California Fruit Tree, Nut Tree and Grapevine Improvement Advisory Board (IAB)

2019 Research Progress Report

Nursery Services Program
California Department of Food and Agriculture
Sacramento, CA 95814

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Reports

Progress report on a research grant proposal to: Fruit Tree, Nut Tree and Grapevine Improvement Advisory Board (IAB)

February 24, 2020

Project Title: Study of the Effects of Little cherry virus-1 and Little cherry virus-2 on Different Cherry Rootstocks

Fiscal Year and Project Duration: Third year of a 4-year project

Project Leader: Maher Al Rwahnih, Academic Administrator, University of California, Davis

Objectives:

1. To test a collection of plants by qRT-PCR to locate infected source material needed for the experiment.

2. To evaluate the effects of LChV-1 and LChV-2 on 16 different popular *Prunus* rootstocks. All rootstocks will be grafted with the same cherry scion cultivar, 'Bing'.

3. To test the inoculated plants in year 2 for the selected viruses and monitor the virus movement and record the symptom observation.

Accomplishments:

Objective 1:

In 2017 laboratory staff screened 35 FPS positive controls selections as well as accessions from the USDA ARS NCGR Wolfskill collection and UCD Plant Pathology Department Armstrong collection. All trees were tested by a RT-qPCR panel for 16 different viruses, including Apple chlorotic leafspot virus (ACLSV), American plum line pattern virus (APLPV), Apple mosaic virus (ApMV), Cherry green ring mottle virus (CGRMV), Cherry leaf roll virus (CLRV), Cherry necrotic rusty mottle virus (CNRMV), Cherry raspleaf virus (CRLV), Cherry virus A (CVA), Hop stunt viroid (HSVd), Little cherry virus-1 and -2 (LCV-1) and (LCV-2), Plum bark necrosis stem pitting associated virus (PBNSPaV), Prune dwarf virus (PDV), Peach latent mosaic viroid (PLMVd), Prunus necrotic ringspot virus (PNRSV), and Peach rosette mosaic virus (PRMV). Two selections were chosen to serve as the inoculation source for LChV-1 and LChV-2. We

identified a single infection source of LChV-1 but were unable to do so for LCHV-2. The inoculation source that we selected is co-infected with CVA.

	Group ID	Disease Profile
Virus Positive Sample	11454	LChV-1
Virus Positive Sample	13157	CVA, LChV-2

We also performed High Throughput Sequencing (HTS) analysis on both selected positive controls to confirm the previous RT-qPCR results and to exclude any possible infection with known or unknown virus/es.

Objective 2:

RT-qPCR was used to verify that all the selected rootstocks were negative for LChV-1 and LChV-2. Trees were also tested for the two common pollen vectored viruses PNRSV, PDV which are already known to cause hypersensitivity reaction in some of the selected rootstocks. Plants were also tested for CVA as it's known to be seed transmitted.

For the first iteration of the trial, previously funded by IAB (funding cycle July 1, 2017 to June 30, 2018), green growing negative control material was sourced from Foundation Orchard Bing trees and positive material from container grown LChV-1 and LChV-2 Bing trees. Material was collected in late May of 2018 and T-bud grafted to container grown rootstocks with 2 buds per rootstock. Bud take success was evaluated post-grafting and additional buds were grafted where success was poor. The virus inoculated, T-budded and non-grafted control trees were planted in a randomized complete block early October 2018.

For the second iteration of the trial, during the 2018-19 funding cycle, material was collected from negative and positive source trees and T-budded to container grown rootstocks in October of 2018. The cultivar Lake was excluded from the fall grafing due to rootstock decilne in the shadehouse. Dormant grafted and non-grafted control trees were planted in the randomized block in April 2019.

The site for the field trial has been cultivated and drip irrigation lines have been installed. Weed and pest control maintenance will continue through the funding of the project.

Objective 3:

Following bud break in 2019, bud graft status, for 1st and 2nd graft iterations, was recorded as either dead, alive or growing. Additionally, in September of 2019, leaf petioles from rootstock branches growing above the graft site were tested by RT-qPCR for graft transmission of LChV-1 or LChV-2. The results of RT-qPCR are shown in Figure 1.

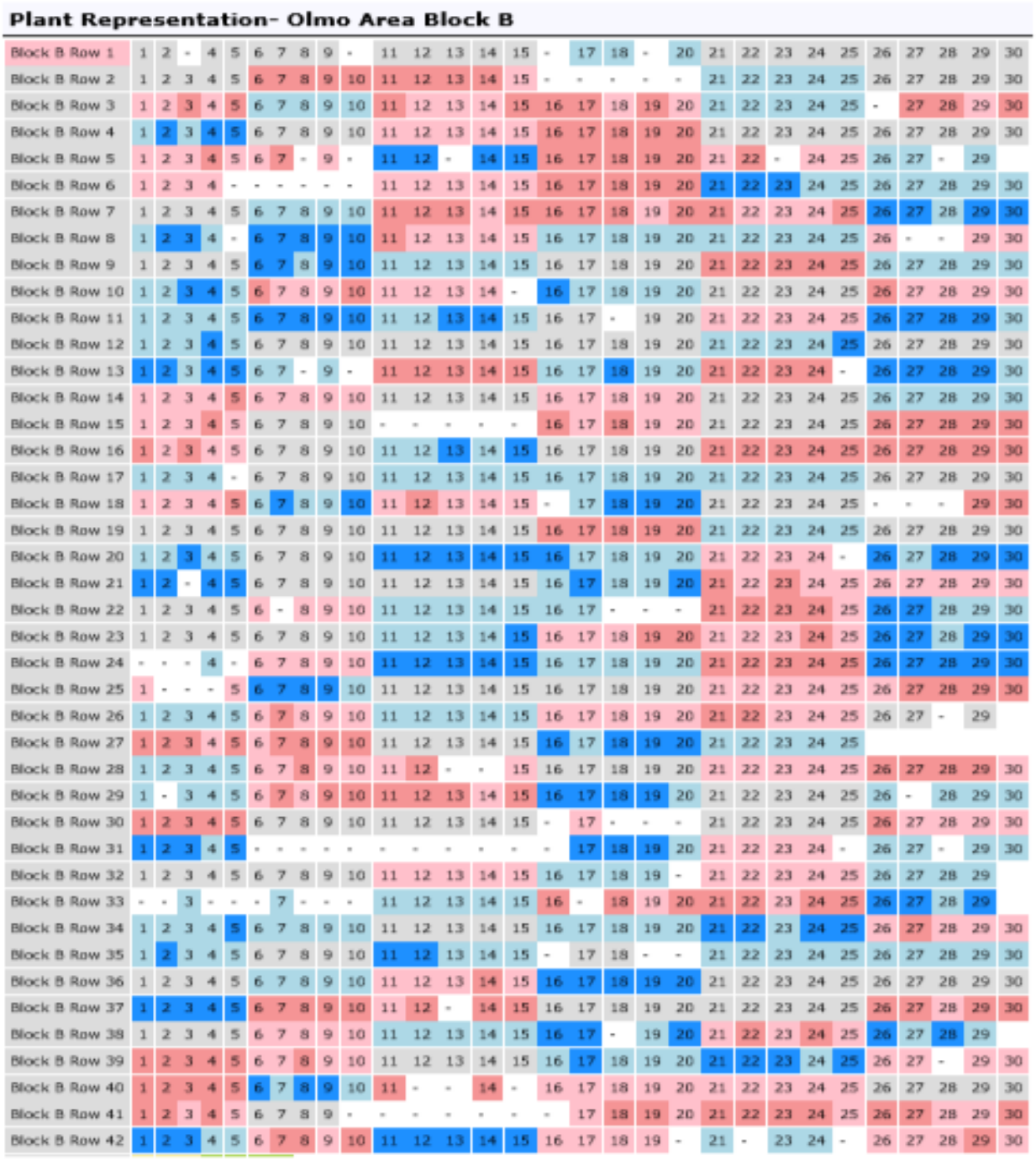


Figure 1. The randomized block of rootstocks grafted with healthy, LChV-1 or LChV-2 infected Bing scion in the spring and fall iterations. The grey boxes represent plants grafted with healthy Bing scion or ungrafted controls. The dark blue boxes represent plants with successful grafting and transmission of LChV-1. The light blue boxes represent plants with unsuccessful grafting and/or negative RT-qPCR results for LChV-1. The dark salmon boxes represent plants with successful grafting and transmission of LChV-2. The light pink boxes represent plants with unsuccessful grafting and/or negative RT-qPCR results for LChV-2. White areas represent plants that died.

Pruning of the root stock growth to promote growth of grafted scion tissue was completed in January 2020. Preliminary observations of root stock response to little cherry infection of successfully grafted trees (the dark blue and dark salmon colored boxes in Figure 1) will occur in late summer 2020. Trees will be observed for death, gumming at the graft site, leaf distortion, leaf color and plant vigor. RT-PCR will be used to test for the presence of LChV-1 or LChV-2.

As shown in Figure 1 (light blue and light pink boxes), for many plants grafting was unsuccessful and transmission of LChV-1 or LChV-2 did not occur. We are preparing to redo T-bud grafting on plants already in the randomized block for which bud growth and/or virus transmission did not occur. This is scheduled for May of 2020.

