CALIFORNIA CITRUS NURSERY BOARD

Scope of Work
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Fiscal Year: 2019
This project is: Continuing, year 6 of 5
Proposal funding cycle: Jan 1, 2019-Dec 31, 2019.

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

Collaborators:

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Summary

We are requesting 25% support for a researcher for an additional year (6 of 5) to try to wrap up this project. The biology of the mixed infections and the collection of multiple samples (e.g. petioles, leaves, budwood) from hundreds of trees (over 116) 3 times per year has been proven extremely challenging (objectives 1 & 2). We will know if we will need one more final year (7 of 5) of part time support to close this project depending on the 2019 samples quality.

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. In 2018, the CCPP started the technology transfer to the CDFA laboratory, and in couple of years CDFA should be responsible for all citrus nursery testing. This will streamline the process and reduce cost and time for results reporting.

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, qPCR based 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 proposed work plan is 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.

The PI (sub-agreement Dr. Fatima Osman, UCD) in collaboration with the Project leader (Dr. Georgios Vidalakis, UCR) will be validating the universal and multiplex detection methods for the regulated viral pathogens in CDFA's Citrus Nursery Stock Pest Cleanliness Program.
The PI primary focus is to incorporate the three high throughput detection assays into a routine workflow, starting from the 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. This optimization and fine-tuning of the system as a whole will reduce time and cost as well as offering flexibility for the future direction of a certification program for citrus nursery stock.

Benefit to the Industry

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 thus setting a 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 pave 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.

Work plans & Methods

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

The PI in collaboration with the project leader has developed and validated three universal/multiplex assays:

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 (Vidalakis and Wang, 2013, Chambers et al., 2018). This result in peer reviewed publication on the detection of three different viroids.

- Osman, F., Dang, T., Bodaghi, S., Vidalakis, G. One-step multiplex RT-qPCR for the simultaneous detection of three citrus viroids of different genera and a wide range of woody and herbaceous hosts. Journal of Virological Methods 245, 40-52.

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

- Osman, F., Hodzic, E., Kwon, S-J, Wang, J., and Vidalakis, G. Development and validation of a multiplex reverse transcription quantitative PCR (RT-qPCR) assay for the rapid detection of Citrus tristeza virus, Citrus psorosis virus, and Citrus leaf blotch virus. Journal of Virological Methods, 220, 64-75.

c) Multiplex detection of DNA pathogens: Candidatus Liberibacter sp. (asiaticus, americanus, africanus) and Spiroplasma citri. In 2019, we will utilize the 9 S. citri full genome sequences we developed in 2018 to improve and continue validating the multiplex detection assay for C. Liberibacter species and S. citri. Manuscript in preparation:

Osman, F., Pagliaccia, D., Bodaghi, S., Dang, T. Vidalakis, G. Multiplex qPCR detection of all Candidatus liberibacter spp. (asiaticus, americanus and africanus).

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.

In spring 2015, we received from the CCPP Foundation Operations at the Lindcove Research and Extension Center 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.

In winter 2016, in order to avoid the high summer temperatures, we executed the mix infection experiment. The trees were tested prior to inoculation for the presence of graft-transmissible pathogens.

In spring 2017, we collected the first set of samples including bark and leaf petioles, from the non-inoculated and mixed inoculated controls for viruses and viroids in order to confirm pathogen establishment. We also monitored temperatures inside the protective structure 24 hours a day.

As you can see in the enclosed scheme of experimental design (Fig. 1), sample collection entails carful labeling, collection of leaves petioles/midveins and budwood from the upper and lower parts of the citrus stems. Subsequently nucleic acids are extracted, purified and analyzed for quality from each sample and run in duplicates with qPCR to detect 3 RNA viruses, 1 DNA pathogen, and all known citrus viroids. As a result this experiment was proven to be time consuming and that is why we have exceeded the original 5 years.

