CALIFORNIA CITRUS NURSERY BOARD
Implementation and streamlining of the newly developed high throughput diagnostic system for citrus nurseries registration.
Annual Report (year 1 of 2)
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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;

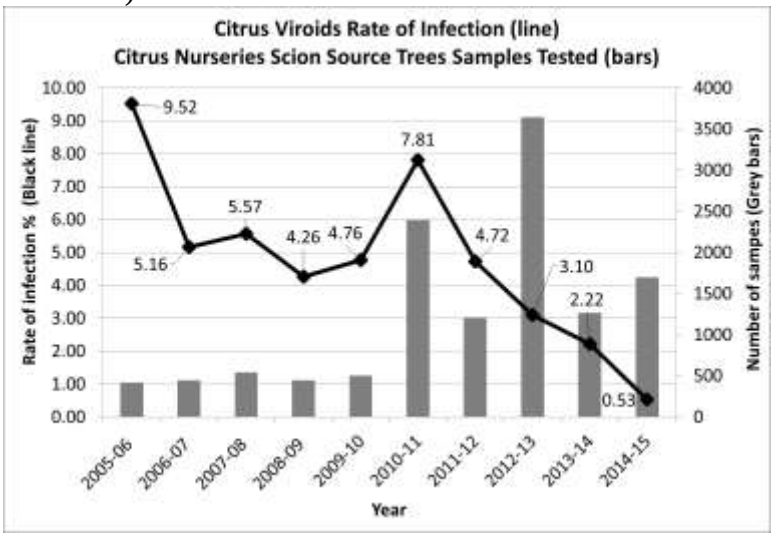
1. Streamlining and minimizing the chance for false results of the viroids, RNA viruses, and DNA pathogens developed methods.
2. Optimizing the time and frequency of sampling in regard to time of the year, temperature, and tree phenological stage.
3. Building a cohesive efficiently working Laboratory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

This year we made significant progress towards the incorporation of three high throughput detection assays into a routine workflow for the multiplex detection of RNA and DNA pathogens.

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

   a. Streamlining diagnostic protocols.

   Two universal/multiplex assays for the detection of citrus viroids and Citrus tristeza virus, Citrus psorosis virus (CPsV), and Citrus leaf blotch virus (CLBV) were validated and streamlined and used successfully in the Citrus Nursery Pest Cleanliness Program in the 2014-15 testing cycle. As a result the viroid infection in the nurseries tested was less than 1% while the virus infection was less than 0.05% (see figure below).



b. Minimizing the chance for false results of the viroids, RNA viruses, and DNA pathogens developed methods.

i. Universal detection of citrus viroids

We designed two multiplex qPCR assays using TaqMan Probes Fluorophore based on the "Apsca" and "Non-Apsca" viroid groups as in the CDFA approved SYBR green protocol (Table 1). We also twigged the primers of the approved SYBR green method in order to improve its performance (e.g. fine-tuning of annealing temperatures). During this year the newly designed TaqMan qPCR assays were tested and compared using both viroid controls and previously tested citrus nurseries samples to determine their capacity of detecting all known citrus viroids in two reactions. We acquired preliminary results that we will need to repeat in the second and third year of the study.

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

ii. Multiplex detection of RNA viruses

We developed and used in over 4000 reactions (2018 samples) a single real-time multiplex reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay for the simultaneous detection of *Citrus tristeza virus* (CTV), *Citrus psorosis virus* (CPsV), and *Citrus leaf blotch virus* (CLBV) using three different fluorescently labeled minor groove binding qPCR probes (Table 2).

Table 2. Multiplexed qPCR assays for *Citrus tristeza virus* (CTV), *Citrus psorosis virus* (CPsV), and *Citrus leaf blotch virus* (CLBV).