In late summer of 2020, observations of graft take on 2020 grafts will be recorded and RT-qPCR for LChV-1 and LChV-2 will be performed on leaf petioles from leaves growing on the root stock above the graft site.

Observations of trees newly grafted in spring 2020 will continue until spring 2022 to observe for signs of hypersensitive response such as death, gumming at the graft site, leaf distortion, leaf color and plant vigor. Progress of the trees successfully grafted and infected with LChV-1 or LChV-2 as observed by RT-qPCR in 2019 will also continue to be monitored for symptoms and hypersensitive response at the grafting site.

Summary:

Little cherry disease (LCD), associated with Little cherry virus-1 (LChV-1) or -2 (LChV-2), is a common problem of cherries (*Prunus avium*) which occurs worldwide, causes unmarketable fruit and often results in tree or orchard removal (Jelkmann and Eastwell, 2011). Most of the new cherry rootstocks used in cherry production are interspecific *Prunus* hybrids which introduces an increased risk of an adverse reaction (hypersensitivity) to some viruses (Lang and Howell, 2001). Hypersensitive reactions exhibit graft union gum exudation, premature abscission, and tree death within one or two growing seasons and have been shown to occur in *Prunus* when infected with Prunus necrotic ringspot virus (PNRSV) and Prune dwarf virus (PDV) (Howell and Lang, 2001, Lang and Howell, 2001, Lang et al., 1998). We propose to evaluate the effects of LChV-1 and LChV-2 on 16 different popular *Prunus* rootstocks. All rootstocks will be grafted with a scion variety from the same accession. Observations of budtake and tree performance will be recorded and evaluated for two years. Rootstocks will be rated for sensitivity to LChV-1 and LChV-2 and this information will be shared with growers and nurseries to assist in making rootstock selection decisions.

Project's Benefit to Nursery Industry:

In the US, sweet cherry fresh market production totaled 254,906 tons and was valued at $703 million in 2015 (NASS, 2017). Washington, California and Oregon account for more than 90% of sweet cherry industry in the US, with 34,786, 34,742, and 13,416 acres planted to sweet cherries in 2012, respectively (NASS, 2017). Interest in sweet cherry production has increased in recent years due to the high value of fresh market cherries and the increasing availability premium quality varieties and new rootstocks with exciting horticultural traits (Lang and Howell, 2001).

Little cherry disease is a concern to growers wherever cherries are grown. LCD is associated with LChV-1 or LChV-2, which can be found in single and mixed infections. Trees with LCD produce cherries of small size and poor color making fruit unmarketable. The problem results in unpicked limbs or trees, tree removal and even orchard removal. The disease is readily transmitted by grafting and LChV-2 is vectored by mealybugs (Jelkmann and Eastwell, 2011). To date, no breeding programs have been successful in finding resistance to the disease.

In orchards worldwide, cherries (*P. avium*) are either budded or grafted onto rootstocks. Rootstocks provide protection from soil-borne pests and improved tolerance to abiotic stresses, such as heavy soils, drought conditions, salinity, and cold winter temperatures, thus, increasing the survival of the scion material. Traditionally, cherries in the US were grown on Mazzard or Mahaleb rootstocks or clonally-propagated 'Colt' which are generally tolerant of infection by pollen-borne viruses, PDV and PNRSV (Lang et al. 1998). It has been increasingly well-documented that new *Prunus* rootstock selections can show hypersensitive reactions to viruses that have been typically well tolerated by traditional rootstocks (Lang et al. 1997, Lang et al. 1998, Lang and Howell 2001, Howell and Lang 2001). These new rootstock selections are derived from species other than or are hybrids with *P. avium* which offers genetic diversity and novel horticultural traits, but with an increased risk of hypersensitivity. Hypersensitive (rapid and lethal) reactions exhibit graft union gum exudation, premature abscission, and tree death within one or two growing seasons. Viruses with documented hypersensitivity include PNRSV and PDV (Howell and Lang, 2001). It is not currently known if LChV-1 and LChV-2 can cause similar hypersensitive reactions in the common *Prunus* rootstocks.

We plan to conduct a field trial to investigate hypersensitivity reactions to LChV-1 and LChV-2 in the top *Prunus* rootstocks. Currently, we anticipate using GiSelA®3, GiSelA®5, GiSelA®6, GiSelA®12, Krymsk®5, Krymsk®6, Krymsk®7, EMLA Colt,' MaxMa®14, Cass, Clare, Clinton, Crawford, Lake and seedlings of Mazzard and Mahaleb in the trial. We will assess the sensitivity of these rootstocks to LChV-1 and LChV-2 and share the results of our research.

This research has a great benefit to the cherry growing industry as the results of our research will assist growers and nurseries in rootstock selection for new plantings. Informed rootstock selection will result in healthier, more productive cherry trees.

Progress report on a research grant proposal to: Fruit Tree, Nut Tree and Grapevine Improvement Advisory Board (IAB)

February 26, 2020

Project Title: Advancing our knowledge on the detection, sampling and epidemiology of grapevine Pinot gris virus

Project Leader: Maher Al Rwahnih, Academic Administrator, University of California, Davis

Project Duration: 07/01/2019 to 06/30/2020

Objectives:

1. Screen select grapevine populations for GPGV to compile a set of diverse isolates and investigate the possibility of a variant-specific assay.
2. Empirically test and validate detection assays using positive controls.
3. Investigate potential reservoirs of GPGV in wild plants from riparian areas adjacent to vineyards.
4. Determine distribution of GPGV in canes and other grapevine tissues over time.
5. Disseminate research results to farm advisors and growers.

Accomplishments:

Objective 1:
Stored total nucleic acid samples (1,206) from the USDA National Clonal Germplasm Repository (NCGR) in Winters, CA were analyzed for GPGV presence; such samples were collected during a previous study about grapevine leafroll-associated virus 3 and represent different grapevine selections originated from around the world (Diaz-Lara et al., 2018). As a result, 8 samples (Table 1) tested positive for GPGV by our developed real-time RT-qPCR assay, subsequently, GPGV infection was confirmed by conventional PCR and Sanger sequencing.

Table 1. Selections located at the USDA-NCGR and tested positive for GPGV.

Sample	Cultivar	Origin
6489	J5-58	United States
6235	Touriga	Portugal
6203	Mosho Patata	Greece
5307	Victoria's Choice 4x	United States
5239	Olmo 672	United States
5363	Moscato Di Terracina	United States
5568	Aspruda Ariloghi	Greece
4253	Khalili	Afghanistan

In addition, we already contacted collaborators to obtain allegedly GPGV-infected plant material from the North Coast and Central Sierra regions in California. This material will be collected by growers and farm advisors, and later shipped to Foundation Plant Services (FPS) using Dr. Al Rwahnih's USDA-APHIS permit.

Objective 2:
Collected grapevine samples for Objective 4 were analyzed by the GPGV ELISA kit (Bioreba) to investigate the reliability of this detection assay. Thus, the new GPGV ELISA kit was challenged against samples previously analyzed by real-time RT-qPCR. As a result, contradicting results (i.e. real-time RT-qPCR positive, ELISA negative) were obtained, we are in the process of further investigate these samples by high throughput sequencing (HTS).

Objective 3:
To determine the extent to which free-living *Vitis* spp. harbor GPGV and serve as a reservoir for the virus, we surveyed 8 different riparian habitats in close proximity to vineyards with GPGV infection. In total 60 free-living vines (Figure 1) were sampled and tested by our developed RT-qPCR for the presence of GPGV; all these vines were marked, and GPS coordinates were recorded to facilitate resampling. Consequently, 23 samples (Table 2) tested positive for GPGV and later confirmed by conventional PCR and Sanger sequencing. We are in the process of recollecting these samples and identify the involved *Vitis* spp. using molecular markers.

Figure 1. Examples of free-living vines observed during the survey.

Table 2. Sampled free-living *Vitis* spp in close proximity to vineyards with GPGV infection.

Riparian Area/Vineyard	Number of Collected Samples	GPGV Positive Samples
1	15	2
2	5	1
3	4	1
4	4	3
5	3	1

6	5	3
7	2	1
8	22	11

Objective 4:
To develop an optimal sampling strategy for GPGV, we are investigating the spatiotemporal fluctuations in virus titer. We hypothesized that distribution of GPGV in infected grapevines varies over time and tissue type, which can affect the efficient detection of the virus. Thus, during the fall 2019, 30 GPGV-infected grapevines located in the Davis Virus Collection, FPS introduction pipeline and the NCGR were sampled and tested by the real-time RT-qPCR assay. Additionally, 35 plants from a commercial vineyard with high incidence of GPGV and located in Napa County were also analyzed. This time, tissues collected included 12 mature expanded leaves per sample (vine). Later, during the dormant season and early sprig (2020) we will collect canes and emerging leaves, respectively. Finally, testing results will be compared, specifically, the quantification of virus titer.

Objective 5:
Preliminary results have been communicated to stakeholders at growers' meetings organized by UCCE. Information will also be disseminated through newsletters, journals, reports and peer-reviewed scientific articles. In addition, results will be shared with the Grape Clean Plant Network for dissemination of information at the national level. Eventually, we will produce an easy-to-read fact sheet for interested parties.

