CALIFORNIA CITRUS NURSERY BOARD

Scope of Work
Dr. Fatima Osman
Department of Plant Pathology
University of California
Davis, CA 95616
Phone: (530) 752-2942
E-mail: fmosman@ucdavis.edu

Fiscal Year: 2020
This project is continuing, year 7 of 5
Proposal funding cycle: Jan 1, 2020-Dec 31, 2020.

Project Leader: Georgios Vidalakis
Department of Microbiology & Plant Pathology, University of California, Riverside, CA 92521. **Phone:** (951) 827-3736, **FAX:** (951) 827-4294, **E-mail:**vidalg@ucr.edu

Sub-agreement: Fatima Osman
Department of Plant Pathology, University of California, Davis, CA 95616, **Phone**: (510) 220-5503, **E-mail**: fmosman@ucdavis.edu

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

Collaborators:

1. Sohrab Bodaghi Department of Plant Pathology & Microbiology, University of California, Riverside, CA 92521. **Phone**: (951) 827-4932, **FAX**: (951) 827-4294, **E-mail**: sohrab.bodaghi@ucr.edu
2. Irene Lavagi, Department of Plant Pathology & Microbiology, University of California, Riverside, CA 92521. **Phone**: (951) 827-4932, **FAX**: (951) 827-4294, **E-mail**: irene.lavagi@ucr.edu

Summary
We are requesting 25% support for a researcher for the final year (7 of 5) to wrap up this project. As we discussed last year, 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 need this final year (7 of 5) of part time support to close this phase of the project. In Dec-Jan 2020 we will make the final sample collection that will give us 2 years worth of sampling points, minimum replications for a peer reviewed publication. The rest of the 2020 will be spent on sample processing and testing and sampling as needed (full or focused) for troubleshooting and data strengthening and interpretation. The preliminary analysis of the data will point us to any future research directions that we may bring to CCNB's attention. In addition, in 2020 the screenhouse currently housing the trees of this experiment at the Citrus Clonal Protection Program (CCPP) Rubidoux Quarantine Facility (RQF) will be converted to a greenhouse, thus this phase of the project needs to be concluded. The mixed

infected trees are of tremendous value and we will relocate them in a lath or screenhouse at a new UC Riverside location. When the location and growing conditions have been defined, we will co-evaluate the new parameters with the 2020 data and plan for the future.

The Citrus nurseries depend on their disease-tested scion and seed sources for the propagation of high quality nursery trees. In California, the 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. Data from this project will make sure that this transmission is smooth and that the CDFA diagnosticians will have a high level of confidence in the CCPP developed protocols, regardless of mixed infections, citrus type/variety, age or type of tissues, and environmental conditions.

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. A few basic questions 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

- **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 was published 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 2020, we will utilize the 15 S. citri full genome sequences we developed in 2018 and 2019 to improve and continue validating the multiplex detection assay for *C.* Liberibacter species and S. citri. Manuscript in preparation:

An updated multiplex qPCR detection assay for the detection of three *Candidatus* Liberibacter species associated with citrus Huanglongbing. Fatima Osman *et al.* 2020.

- **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.

