLIMS subscription (all Elab LIMS SQL modules) including LIMS Studio; Implentation services (configurations, project management, Training, instrument interface engine); 3 users
Silbmed	Silabmed LIMS Core Engine, Configuration, Installation, Customization, Specimen Management, Barcode Label Management, Data transfer, Test Result Data Entry, Data Warehouse, Reporting, Instrument Interface Engine, Technical Support, Software Maintenance; 3 user
Autoscribe	Full Matrix Gemini, Instrument Calibration and Maintenance Module, Integrated Competency Manager module, Stand-alone, single-user Competency Management module, Crystal Reports Professional Edition, on-site to finalize detailed requirements, project management, finalize setup & install; 3 users

Project Management and Evaluation

Dr. Georgios Vidalakis is the Director of CCPP and has long experience with pathogen detection and management in citrus germplasm. He is working closely with the researchers of this project.

Dr. Fatima Osman is an assistant project scientist at the University of California Davis with an appointment in the CCPP at the University of California, Riverside. Her main research work focuses on the design of novel sensitive detection techniques of Citrus pathogens. Dr. Osman has more than twelve years' experience in developing advanced real-time quantitative RT-qPCR singleplex and multiplex assays for the detection of grapevines, fruits and citrus.

Dr. Sohrab Bodaghi is an associate research scientist with CCPP at the University of California Riverside. Dr. Bodaghi has more than 15 years' experience in Microbiology and Plant Pathology fields with expertise in general, molecular and plant virology. He has been involved with regulated pathogens laboratory testing (dsRNA, sPAGE, RT-qPCR) of quarantined citrus varieties and thousands of samples from nursery as well as CCPP's citrus germplasm sources for commercial use.

Dr. Maher Al Rwahninh has extensive experience using Next Generation Sequencing (NGS) in identifying pathogens strains, and will be instrumental in processing samples for NGS, as well as analyze the vast sequencing information derived from the Bioinformatic analysis to screen for proper pathogen's sequence required for designing the multiplex qPCR assays.

Budget Proposal

Project Title: Implementation and streamlining of the newly developed high throughput diagnostic system for citrus nurseries registration.

Project Leaders: Georgios Vidalakis and Fatima Osman

Proposed Fiscal Year: 2016

A.	**PERSONNEL SERVICES**:	
	Assistant Project Scientist @ 70%	$49,500
	Benefits 39%	$19,305
	TOTAL PERSONNEL SERVICES	$68,805
B.	**OPERATING EXPENSES:**	
	Laboratory Supplies (kits, sequencing, etc.)	$18,000
	Travel	$ 1,500
C.	**TOTAL OPERATING EXPENSES**:	$19,500
D.	**TOTAL BUDGET REQUESTED**:	$88,305

Signatures of Requestors:  Date: 11-17-15

_____ Date: _____

Signature of Cooperator: _____ Date: _____

Department Chair: _____ Date: _____

LIAISON OFFICER _____ Date _____

Literature Review

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