Summary:
Grapevine Pinot gris virus (GPGV) was first discovered in 2012 by high throughput sequencing (HTS) in Italian Pinot gris vineyards. In 2015, this new member of the genus *Trichovirus* (family *Betaflexiviridae*), was first reported in the US in a study of the collections at Foundation Plant Services (FPS, University of California-Davis) in Davis, California. Later, GPGV was also reported by a private virus testing laboratory, which was the first detection of GPGV in the Napa Valley vineyards. In California, GPGV has been identified in symptomatic and symptomless vines from different varieties. The relationship between GPGV infection and symptoms remains complex. Characterized California GPGV isolates share close homology with asymptomatic reference isolates and when symptoms were observed in GPGV-positive vines, those vines were also infected with other viruses or viroids. Thus, the primary goal of this proposed research is to advance our knowledge of the newly reported GPGV in California. We will investigate the virus distribution in grapevine tissues (including canes) over time and the possibility of alternative hosts in California vineyards; in addition, we will investigate the option of a molecular assay with the capacity to distinguish between virulent and latent GPGV variants and test a recently released ELISA kit against different GPGV isolates. Consequently, the proposed research will address detection and biology of an emerging grapevine viral disease in California and will support the development of management strategies.

Progress report on a research grant proposal to: Fruit Tree, Nut Tree and Grapevine Improvement Advisory Board (IAB)

February 26, 2020

Project Title: Development and validation of real-time quantitative PCR assays for the detection of fruit tree viruses

Project Leader: Maher Al Rwahnih, Academic Administrator, University of California, Davis

Project Duration: 07/01/2019 to 06/30/2020

Objectives:

1. Evaluate currently available real-time qPCR assays and screen select fruit tree populations for targeted pathogens to compile a representative set of isolates.
2. Incorporate new genetic data into a more complete characterization of genetic variation across the targeted pathogens to inform assay design.
3. Construct improved assays utilizing multiple primers/probes sets for detecting all existing targeted pathogen variants.
4. Empirically test and validate proposed assay designs using positive controls.
5. Disseminate research progress and results.

Accomplishments:

Objective 1:
Previously published real-time quantitative PCR (qPCR) assays for targeted pathogens (Table 1) were evaluated *in silico* to determine their detection capacity (i.e. number of isolates that they can detect) using the available sequence data in GenBank and bioinformatics programs. In the case of ACLSV, ApMV, ASGV, ASSVd, CGRMV, CRLV, LChV-1, LChV-2, PDV, PNRSV and ToRSV assays (Table 2), sequence comparisons showed imperfect complementary between primers/probes of corresponding assays and the alignment generated for each pathogen. Nucleotide mismatches observed during these analyses ranged from 1 to 10, suggesting the need of updating with additional primers/probes or redesigning to avoid a potential detection failure. Contrasting, the pear decline phytoplasma and CLRV assays (Hodgetts et al. 2009; Osman et al. 2014) did not display nucleotide mismatches, which indicates that not modifications are needed. Lastly, for ASPV, ArMV and TRSV there is not published real-time qPCR assay.

Table 1. Targeted pathogens of fruit trees and included in the initial research proposal.

Disease	Disease Agent	Host
Apple chlorotic leaf spot	Apple chlorotic leafspot virus (ACLSV)	Pome, *Prunus*

Apple mosaic	Apple mosaic virus (ApMV)	Pome, *Prunus*
Apple stem grooving	Apple stem grooving virus (ASGV)	Pome
Apple stem pitting, Pear stem pitting, Pear stony pit disease	Apple stem pitting virus (ASPV)	Pome
Flat apple disease	Cherry rasp leaf virus (CRLV)	Pome, *Prunus*
Tobacco ringspot	Tobacco ringspot virus (TRSV)	Pome
Apple union necrosis	Tomato ringspot virus (ToRSV)	Pome, *Prunus*
Pear decline	Pear decline Phytoplasma	Pome
Apple scar skin/Dapple apple disease	Apple scar skin viroid (ASSVd)	Pome
Pear blister canker	Pear blister canker viroid (PBCVd)	Pome
Cherry green ring mottle	Cherry green ring mottle virus (CGRMV)	*Prunus*
Cherry leafroll	Cherry leafroll virus (CLRV)	*Prunus*
Little cherry	Little cherry virus 1 (LChV-1)	*Prunus*
Little cherry	Little cherry virus 2 (LChV-2)	*Prunus*
Prune dwarf	Prune dwarf virus (PDV)	*Prunus*
Prunus necrotic ringspot	Prunus necrotic ringspot virus (PNRSV)	*Prunus*
Arabis mosaic	Arabis mosaic virus (ArMV)	*Prunus*

Table 2. Published real-time qPCR assays for targeted pathogens of fruit trees.

Pathogen	**Citation of Published Assay**
ACLSV	Osman et al. 2016
ApMV	Osman et al. 2014
ASGV	Gadiou and Kundu 2012
ASSVd	Kim et al. 2010
CGRMV	Osman et al. 2016
CRLV	Osman et al. 2016
LChV-1	Katsiani et al. 2018
LChV-2	Jelkmann et al. 2006
PDV	Osman et al. 2014
PNRSV	Osman et al. 2014
ToRSV	Osman et al. 2014

In addition to the 17 different pathogens included in the initial research proposal, during this second year, 11 additional pathogens (Table 3) and their corresponding detection assays were also investigated *in silico*. Likewise, these assays (i.e. CNRMV, CVA and PBNSPaV) displayed nucleotide mismatches that could result in false negatives (Figure 1) and called for an improvement. Even more, in most cases, not published real-time qPCR assay exist for these pathogens.

Table 3. Pathogens added during the second year of the project and currently available real-time qPCR assays.

Pathogen	**Acronym**	**Assay Citation**
Apple green crinkle associated virus	AGCaV	Not available assay
Apple hammerhead viroid	AHVd	Not available assay

Apple rubbery wood virus 1 & 2	ARWV 1 & 2	Not available assay
Cherry necrotic rusty mottle virus	CNRMV	Osman et al. 2016
Cherry rusty mottle associated virus	CRMaV	Not available assay
Cherry virus A	CVA	Osman et al. 2016
Citrus concave gum-associated virus	CCGaV	Not available assay
Nectarine stem pitting-associated virus	NSPaV	Not available assay
Nectarine virus M	NVM	Not available assay
Peach mosaic virus	PcMV	Not available assay
Plum bark necrosis stem pitting-associated virus	PBNSPaV	Lin et al. 2013

Figure 1. Potential false negatives caused by nucleotide mismatches. Example CNRMV.

Number of CNRMV Isolate Sequences	Number of Mismatches in CNRMV Assay	Assay Sequence
65	0	CTCAACATTGCATCTGAT
1	1	CTCAACATTGCA**A**CTGAT
8	1	CTCAACATTGCA**G**CTGAT
56	3	CTCAA**T**AT**C**GC**C**TCTGAT
2	3	CTCAA**T**AT**C**GC**T**TCTGAT
19	3	CT**T**AACATTGC**C**TCTGA**A**
28	4	CT**T**AA**T**AT**C**GC**C**TCTGAT
1	5	CT**T**AA**T**AT**C**GC**CC**CTGAT

Plant material allegedly infected by all the targeted pathogens (Table 1 and 3) was obtained from the Foundation Plant Services (FPS) and the Clean Plant Center Northwest (CPCNW) introduction pipelines. Both collections include foreign and domestic selections of *Prunus* and pome fruit trees. In total, 214 samples (Table 4) were obtained and included in the pathogen screening via high throughput sequencing (HTS). As a result, multiple isolates were identified for all the pathogens (Table 5), with the exception of NVM, PcMV, CRMaV, ToRSV, TRSV and ARWV 1 & 2 with a limited number of detected isolates (i.e. one isolate for each virus).

Table 4. Fruit tree samples analyzed by high throughput sequencing (HTS).

Fruit Tree	Number of Samples
Almond	3
Apple Rootstock	17
Apricot	2
Cherry	35
Cherry Rootstock	5

Miscellaneous Prunus Species	12
Nectarine	8
Peach	34
Pear	21
Pear Rootstock	5
Plum/prune	22
Apple	50

Table 5. Number of isolates obtained for each targeted pathogen of fruit trees.

Acronym	**Number of Isolates**
ACLSV	24
AGCaV	18
AHVd	8
ApMV	5
ASGV	24
ASPV	39
ASSVd	2
ARWV 1 & 2	1
ArMV	2
CGRMV	9
CLRV	2
CNRMV	4
CRLV	2
CRMaV	1
CVA	24
CCGaV	3
LChV-1	3
LChV-2	3
NSPaV	2
NVM	1
PcMV	1
Pear decline phytoplasma	2
PBCVd	8
PBNSPaV	10
PDV	16
PNRSV	19
ToRSV	1
TRSV	1

Objective 2:
All the new HTS data generated during this fiscal year was analyzed using the FPS' in-house bioinformatics pipeline; such pipeline is a local UNIX server based high throughput viral meta-genomics pipeline. As a result of this analysis, several complete and near-complete genomes of viruses and viroids were obtained from pome and *Prunus* tree samples. These sequences included ACLSV, ApMV, ASSVd, ASGV, ASPV, PBCVd, PNRSV, LChV-1, LChV-2, PBNSPaV and CGRMV. We are in the process of submitting this new sequence data to GenBank. The addition of new genetic data will result in a more complete characterization of genetic variation across the targeted pathogens.