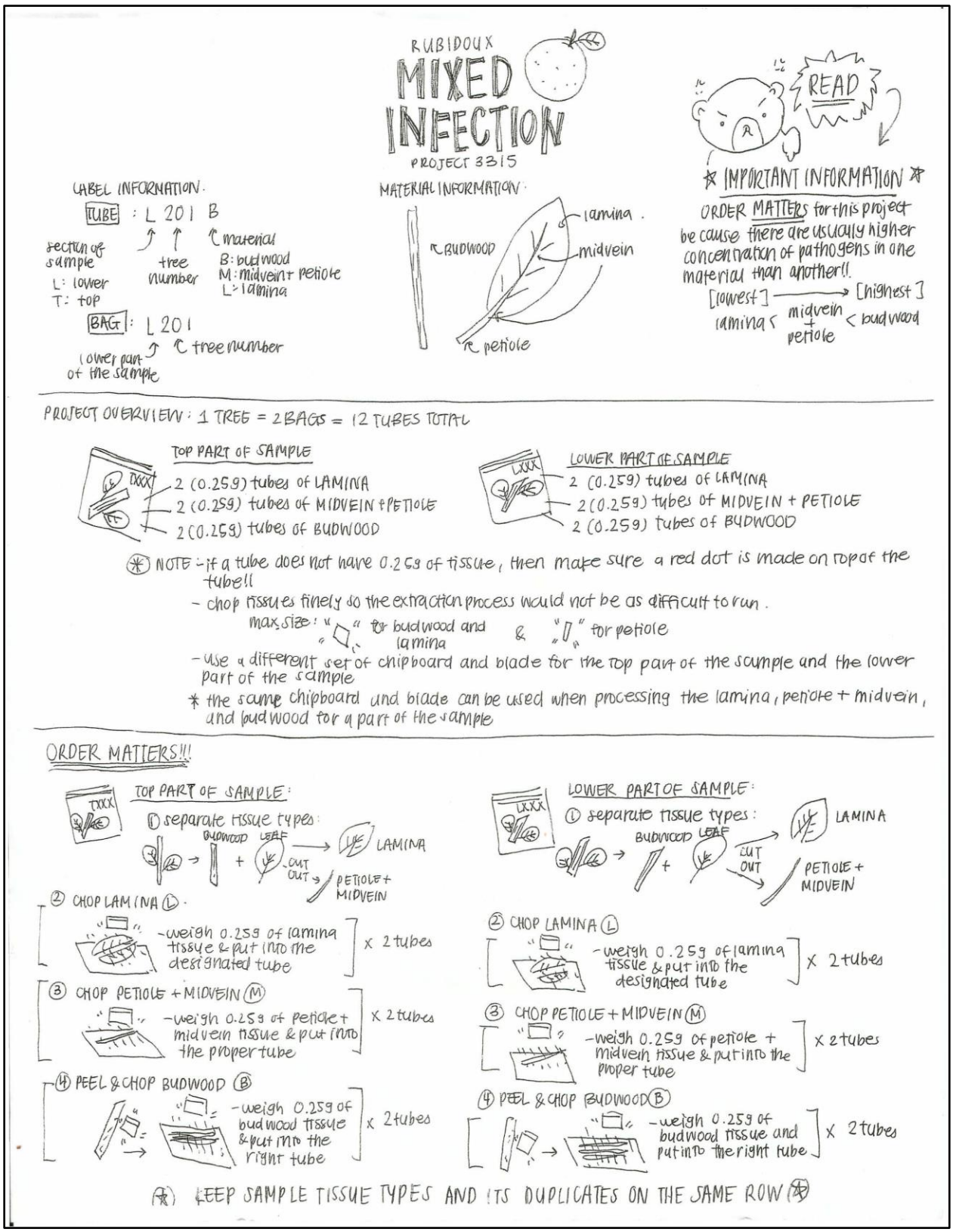


Fig 1. Experimental Scheme.

Table 1: All different pathogens used in the mixed infection experiment

Treatment Code	Treatment	Treatment-Full name	Flag Pattern	Flag Color
1	H	Healthy-Not-inoculated	Striped	Yellow / Black
2	CTV	**Citrus tristeza virus (CTV)**	Striped	Blue / White
3	Ps	**Citrus psorosis virus (CPsV)**	Striped	Green / Black
4	LB	**Citrus leaf blotch virus (CLBV, Dweet mottle)**	Striped	Green / White
5	Sc	**Spiroplasma citri**	Striped	Orange / Black
6	Vds	**Citrus viroid mix (8-14)**	Striped	Red / Black
7	ALL	All pathogens (1-6)	Striped	White / Black
8	CEVd	**Citrus exocortis viroid (CEVd)**	Checkered	White / Black
9	Ia	**Citrus bent leaf viroid (CBLVd, CVd-Ia)**	Checkered	Blue / Black
10	IIa	**Hop stunt viroid (HSVd, CVd-IIa, non-cachexia)**	Checkered	Blue / White
11	IIb	**Hop stunt viroid (HSVd, CVd-IIb, cachexia)**	Checkered	Green / Black
12	IIIb	**Citrus dwarfing viroid (CDVd, CVd-IIIb)**	Checkered	Orange / White
13	IV	**Citrus bark cracking viroid (CBCVd, CVd-IV)**	Checkered	Pink / White
14	V	**Citrus viroid V (CVd-V)**	Checkered	Red / Black
15	VI	**Citrus viroid VI (CVd-VI)**	Checkered	Red / White

Table 2. Healthy controls used

Flag Color	Citrus Species - Swingle	Count
Black	Citrus aurantifolia	13
Blue	C. aurantium L.	4
Fluorescent Yellow	C. hybrid-C. maxima (Burm.) Merr. X C. reticulata Blanco	12
Green	C. limon (L.) Burm.f.	12
Orange	C. paradisi Macf.-Grapefruit	12
Pink	C. reticulata Blanco-Clementine	29
Purple	C. reticulata Blanco-Mandarin	68
Red	C. reticulata Blanco-Satsuma	29
White	C. reticulata Blanco-Tangor	4
Yellow	C. sinensis (L.) Osb.-Navel	91
Black Dots / Pink	C. sinensis (L.) Osb.-Sweet orange	8
Orange dots / White	C. sinensis (L.) Osb.-Valencia	7
Red Dots / Yellow	Citroncirus J.W. Ingram & H.E. Moore	45
No Flag	C. hybrid- C. reticulata Blanco X C. tangelo J.W. Ingram & H.E. Moore	1
No Flag	C. maxima (Burm.) Merrill.	2
No Flag	C. medica L.	2
No Flag	C. paradisi Macf.-Pummelo	1
No Flag	C. sinensis (L.) Osb.-Blood orange	2
No Flag	C. sinensis (L.) Osb.-Tangor	1