Primers/probes	Amplicon size (bp)
CTV CA-CP-16678 F CTV CA-CP-16679 F2 CTV CA-CP-16813 R CTV CA-CP-16763 **p-TET**	136
CLBV CP 7711 F CLBV CP 7872 R CLBV CP 7738 **p-FAM**	163
CPsV -792 F1 CPsV-791 F2 CPsV 946 R1 CPsV 946 R2 CPsV-851 **p-VIC**	156

F: Forward primer. R: Reverse primer. p: qPCR probe

The capacity of the multiplex RT-qPCR assay in detecting the viruses was compared to singleplex RT-qPCR designed specifically for each virus and was assessed using multiple virus isolates from diverse geographical regions and citrus species as well as greenhouse created graft inoculated citrus plants infected with various combination of the three viruses. No significant differences in detection limits were found and specificity was not affected by the inclusion of the three assays in a multiplex RT-qPCR reaction (Table 3). Comparison of the viral load for each virus using singleplex and multiplex RT-qPCR assays, revealed no significant differences between the two assays in virus detection. No significant difference in Cq values detected when using one-step and two-step multiplex RT-qPCR detection formats. Results showed that the developed multiplex RT-qPCR can streamline viruses testing of citrus nursery stock by replacing three separate singleplex assays, thus reducing time and labor while retaining the same sensitivity and specificity. We also developed a special detection assay for a unique group of psorosis isolates reported from Argentina.

Table 3. Cq values and comparison between singleplex and multiplex RT-qPCR assays in detecting *Citrus tristeza virus* (CTV), *Citrus psorosis virus* (CPsV), and *Citrus leaf blotch virus* (CLBV) mix infected citrus trees.

		Singleplex qPCR			Two-step multiplex qPCR			One-step multiplex RT-qPCR		
Sample Description*	Cox	CTV	CPsV	CLBV	CTV	CPsV	CLBV	CTV	CPsV	CLBV
CPsV-P213+CTV+CLBV	21.43	19.24	26.65	20.68	20.85	26.86	21.00	17.80	23.50	20.16
CPsV-P1365+CTV+DMV930	20.75	19.54	24.95	28.38	22.45	26.78	29.64	19.12	22.15	26.62
CPsV-P1365+CTV+CLBV	19.89	19.70	25.15	29.20	22.58	25.80	30.12	19.74	22.32	28.49
CTV-SY558+CPsV-P203A+CLBV	21.86	16.93	27.79	32.52	20.41	30.79	34.54	16.07	26.78	31.98
CTV-SY558+CPsV-P250+CLBV	21.39	16.74	27.82	28.42	19.76	29.92	30.31	16.17	28.13	28.92
CTV-SY568+CPsV-P203A & P250+CLBV	21.36	19.41	26.00	33.79	21.33	31.91	35.79	18.91	24.50	33.89
Pooled RNA positive controls	24.69	22.38	27.35	27.14	24.55	28.33	29.73	23.17	25.76	22.03

*All virus combinations were grafted onto Madam Vinous sweet orange. RNA and cDNA samples were used as positive control for one-step and two-step multiplex RT-qPCR, respectively. Virus isolate name is provided after virus name whenever applicable.

iii. Multiplex detection of DNA pathogens

A multiplex qPCR assay was designed to detect all three *C.* Liberibacter species as well as *S. citri* (Table 4). GenBank sequences (16 S rRNA) of three *C.* Liberibacter species (asiaticus, africanus, and americanus) associated with Huanglongbing (Table 4).

Table 4. Multiplexed qPCR assays for singleplex and multiplex qPCR detection of *Candidatus Liberibacter spp.* and *Spiroplasma citri*

Primers/probes	Amplicon size (bp)
HLBas.2 F HLBas.3 F HLBaf.2 F HLBam.2 F HLB3 R HLB **p VIC**	162
S.C.-114371-F1 S.C.-114371-F2 S.C.-114484-R S.C.-114434 **p FAM**	113

HLBas (*Candidatus* Liberibacter asiaticus), HLBaf (*Candidatus* Liberibacter africanus) HLBam (Candidatus Liberibacter americanus). *Spiroplasma citri* (S.C.). F: Forward primer. R, r: Reverse primer. p: qPCR probe.

This new universal *C.* Liberibacter HLB qPCR assay was validated against many *C.* Liberibacter samples and it was able to detect all three *C.* Liberibacter species. Most importantly this HLB assay was able to detect the HLB-CA isolate that was found in a backyard tree in Hacienda Heights, Los Angeles, CA in 2012. Collaborators from around the world kindly supplied *C.* Liberibacter nucleic acids used in the validation experiments. Samples have been sent by: Svetlana Folimonova-Florida, Gerhard Pietersen-South Africa, Nelson Wulff-Brazil, Cristina Paul-Beltsville and Dr. Shagufta-Pakistan. We would like also to acknowledge Dr. MaryLou Polek and Dr. Cynthia LeVesque of the CRB for providing the USDA-APHIS-PPQ permit and laboratory facilities for the importation of CLas infected lyophilized tissue from Florida in order to test the newly developed high throughput robotic nucleic acid extraction protocol approved for use in the registration testing program (Table 5).