Objective 3:
Overall, 24 new or updated real-time qPCR have been developed during this study (Table 6). Additional primers or probes were added to the previously published ACLSV, CRLV, LChV-1, PDV, ToRSV and ApMV assays in order to cover all the known genetic diversity of these pathogens (i.e. variants); one probe or one primer was added when more than 2 nucleotide mismatches were detected during the sequence comparison. Hence, adjustments to these assays mainly involved one extra probe or up to two extra primers.

Table 6. Updated or newly designed assays for detection of pathogens infecting fruit trees.

Pathogen	Type of Primer	Number of Primers	Target Region	Reference	Note
ToRSV	Forward	2	CP/Polyprotein	Osman et al. 2014	Updated
	Reverse	2			
	Probe	1			
ArMV	Forward	2	CP/Polyprotein	This study	
	Reverse	1			
	Probe	2			
ApMV	Forward	4	CP	Osman et al. 2014	Updated
	Reverse	2			
	Probe	2			
ACLSV	Forward	1	CP	Osman et al. 2017	Updated
	Reverse	5			
	Probe	2			
LChV1	Forward	4	CP	Katsiani et al. 2018	Updated
	Reverse	1			
	Probe	2			
LChV2	Forward	2	RdRp	This study	
	Reverse	1			
	Probe	2			
CRLV	Forward	1	RdRp		Updated

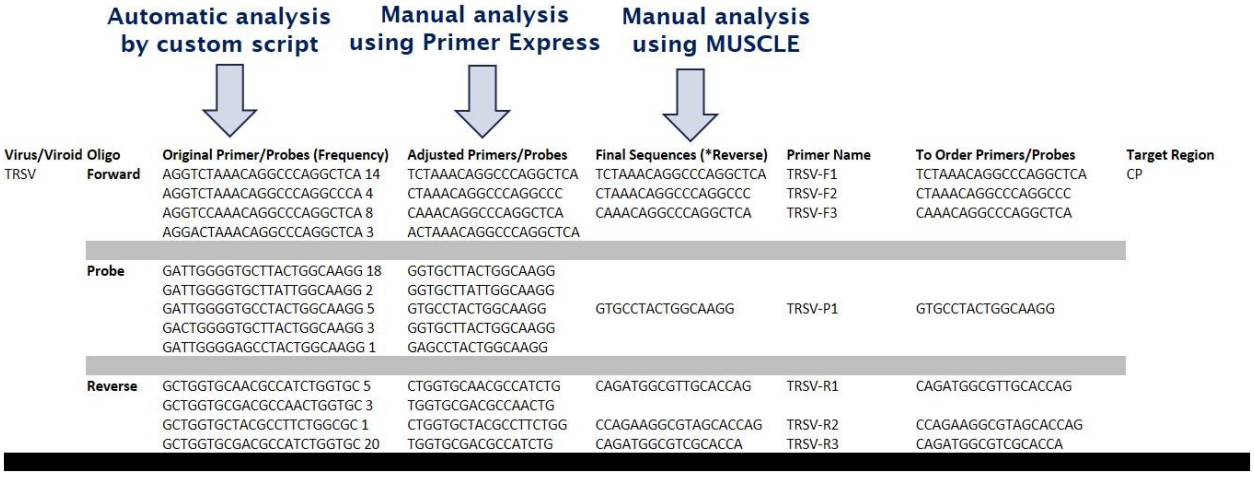
	Reverse	2		Osman et al. 2017	
	Probe	2			
PDV	Forward	4	CP	Osman et al. 2014	Updated
	Reverse	2			
	Probe	2			
PNRSV	Forward	4	CP	This study	
	Reverse	2			
	Probe	2			
CGRMV	Forward	2	TGB1	This study	
	Reverse	1			
	Probe	1			
PBCVd	Forward	3	Viroid genome	This study	
	Reverse	4			
	Probe	2			
ASSVd	Forward	5	Viroid genome	This study	
	Reverse	4			
	Probe	2			
ASPV	Forward	5	CP	This study	
	Reverse	5			
	Probe	2			
TRSV	Forward	3	CP	This study	
	Reverse	3			
	Probe	2			
ASGV	Forward	2	MP/Polyprotein	This study	
	Reverse	1			
	Probe	1			
PBNSPaV	Forward	1	3' UTR	This study	
	Reverse	1			
	Probe	1			
CVA	Forward	3	CP	This study	
	Reverse	2			
	Probe	2			
AHVd	Forward	3	Viroid genome	This study	
	Reverse	3			
	Probe	1			
NVM	Forward	1	RdRp/Polyprotein	This study	
	Reverse	2			
	Probe	1			
NSPaV	Forward	2	CP	This study	

	Reverse	2			
	Probe	1			
CNRMV	Forward	1	CP	This study	
	Reverse	1			
	Probe	1			
ARWV 1&2	Forward	2	RdRp	This study	Multiplex
	Reverse	2			
	Probe	1			
CRMaV	Forward	2	CP	This study	
	Reverse	2			
	Probe	1			
PcMV	Forward	1	RdRp	This study	
	Reverse	2			
	Probe	1			

Given the new sequence data available in GenBank, the *in silico* analysis revealed that the genomic regions targeted by the published LChV-2, CNRMV, CVA, PNRSV, ASGV, ASSVd, CGRMV and PBNSPaV assays are not completely conserved as previously thought. As consequence, novel assays were designed amplifying a different region (Table 6).

Finally, for NSPaV, NVM, AHVd, ASPV, ArMV, PBCVd, TRSV, CRMaV, PcMV and ARWV 1 & 2 not previous real-time PCR assay is available. Thus, using a custom script and a multi-step process (Figure 2), real-time qPCR assays were developed for such viruses and viroids (Table 6). This script identified potential candidates for primers/probes, later, candidate primers/probes were adjusted according to the parameter for TaqMan real-time qPCR (MGB probes).

Figure 2. Design of novel real-time qPCR assays. Example TRSV.



Objective 4:
All the updated or novel assays for pathogens included in Table 6 were challenged using infected material (Table 5) and healthy plants (i.e. virus free). As a result of this initial validation, these assays specifically and efficiently detected the different targeted pathogens, generating Ct values from 12 to 29. Thus, samples with known pathogen infection by HTS, tested positive during the screening using the real-time qPCR assays.

During the Summer 2019, 333 tree samples were collected at the USDA National Clonal Germplasm Repository located near Winters, California. This is a *Prunus* germplasm collection of worldwide origin and contains approximately 4,000 *Prunus* trees representing different accessions. Trees in this collection include almonds, apricots, cherries, peaches, plums and nectarines. We screened this *Prunus* tree population using the improved real-time qPCR assays; as a result, ACLSV, CGRMV, CNRMV, CVA, LChV-1, LChV-2, NSPaV, NVM, PcMV, PBNSPaV, PDV and PNRSV were identified.

Objective 5:
Preliminary results have been presented during growers' meetings organized by the UC Cooperative Extension, and scientific meetings organized by the California Department of Food and Agriculture (CDFA). This work is especially timely as the development of these more robust fruit trees pathogen detection assays concurs with recent changes to the CDFA Pome Registration and Certification Program in anticipation of creating a program at FPS that harmonizes with other state's pome industries. In that sense, FPS is working closely with the CPCNW to standardize the testing process of new domestic and foreign fruit tree introductions.

In addition, the novel detection tools will be shared with diagnostic labs involved in the fruit tree industry in the US, including the National Clean Plant Network. Consequently, any assay we develop as a result of this project will be made available to CDFA and private commercial diagnostic labs and will augment the production of certified propagation material and the effective control of fruit tree pathogens in California and beyond. Finally, a scientific article describing several of these improved assays was recently published ("Comprehensive real-time RT-PCR assays for the detection of fifteen viruses infecting *Prunus* spp.".

Summary:
This project will evaluate the broad-range detection capacity of currently available pome and *Prunus* fruit tree virus, viroid and phytoplasma real-time qPCR assays and update or design new assays if current assays are inadequate or absent. HTS will be used to screen select pome and *Prunus* tree populations for targeted pathogens. The CDFA is currently working to update the Pome Fruit Tree Registration and Certification regulations in order to create regulations that are harmonized with other state's pome industries. Current detection methods for viruses (and other pathogens) identified as targeted viruses by the pome fruit working group, in addition to primary *Prunus* viruses, will be investigated. Our objectives are to screen select pome and *Prunus* tree populations for targeted viruses, viroids and phytoplasma to compile a representative set of isolates, evaluate current published assays, incorporate new genetic data into a more complete characterization of genetic variation across the targeted pathogens to inform assay design, construct improved assays utilizing multiple primers/probes sets for detecting all existing

targeted pathogen variants, empirically test and validate proposed assay designs using positive controls, and disseminate research progress and results. The overarching goal of this work is to design the most robust assays for virus, viroid and phytoplasma detection of pome and *Prunus* fruit trees which will contribute to maintaining the highest quality nursery stock.