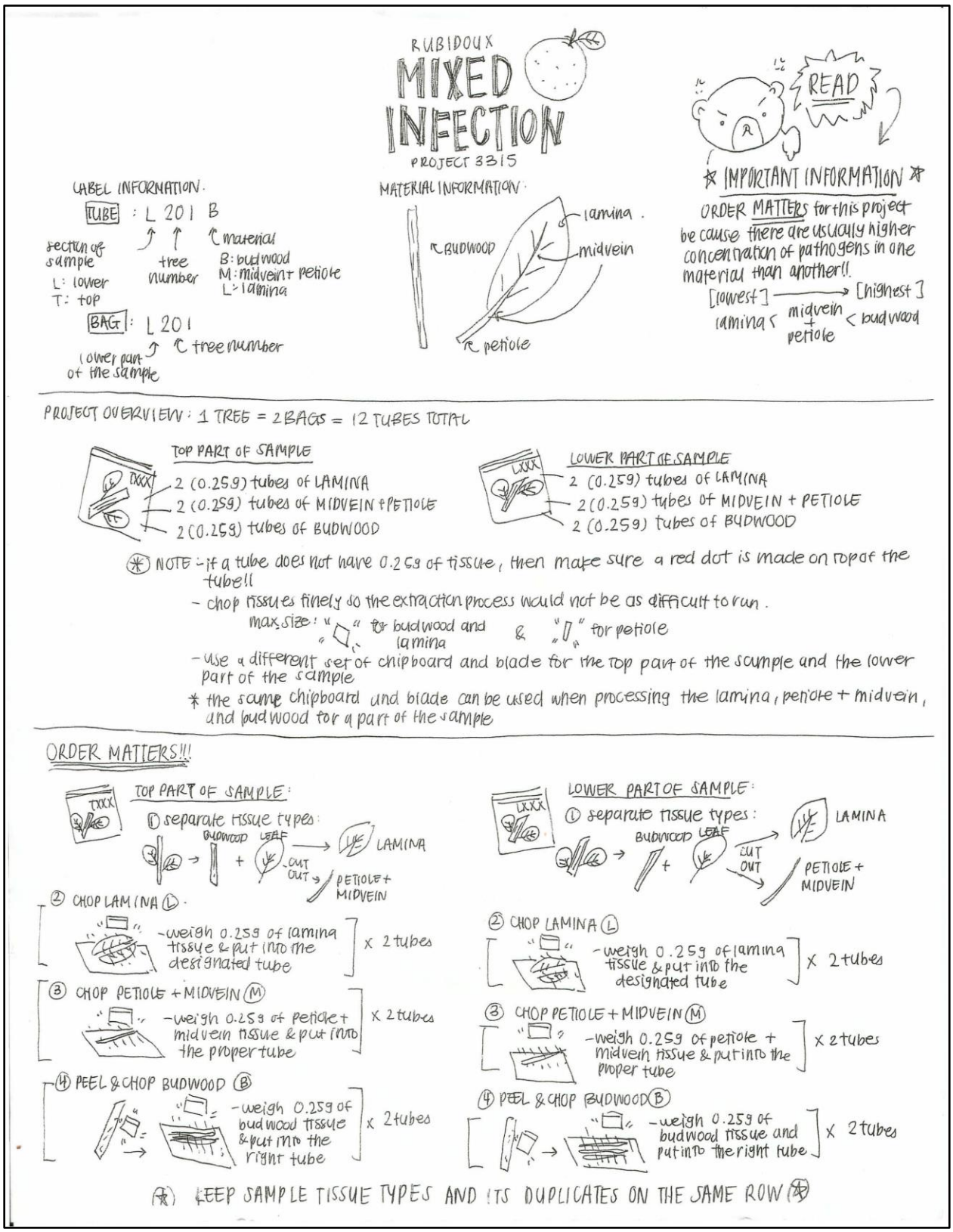


Fig 1. Experimental Scheme.

In 2019, a time course study will be performed in regular sampling intervals (3 times a year) to evaluate the pathogen titer in different seasons within the period of vegetative growth throughout a range of temperatures.