Table 5. Cq values of multiplexed qPCR assays for singleplex and multiplex qPCR detection of *Candidatus* Liberibacter spp. and *Spiroplasma citri*. *C.* Liberibacter samples derived from Florida, Beltsville Maryland, and Pakistan (asiaticus), South Africa (africanus) and Brazil (americanus).

		Singleplex qPCR		Multiplex qPCR	
Sample	Cox	HLB	S. citri	HLB	S. citri
Florida	14.7	28.2	-	27.5	-
Florida	14.9	25.5	-	25.5	-
Florida	15.2	26.0	-	25.3	-
Florida	15.1	23.8	-	23.8	-
Florida	14.9	25.7	-	25.5	-
Florida	15.4	29.0	-	28.4	-
Florida-B437	14.1	24.2	-	24.6	-
South Africa-B432	13.6	38.1	-	39.6	-
South Africa-11-2003	13.4	23.3	-	23.4	-
Brazil-Bebedouro	15.3	33.7	-	34.1	-
Brazil-Bebedouro	15.5	27.9	-	29.2	-
Brazil-Jose Bonifacio	15.9	27.0	-	27.3	-
Brazil-Santa Maria Da Serra	15.3	28.3	-	28.8	-
Pakistan					
Ruby Sweet	14.9	27.2			
Pera rio	14.9	33.3			
Hamiln	15.3	29.2			
Netal	14.0	31.5			
Jafa	16.3	30.5			
Kozan	14.4	27.4			
Blood red	15.3	30.3	Currently under testing		
Ruby blood	15.2	27.5			
Jafa Pure	15.3	26.8			
Tracco 3	15.1	28.4			
Hankley	14.8	27.9			
Gamble valancia	15.7	31.6			
Sulstiana 15	15.5	33.5			

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

We gathered 304 CCPP (Table 6) foundation block trees and made all necessary arrangements (e.g. permits, insecticide treatments, trucking, preparation of screenhouse benches and irrigation

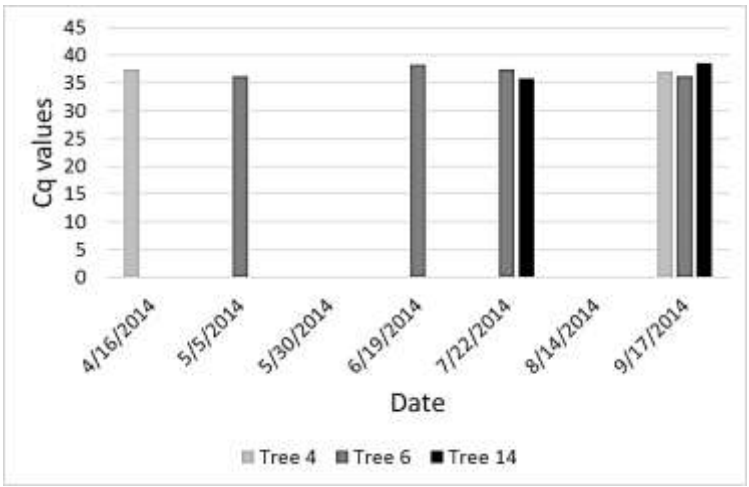
system, etc.) for their transportation from Lindcove to the Riverside quarantine screen house (CDFA-USDA approved structure).

Table 6. Citrus trees gathered for screenhouse experiment

Type	No.
Blood orange	3
Citron	2
Citrumelo	2
Grapefruit	22
Kumquat	2
Lemon	12
Lime	1
Limequat	2
Mandarin	104
Navel	95
Pummelo	1
Rootstock	2
Satsuma	29
Sour orange	4
Sweet orange	9
Tangelo	2
Tangor	5
Valencia	7
Total	304

We initiated the design of the mix infection experiment with citrus nursery regulated pathogens endemic to California (i.e. CTV, CPsV, *S. citri*, and citrus viroids).