Progress report on a research grant proposal to: Fruit Tree, Nut Tree and Grapevine Improvement Advisory Board (IAB)

Project Title: Testing rootstocks of perennial crops for resistance to *Meloidogyne floridensis*, a new species in California.

Project Leader: Andreas Westphal, Department of Nematology, UC Riverside,

Project duration: 2019-2020 Progress Report

This project was aimed at determining the host status of some representative plant lines of walnut, almond, pistachio, and grape toward the newly discovered *Meloidogyne floridensis.* Because of the current quarantine status of the nematode, these greenhouse experiments were conducted under containment conditions at the UC Riverside main campus. The project was encumbered by the Covid-19 pandemic but now is almost complete. One experiment was completed, and a repeat of that is scheduled to be harvested in May/June 2022.

Executive Summary – Rootstocks resistant to root-knot nematodes are a critical management tool against infestations with these soil-dwelling parasites. The high level of resistance to southern root-knot nematodes in peach rootstock 'Nemaguard' has protected plantings of *Prunus* crops, including almonds and stone fruit. This durable resistance has been effective for decades. Further introgression, some in crosses with almonds, has broadened this resistance utility. Recently, *Meloidogyne floridensis*, the peach root-knot nematode (PRKN), was first described from California almond orchards (Westphal et al., 2019). This nematode species has been damaging *Prunus* plantings in Florida for 10-20 years. It can overcome currently deployed resistance against root-knot nematodes in the California commercial rootstock cultivars. Namely, it was found to damage 'Nemaguard', 'Hansen536', and 'Bright's Hybrid 5'. The problem of overcoming currently used resistance in perennial crops is foremost recognized in *Prunus*. Root-knot nematodes typically have wide host ranges, and it is important to know if other perennial crops could be infected by PRKN. At the initiation of this study, there was little information available if grape is a host. Similarly, walnut and pistachio were only poorly characterized.

Objectives

It was the objective of this study to determine the host status to PRKN of *Prunus, Juglans, Pistacia*, and *Vitis*. For Prunus, four breeding lines along with six commercial rootstocks were entered into the testing. For grape, there were three commercial lines, for pistachio, two commercial lines and two breeding lines, and three commercial lines for walnut.

Two greenhouse experiments were conducted. At the Kearney Agricultural Research and Extension Center (KARE), two experiments with four plants of each plant line were planted, one in October 2020 and the second one in November 2020. Liners of the genotypes under investigation were planted in 3-L pots with sandy-sandy loam soil mix with organic matter amendment. Plants were arranged in a lathhouse and were allowed to enter dormancy. The potted plants were transported to the UC Riverside Nematology Quarantine and Containment facilities the following spring. The experiments were placed on greenhouse benches in a randomized complete block design with four replications. A drip irrigation system was installed. The soil in each pot was infested with second-stage juvenile (J2) suspensions of PRKN by pipetting the equivalent of 3,000 J2 into three 1-inch deep depressions surrounding the rootstock. The J2 were obtained from greenhouse cultures on vegetable hosts and had been molecularly confirmed to be *M. floridensis.*

During greenhouse incubation at approximately 25˚± 3˚C and ambient light, plants were fertilized and watered as needed. Occasionally, they were pruned to allow for uniform growth space of the different plant species. After six months of incubation, the plants were uprooted, roots shaken free of soil and carefully rinsed. Nematode-induced galls were counted. To aid in counting the eggmasses, the entire root systems were submerged in erioglaucine solution overnight before stained eggmasses were enumerated. Accessions with no galling or eggmasses on their roots were tentatively classified as resistant. In Prunus, a simple gall rating/counting was sufficient for the host response classification (Maquilan et al., 2018), but other plant species may respond differently. Thus, galling assessment and eggmass counts were used in combination for host response assessments. The entire experiment was completed once, and the second experiment is scheduled for harvest and evaluation in May/June.

Results

Data are presented as box plots following non-parametric analysis using Mood's Median separation test at $P = 0.05$. Only data of one greenhouse experiment were illustrated, experimental lines were coded because these are only putative results that need confirmation by a duplicate experiment. There were plant top weight differences at harvest, one of the pistachio accessions having the highest weight, followed by prunus 'Marianna 2624' (M2624) and walnut 'AX1' (Fig. 1). Roots of three prunus lines (Expt.1, Krymsk 86, and Nemaguard) galled the most severe, followed by four lines ('BB106', 'Hansen 536', 'Lovell', and 'Viking') with fewer galls (Fig. 2). Two prunus lines (Expt.3, 'Marianna 2624') had no galls, and one had just a few (Expt.4). One line each of the grapes ('Zinfandel'), pistachio (Expt.14), and walnut ('VX211') had some galls but at low levels (Fig. 2). Eggmass counts corroborated the root galling information with the exception of pistachio, in which virtually no eggmasses were detected (Fig. 3). Galling on lines resistant to southern root-knot nematodes was severe (Fig. 4).

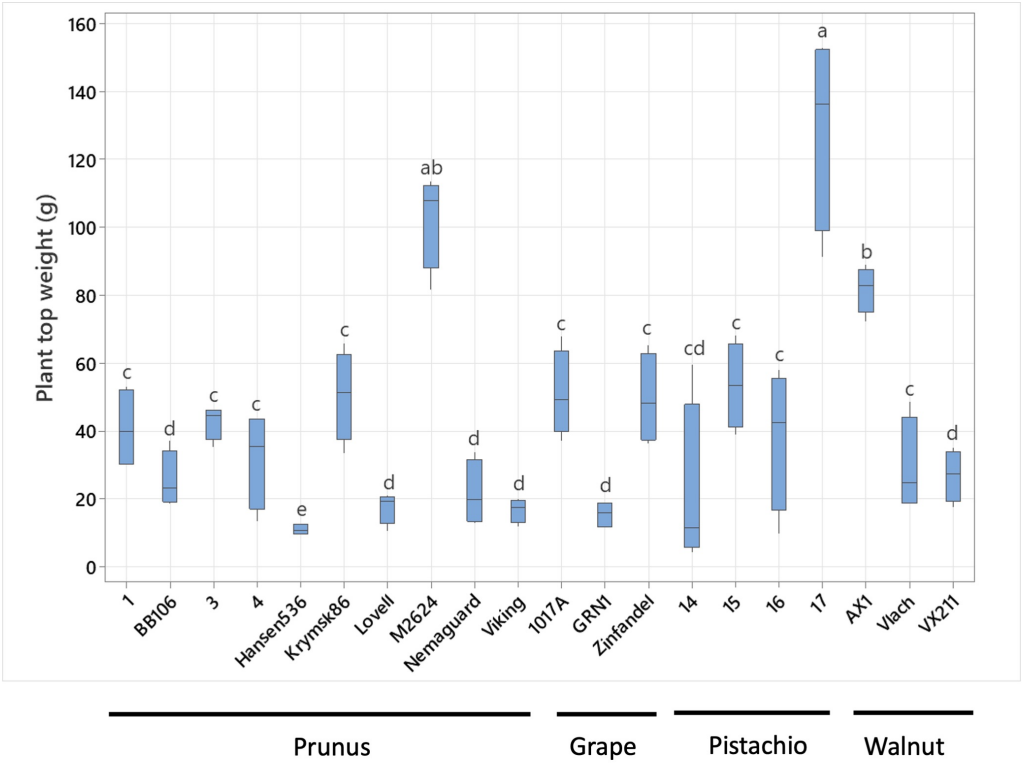


Fig. 1. Top plant weight of prunus, grape, pistachio, and walnut that grew 6 months in *Meloidogyne* floridensis-infested soil in a greenhouse experiment. Boxes with the same letter were not significantly different when tested with Mood's Median separation test at $P = 0.05$.

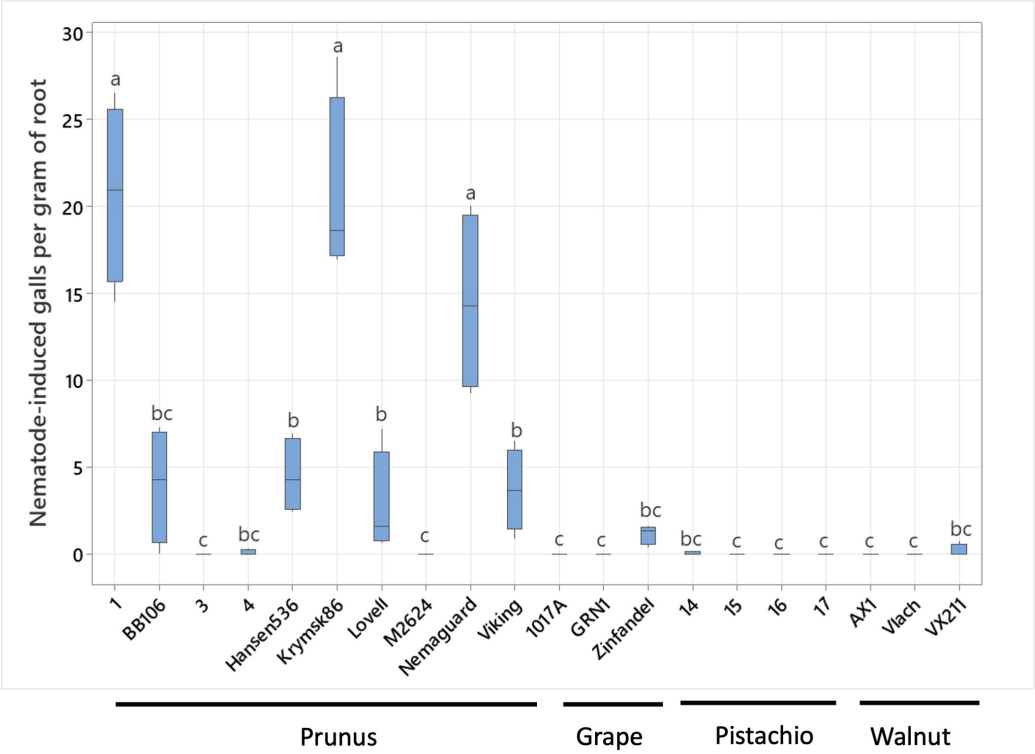


Fig. 2. Nematode-induced galling of prunus, grape, pistachio and walnut that grew 6 months in *Meloidogyne* floridensis-infested soil in a greenhouse experiment. Boxes with the same letter were not significantly different when tested with Mood's Median separation test at $P = 0.05$.