No Flag	C. tangelo J.W. Ingram & H.E. Moore		2
No Flag	Fortunella margarita (Lour.) Swingle		2
No Flag	X Citrofortunella sp.		2
No Flag	X Citroncirus spp.-1		1
No Flag	X Citroncirus spp.-2		1
No Flag	X Citrondarin spp.		1
		Total	352

Table 3. Collection Season, Nucleic acid extraction and qPCR performed

Collection Season	Collection	Nucleic acid Extraction	qPCR
February 2018	complete	√	√
May 2018	complete	√	√
Sept 2018	complete	√	
Dec 2018	complete	√-plate 1-4*	
February 2019	complete	√-plate 1-4*	
May 2019	complete	√-plate 1-4*	
Sept 2019	80% collected		

*Plate 1-4 are the infected samples

In 2018-2019, we continued collection for the time course study, which was 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. **1,392 samples** are collected each season **in duplicates** including the infected samples with the different pathogens listed in the table 1and healthy controls (Table 2). Each season we collect a total of 2,784 samples due to duplicates.

We have secured collections from February 2018 through September 2019. All samples were collected in duplicates and were subjected to freeze drying as part of our established sample processing protocol. Samples were either kept in freeze dry conditions for storage or subjected to RNA extraction and qPCR testing. All collections have been freeze dried as per our sample processing citrus protocol established by CCPP.

Due to the complexity of the high number of tested samples, a decision was made to give priority for testing infected samples (plate 1-4) over healthy samples as indicated in Table 3.

The infected samples (plate 1-4, Table 3) for seasons (Dec 2018, Feb 2019 and May 2019) were processed and tested via qPCR. Healthy samples for these respective seasons were stored in freeze dried conditions until tested. Partial collection for season Sept 2019 is now complete. No further processing has been performed on the collection from September 2019.

2020-Extra Year experiment: Rationale

- The 2019 sampling (year 6 of 5) provided 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 (processing 2,784 samples/season) and the

changing weather patterns we feel that a final year (7 of 5) of full or selective sampling (2018, 2019, and 2020 / 36 months) will significantly strengthen our results.

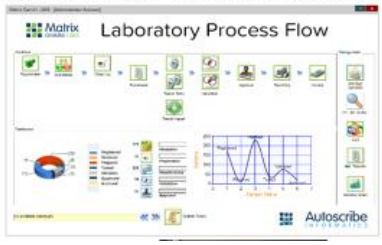
- After preliminary data analysis indicate a specific time period, temperature range, or phenological tree stage that is preferable for pathogen detection, the protocols will be validated on citrus nursery regulated pathogens exotic to California (future project).

  For example, if our data indicate that temperatures 25-28ºC are optimum for sample collection and testing of CTV, CPsV and viroids we can contact fellow scientists in Florida (or international partners) 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.

- We apologize for going 2 years 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.

Customization Face to Face Kick Off meeting with Autoscribe LIMS staff – March 27-28, 2018 @ UCR



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 software "Matrix Gemini LIMS" from Autoscribe Informatics was installed in the UCR Servers and we initiated its customization (March 2018) for this CCNB and other CCPP projects.

In 2019, Autoscribe delivered initial LIMS configurations which were later tested by CCPP. The test assessment was reported to Autoscribe and there will be a second iteration of LIMS to ensure LIMS is able to cope with various use cases and scenarios before moved into production. A phase two development cycle will also be carried out after LIMS goes live, aiming to integrate LIMS with qPCR machines in CCPP Citrus Diagnostic and Research Lab so qPCR results will be preprocessed and imported into LIMS directly. We will continue with the customization and implementation of the Autoscribe LIMS in samples processing to fit LIMS into different workflows, including Rubidoux mixed infection experiment.

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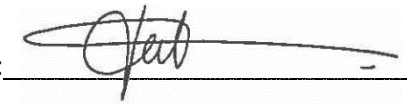
Osman, F., Pagliaccia, D., Bodaghi, S., Dang, T. Vidalakis, G. Multiplex qPCR detection of all Candidatus liberibacter spp. (asiaticus, americanus and africanus).

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%	$20,546
	Benefits 52%	$10,684
	TOTAL PERSONNEL SERVICES	$31,230
B.	**OPERATING EXPENSES:**	
	Laboratory Supplies (kits, sequencing, etc.)	$0
	Travel	$0
C.	**TOTAL OPERATING EXPENSES**:	$0
D.	**TOTAL BUDGET REQUESTED**:	$31,230

Signatures of Requestors:  Date: 11-18-19

Signature of Cooperator:  Date: 11-18-19

Department Chair: _____ Date: _____

LIAISON OFFICER _____ Date _____