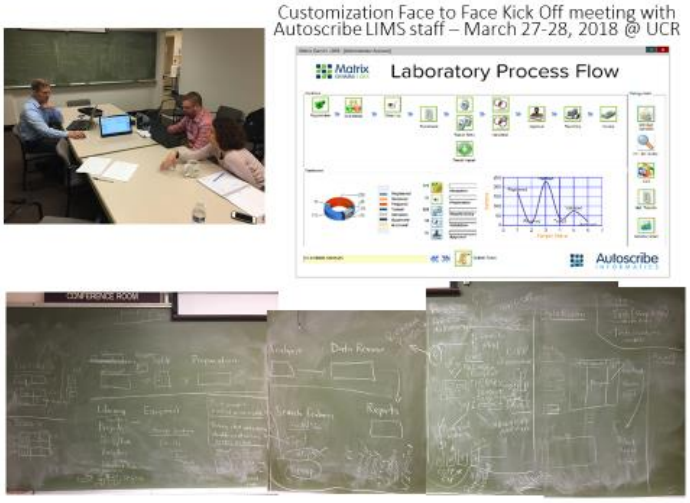
The 2019 sampling (year 6 of 5) will provide the minimum number of time replications for the experiment (i.e. two separate years 2018 & 2019/24 months) to withstand peer review. However, given the complexity of the experiment and the changing weather patterns we feel that one more year of sampling (2018, 2019, and 2020 / 36 months) will significantly strengthen our results.

After we evaluate the quality of the 2019 samples and if you agree, we would like to request one more year (2020 year 7 of 5) of part time support (20-25%) so we can wrap up this project and provide well needed data in support of the mandatory "Citrus Nursery Stock Pest Cleanliness Program" sampling and testing protocols. We apologize for going over the estimated 5 years of the project but this large and complex experiment has been proven challenging.

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

Incorporation of a Laboratory Information Management System (LIMS) for accurate sample tracking and documentation, and timely results reporting will address many of regular laboratory concerns. Different products were evaluated and in 2018 the system "Autoscribe Informatics" was installed in the UCR Servers and we initiated its customization (March 2018) for this CCNB and other CCPP projects.

In 2019 we will continue with the customization and implementation of the Autoscribe LIMS in samples processing.



Project Management and Evaluation

Dr. Georgios Vidalakis (Project Leader) 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 (sub-agreement) is an associate project scientist at the University of California Davis with an appointment in the CCPP at the University of California, Riverside and she is one of the two administrative coordinator of the National Clean Plant Network for Citrus. Her main research work focuses on the design of novel sensitive detection techniques of Citrus pathogens. Dr. Osman has more than fifteen years' experience in developing advanced real-time quantitative RT-qPCR singleplex and multiplex assays for the detection of grapevines, fruits and citrus.

In specific, her research involves the development of comprehensive qPCR-based multiplex diagnostic methodology to streamline detection of CDFA regulated pathogens infecting Citrus and integrate this methodology in routine CCPP diagnostics using platforms in which 364 samples can be tested. Thus facilitating the testing of imported new citrus stocks varieties and decertifying contaminated nursery stocks of citrus before they can be distributed in the United States for the purpose of maintaining disease free status of clean citrus budwood and seed sources.

Establishment of collaborative research work between UC Davis and UC Riverside is a highly desirable goal for the academic enrichment of both UC campuses.

References

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Vidalakis, G., Wang, J., 2013. Molecular method for universal detection of citrus viroids. US Patent Publication number 20130115591.

Budget Proposal

A.	**PERSONNEL SERVICES**:	
	Assistant Project Scientist @ 25%	$19,366
	Benefits 52%	$10,089
	TOTAL PERSONNEL SERVICES	$29,455
B.	**OPERATING EXPENSES:**	
	Laboratory Supplies (kits, sequencing, etc.)	$0
	Travel	$0
C.	**TOTAL OPERATING EXPENSES**:	$0
D.	**TOTAL BUDGET REQUESTED**:	$29,455