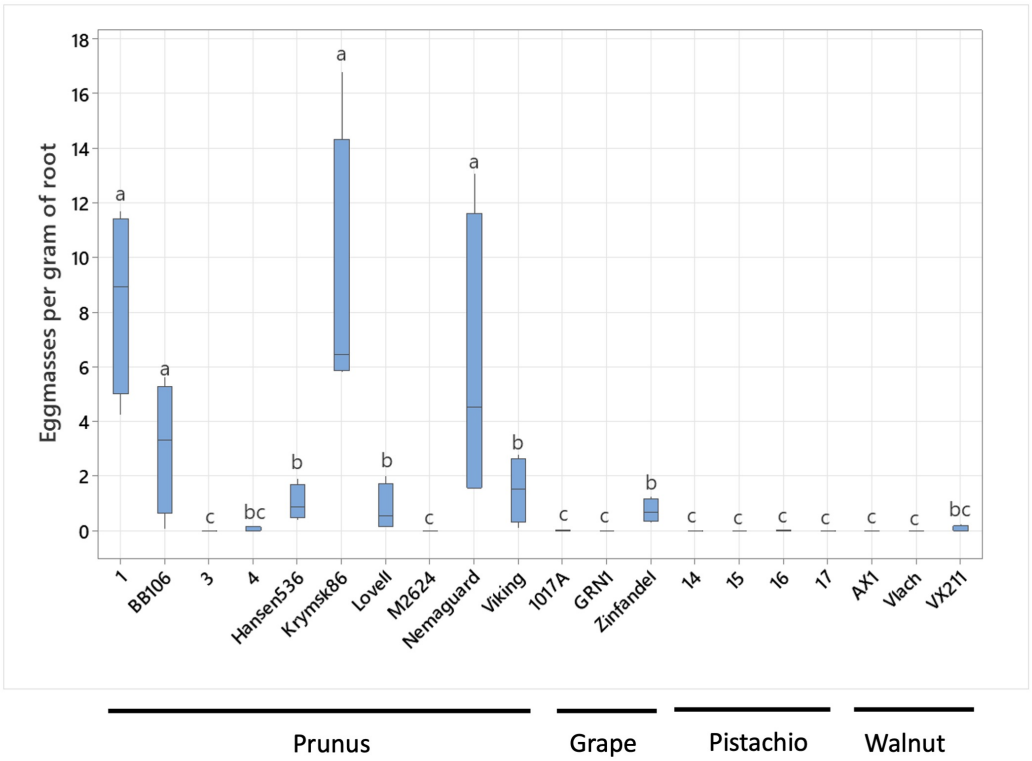


Fig. 3. Eggmasses on roots of prunus, grape, pistachio and walnut that grew 6 months in *Meloidogyne* floridensis-infested soil in a greenhouse experiment. Boxes with the same letter were not significantly different when tested with Mood's Median separation test at $P = 0.05$.

Fig. 4. Peach root-knot nematode induced galling on 'Nemaguard' rootstock resistant to southern root-knot nematodes (Photo: Z.T.Z. Maung)

Discussion

In this experiment, *Meloidogyne floridensis* infected prunus lines the most aggressively. Several prunus breeding lines that had lower infection and reproduction levels. Foremost, two lines did not show symptoms nor had any eggmasses after inoculation with *M. floridensis*. This provided some potential for finding resistance in prunus rootstock material grown in California. In grape, one of the lines was susceptible to *M. floridensis.* In pistachio, there was some galling detected, but nematode infection and reproduction were not corroborated by eggmasses. In walnut, there was some galling and also some eggmass detection. The initial hypothesis that more perennial crops may harbor lines that are susceptible to *M. floridensis* needed to be accepted. The risk for damage to these crops exists, and a better understanding of how widespread this nematode species occurs is urgently required.

The peach root-knot nematode, *Meloidogyne floridensis*, is a production problem in peach in Florida. It was first discovered in 1966 on peach rootstocks that otherwise were known to be resistant to multiple root-knot nematode species (Maquilan et al., 2018). This species was initially considered a population of the southern root-knot nematode until it was described as a separate species (Handoo et al., 2004). This nematode is a major production restrictor in Florida, because it infects the current preferred rootstocks 'Nemaguard', 'Nemared', 'Okinawa', and 'Guardian'. Intermittently, there seemed some hope that 'Flordaguard' would protect from *M. floridensis*, but soon nematode populations were identified that overcame that type of resistance.

More recently, *M. floridensis* has been confirmed in South Carolina and Georgia, making it a possibly widespread production risk. In the Florida stone fruit rootstock breeding program, resistance was identified in *Prunus kansuensis*, a species used as seed rootstock in its origin in North-west China. The here putatively resistant prunus line does not contain this species as a parent, and it will be of utmost curiosity to corroborate this plant response. It remains unknown how the populations of *M. floridensis* in California relate in terms of virulence with populations in Florida. Mining the California germplasm pool for resistance to *M. floridensis* seems fruitful when developing the "next generation" of rootstocks. Public and private breeding material should be tested against this nematode if further distribution of the nematode is confirmed.

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Progress report on a research grant proposal to: Fruit Tree, Nut Tree and Grapevine Improvement Advisory Board (IAB)

Project Title: Development of an Armillaria resistance screen for clonal walnut rootstocks

Project leader: Pat J. Brown, Chuck L. Leslie, and Wes Hackett

Project duration: 2019-2020 Progress Report

Objective 1: Using paradox wood chips as a matrix in vitro, we tested the growth of Armillaria mellea walnut strain Yolo 308 using water, potato dextrose medium (PDM) or sucrose (3%) as additives. Observations after incubation at 25C in fluorescent light(75-125 umol/m-2/s-1) or dark for eight weeks indicate that growth of mycelium and rhizomorphs is no faster on 3% sucrose than on PDM or water and if anything slightly slower. Both mycelium and rhizomorphs grew better in the dark than in light on all three additives. These observations indicate that the rapid growth of Armillaria mellea mycelium and efficient infection of rooted walnut microshoots on Driver and Kuniyuki Walnut medium in vitro is not likely to be due to the high concentration of soluble carbohydrates (3% sucrose) in this medium. The observations do indicate that it might be advantageous to exclude light from the inoculation medium while trying to infect plant roots with Armillaria mellea. In another experiment performed in vitro, we compared the growth of Armillaria mellea Yolo 308 on paradox wood chips using water or PDM as additives that had been autoclaved for 30 minutes or on similarly treated chips that had not been autoclaved. After inoculation, cultures were incubated in the dark at 25C for eight weeks. Observation at that time, showed that autoclaved cultures had vigorous growth of mycelium and rhizomorphs while not autoclaved cultures had no evidence of Armillaria growth but did have growth of other microorganisms in the wood chip matrix. These observations have two possible explanations: 1. Armillaria mellea strain Yolo308 is suppressed by fungal and /or bacterial contaminants growing in the not autoclaved paradox wood chips or 2. Autoclaving releases some substance(s) required for growth of Armillaria mellea but not for growth of contaminating fungi or bacteria. If explanation #1 is correct, these observations suggest that one reason infection of roots of walnut microshoots in vitro is fast and efficient is the absence of competing microorganisms in vitro. We have observed with rooted pear microshoots infected with Armillaria mellea in vitro that growth of mycelium is completely inhibited by the presence of bacterial contamination.

Objectve 2: Using Magenta GA7 clear plastic, autoclaveable containers, we have developed a system for inoculating soil grown liner sized clonal walnut plantlets using paradox wood chips colonized with Armillaria mellea strain Yolo 308 mycelium and rhizomorphs. This involves modifying the Magenta container by drilling a 3/16 inch drain hole in the bottom which is initially plugged with silicone glue. The Magentas are filled with paradox wood chips using a sleeve like form to localize the chips in the periphery of the Magenta and to leave a tapered hole that accommodates the plantlet soil-root ball or roots when inserted at the time of inoculation. The chip filled Magentas are saturated with water and autoclaved. After autoclaving they are inoculated with a measured amount of Armillaria mellea strainYolo308 in water or PDM. The inoculated, paradox wood chip filled Magentas are incubated in the dark at 25C for up to eight weeks. At the time plants are to be inoculated, the silicon plug is pulled out of the drain hole and the form is removed from the Armillaria colonized chips. The plantlet soil-root ball or bare roots can then be inserted into the hole surrounded by chips filled with mycelium and rhizomorphs. Inoculated plantlets are grown in a controlled environment room with temperature at

24C, 85- 95% relative humidity and 16 hours of light (250-300umol/m-2/s-1) from sodium vapor lights. By using an aluminum foil wrap, the inoculated chips and plant soil root ball can be shielded from light or not. This system allows easy non-destructive observation of growth of mycelium and rhizomorphs as well as plantlet roots over time.