The time course study for the detection of *Spiroplasma citri* in known infected and uninfected field trees initiated on April 2014 was continued. *S. citri* samples were collected at University of California Riverside Agricultural Operations. Sixteen citrus trees were sampled every three to four weeks to determine *S. citri* titer in different seasons within the period of vegetative growth throughout a range of temperatures. Four budsticks (including the leaves) were collected from each of the four tree quadrants. The samples were grouped and stored with their respective quadrants and tree. This process was repeated for each tree and sampling time.



Each sample was separated into 3 parts: Budwood-bark peels, Mid vein and petiole, and Leaf blades. Samples were extracted with the CDFA approved semi-automated magnetic bead MagMax system stored at -80C and tested with qPCR.

Preliminary analysis of budwood

samples has not yet revealed any important differences or patterns on *S. citri* concentrations and more experiments need to be performed in years 2 and 3 of this project.

Finally, a study on sanitation practices was initiated during year one of this project and will continue in years 2 & 3. We are testing a series of different sanitation practices (Table 7) following up with qPCR and biological testing.

Table 7. Sanitation treatment and products being tested

Treatment
Bleach
Flame
Lysol wipe
Clorox wipes
Santicloth HB (green)
Santicloth Plus (red)
409 Spray
Simple Green
Next Gen wipes
Green works wipes

Preliminary experiments have indicated the *Citrus exocortis viroid* (CEVd) RNA was not detectable by RT-qPCR on surfaces, razor blades, or healthy citrus tissue after treatment with 10 & 20% bleach solutions. On the other hand CEVd RNA was detectable by RT-qPCR after cleaning surfaces twice with sanitizing wipes (Table 8).

Table 8. Cq Values of *Citrus exocortis viroid* RT-qPCR on surfaces swabs after processing infected citrus tissues and use of different sanitation treatments

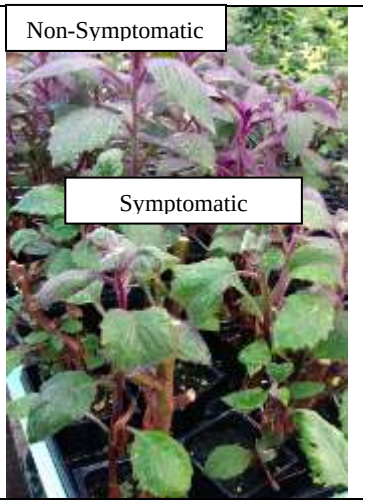
	Treatment		
Razor blade	Bleach 10%	Bleach 20%	No Treatment
	-	-	26.07
	-	-	24.42
	-	-	26.23
	-	-	26.28
	-	-	24.22
	-	-	25.88
Healthy citrus tissue	Bleach 10%	Bleach 20%	No Treatment
	-	-	28.52
	-	-	27.65
	-	-	26.36
	-	-	29.89
	-	-	27.25
	-	-	30.33
	-	-	28.76
	-		29.26
	-	-	35.32
Petri dish	Wipe Once*	Wipe Twice	No Treatment
	28.76	30.99	22.86
	27.30	32.01	23.23
	24.16	30.15	21.33
	24.50	-	20.26

*Santicloth HB (green)

Similarly, bleach was the only treatment that eliminated CEVd transmissibility to gynura (Table 9). Once more these are results from preliminary experiments with limited replications that we will repeat in larger scale in years 2 & 3 of this project.

Table 9. *Citrus exocortis viroid* transmission to *Gynura aurantiaca* after processing healthy and infected citrus tissues and use of different sanitation treatments

Treatment	Symptomatic Plants/Plants inoculated
Healthy citrus tissue	0/3
Water	1/3 (2 dead)
Bleach 10%	0/3
SantiCloth (green)	1/3
Lysol	2/3



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.

Limited funds has delayed progress on this objective. This year we identified a company specializing in barcoding products and software and we have been experimenting with different kinds of labels for the samples. We are also in contact with CDFA and we exchange data in electronic format to minimize human error. Finally, we received support by the Citrus Research Board and National Clean Plant Network to complete the CCPP LIMS system. Phase II was completed this year (https://ccppdms.ucr.edu/ccppdms/user_login.login).