Objective 3: Four experiments have been initiated with the paradox wood chips inoculation system described under Objective Two above. One of these experiments has been completed with data taken eight weeks after inoculation. These observation indicate that much of the Armillaria mellea Yolo 308 mycelium in the wood chips has developed into thick dark fungal cells (black lines) called zone lines which may play role in protecting Armillaria from unfavorable environmental conditions or other fungi. White plantlet roots had grown into the Armillaria colonized paradox chips and in many case out the drain hole. There was little or no evidence of mycelium or rhizomorphs growing into the plantlet soil root ball. There were no root or crown disease lesions (mycelial plaques under the bark) apparent and no symptoms of stress in the leaves or stem except that some of the leaves had yellowed and ultimately abscised. None of the plants were dead after eight weeks and have continued to grow up to the present. Samples of the smallest white root tips in the paradox chips and soil were collected for analysis for evidence of Armillaria mellea strain Yolo303 DNA using qPCR technology by Dr. Kendra Baumgartner. This analysis provided evidence of strain Yolo303 DNA in in roots of samples of 10 of the 22 inoculated plants and none of the 10 control non-inoculated plants. In a second experiment, observations at 12 weeks after inoculation were very similar to those described above except that mushrooms up to the size of a dime were growing on the top surface of the soil root ball and paradox wood chips in both Armillaria inoculated and control Magentas. According to Dr. Kendra Baumgartner, the mushrooms observed were not from Armillaria species. Results of qPCR analysis for the presence of strain Yolo303 DNA in the roots are not complete so we do not know about the rate of infection if any. In two other ongoing inoculation experiments, we've observed that the development of mycelial into zone lines begins very soon after insertion of soil root balls into the strain Yolo303 colonized paradox chips. The observations described above, suggest that the Yolo strain303 of Armillaria mellea is very sensitive to the controlled environment being used or to the competition from microorganisms being introduced on the plantlet soil root ball. We have an ongoing experiment to try to distinguish between these possibilities.

Based on the results and observations summarized under Objectives one and two, we tentatively conclude and hypothesize that: 1. Yolo 303 strain of Armillaria mellea is very sensitive to competition from microorganisms carried on the paradox wood chips used as a matrix and substrate for growing inoculum and those being introduced on the plantlet soil root ball we are trying to infect and 2.the main reason that Armillaria mellea mycelium growth in vitro is very vigorous and infection of rooted walnut microshoots is highly efficient is due to the sterile conditions provided. Therefore, in order to reach our goal of devising a method for screening for genetic resistance to Armillaria root disease, we need to: 1. Identify a more virulent or competitive strain of Armillaria mellea and/or 2.develope procedures for reducing or suppressing the population of microorganisms that exist on the soil root ball or roots of the plantlets being screened for resistance.

Final report on a research grant proposal to: Fruit Tree, Nut Tree and Grapevine Improvement Advisory Board (IAB)

Project Title: Rootstock improvement

Project Leader: Tom Gradziel Department of Plant Sciences, UC Davis

Project duration: 2019-2020

A. Summary

Notable achievements for 2020 include the evaluations for vigor/robustness and disease tolerance for over 300 genotypes including the identification of species sources conferring potential resistance as well as selection of promising individuals within those sources for propagation to allow advanced testing and use as parents for the next round of hybridizations. Based on similar field and lab-based assessments from 2019, over 860 interspecies hybrids and over 210 backcross and self-progeny were generated among selected peach, plum and almond parents with harvested seed currently being prepared for greenhouse planting. Fifty of the most promising earlier interspecies selections involving crosses among peach, almond and plum species were clonally propagated to allow replicated testing by cooperators of a predicted resistant subset (based on preliminary evaluations) of these selections for nematode, phytophthora, oak root fungus and salinity, as well as in separate field test-plantings in our UCD disease evaluation plots for oak root fungus, crown gall and waterlogging. To accelerate breeding progress, we are pursuing a modular 'plug-and-play' type breeding strategy where parents are selectively chosen to contribute specific resistance/tolerance to a recurrent California-adapted rootstock. This approach would allow more strategic flexibility in pursuing specific rootstock-site solutions. For example, current goals include the introduction of ring nematode resistance to an otherwise well-adapted Hansen-type rootstock for regions in the San Joaquin where ring nematode is becoming a problem, and the introduction of root knot nematode immunity to a Krymsk-type rootstock otherwise well adapted to the heavier soils typical of the Sacramento Valley. Success depends on our ability to identify, characterize and incorporate major resistance/tolerance genes such as the *Mi* genes for root-knot nematode immunity. A crucial breeding obstacle has been our capacity to generate the large numbers of progeny required for genomic characterization and subsequent trait incorporation for these inherently difficult interspecies crosses and introgression lines. We have been able to largely overcome this barrier through appropriate choice of breeding parents and crossing strategy combined with the use of embryo-rescue for particularly difficult hybrids such as plum by almond. The remaining barrier was the challenge of clonally propagating these complex interspecies hybrids for the necessary replicated regional trials. This has been overcome to a degree through the selection and refinement of specialized propagation methods, ranging from air-layering to sterile shoot culture, depending upon species hybrid background. Individual selections and populations segregating for resistance are currently being evaluated by ourselves and cooperators for multiple traits including resistance/tolerance to root-knot and ring nematode, oak root fungus, phytophthora, high soil and/or irrigation water salinity, waterlogging, wood rots, drought, heat tolerance and hybrid vigor. Breeding populations segregating for potential resistance/tolerance genes have been made available to other public and private breeders to accelerate overall rootstock breeding progress for California almond. In addition, public and private breeders are given breeding access (i.e. use as parents for future

crosses) of UCD released rootstocks so that this increased availability of advanced resistance germplasm might further facilitating statewide progress in rootstock development and deployment. *[In 2019/2020 this project was jointly funded by IAB and the almond board of California (ABC) with ABC assuming or funding in 2021].*

B. Objectives

Develop a series of specialized rootstocks possessing the specific combination of essential traits for continued profitable production in the diverse California almond production environments as well as enabling expanded production into previously marginal soils and climates.

C. Annual Results and Discussion

Three main activities dominate breeding efforts: a) generate breeding populations; b) grow and select the most promising seedling for further testing, and c) propagate selected genotypes for replicated, regional evaluations. The core of the breeding operation: generating recombinant populations, takes place during the very narrow window of crop bloom which can be further limited by inclement weather. Flowering and fruit-set conditions in spring, 2020 were relatively good allowing very good seed recovery from these inherently difficult crosses (Table 1). Seed have been cleaned and harvested and are about to be stratified for spring 2021 field planting. Plum seed, including plum by almond and plum by peach hybrids were shipped to Clemson University immediately after harvest to allow embryo-rescue and culture required for the in-vitro screening for oak root fungus resistance as described below.

Table. 1 2020 Hybridizations

	Number seed (From all crosses)
Almond by peach	527
Almond by plum	64
N emaguard by almond	21
Plum by almond	53
Plum by peach	18
Multi-species cross	179

Over 50 promising selections were propagated, both in-house and with cooperating nurseries, and provided to regional cooperators for intensive disease and environmental stress testing as summarized in Table 2. To meet the needs of all cooperators, some advanced selections required over 50 clones per selection to be propagated, which presented particular challenge as many of the wide species-crosses proved recalcitrant using traditional methods such as hardwood cuttings (see publication 1). Through trial and error, and by tapping into nursery expertise, we were able to develop strategies to successfully propagate the desired numbers for most clones allowing this diverse germplasm to begin thoroughly and expert evaluation. In 2020, we also finished the first round of in-field UCD evaluations for oak root fungus, crown gall and asphyxia from saturated soils and receive the first round recommendations from nematode trials run by Andreas Westphal, which will be the focus of this progress update.

Figure 1. Generalized rootstock breeding strategy with 2020 progress for nematode resist.

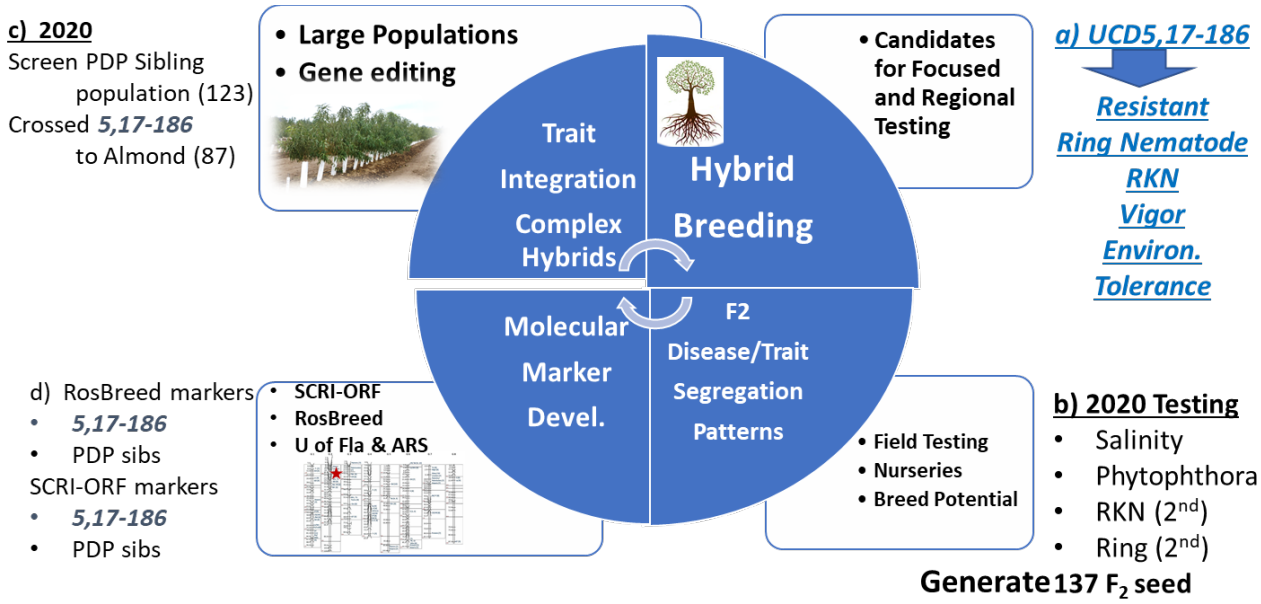


Figure 1 plots the generalized breeding plan being used for UCD rootstock breeding with examples from the 2020 nematode resistance program. The modular nature of this strategy allows us to quickly develop candidate rootstocks for different resistance goals while concurrently generating next-generation progeny which allow both further improvement of that rootstock line (such as incorporating regional adaptability or additional resistance), as well as providing segregating progeny populations with sufficient quantity and genetic quality to identify molecular-markers for that resistance, (thus allowing more efficient selection in the next round of breeding). For example, selection UCD5,17-186 was identified as showing promising levels of resistance to both root-knot (RKN) and ring nematode. [Earlier research had shown peach and related species to be the most promising source of nematode resistance (Figure 2)]. The seed parent for UCD5,17-186 was a (peach by *P. davidiana*) species hybrid which appeared to inherit RKN resistance from the *P. davidiana* parent. (While the RKN immune rootstock Nemaguard is usually considered a peach, our previous molecular analysis has shown that RKN immunity was more likely derived from *P. davidiana*). For the recurrent peach parent we used the variety *Loadel* which was an open pollinated seedling of *Lovell* peach which is reported to have resistance to ring nematode. We had also advanced

UCD5,17-186 for screening by the Westphal lab because it had shown desirable levels of both vigor and environmental tolerance (including drought tolerance) during initial seedling evaluations. Once promising nematode resistance levels were verified in 2019, we propagated an additional 57 clonal trees to allow a second round of nematode resistance testing while also allowing concurrent testing for tolerance to salinity and phytophthora (Figure 1.b). To facilitate molecular marker development, 137 F2 seed were generated to provide a population segregating for resistance, and an additional 123 seedlings were recovered from the original [Loadel peach x (peach x P. davidiana)] (PDP) cross to provide an additional population for molecular marker development as well as perhaps identify additional UCD5,17-186-like resistant rootstock candidates (Figure 1.c) but adapted to additional growing regions or climates (see publication 2). [The wide range in seedling tree vigor (measured as stem diameters) observed in this interspecies introgression, is shown in the lower graph of Figure 2 and ranges from highly vigorous to dead seedlings (blank spaces in graph)].

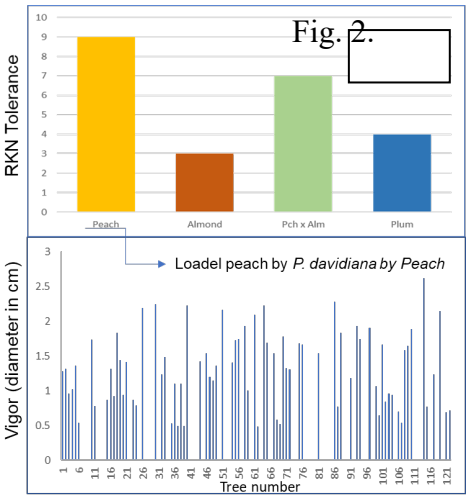


Fig. 2.

Finally, 5,17-186 was crossed with almond with the objective of selecting a Hansen-type rootstock having added resistance to ring nematode (Figure 1.c) with resultant seed now being prepared for planting and next-generation evaluation.

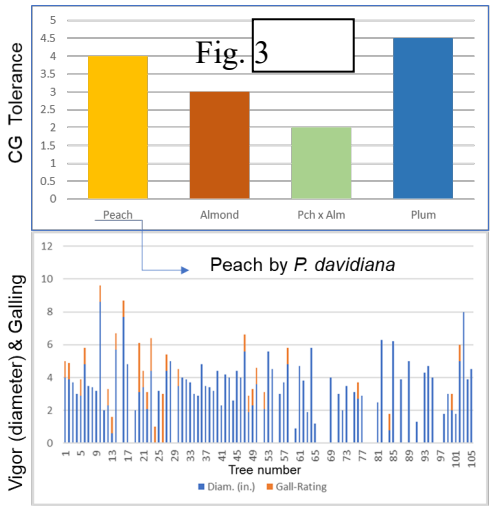


Fig. 3

The F2 seedling population has now been planted for future disease screening/marker development. Both 5,17-186 and its PDP sibs are currently being screened for RKN and ring nematode resistance and simultaneously for disease resistance markers based on earlier RosBREED marker analysis of these trees and more recently markers from the multistate Oak Root Fungus (ORF) rootstock breeding project recently funded by SCRI and based out of Clemson University. [The inset in (Figure 1.d) shows a recently compiled molecular map for almond (publication 3) where the red star designates the expected location (based primarily on peach studies) of the root not nematode immunity gene (*Mi*)].

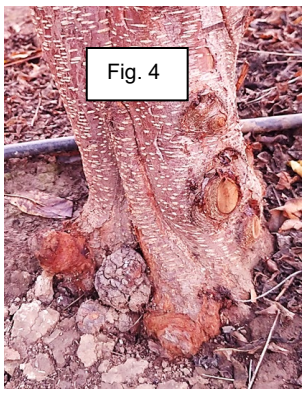
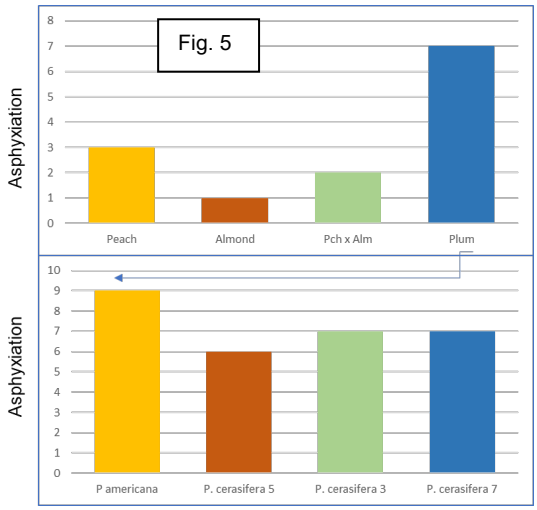


Fig. 4

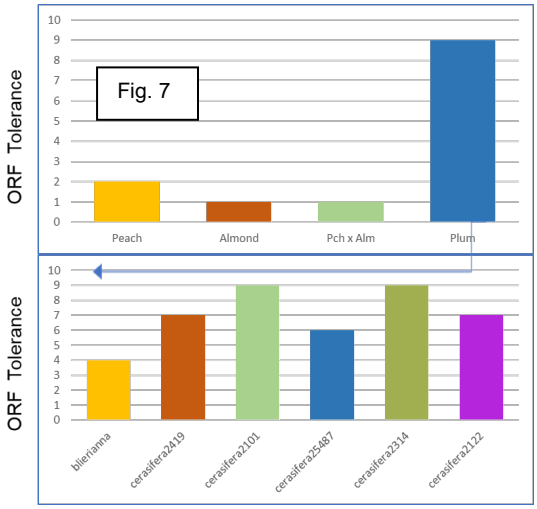
Crown gall. Previous research had shown that plum and, to a lesser extent, peach species offered the best opportunities for crown gall (CG) resistance (Figure 3). The Kluepfel lab had also identified the almond species *P. tangutica* as potentially harboring resistance. At our UC Davis crown gall evaluation plot, most peach and almond selections proved susceptible. A segregating peach by *P. tangutica* F2 population is so far not showing any symptoms of disease but neither are interplanted susceptible controls, perhaps because the planting was on the edge of the crown-gall hotspot. Several populations from peach by P. davidiana crosses appear to be segregating for crown gall as well as hybrid vigor (lower graph in figure 3 where higher gall rating indicates higher disease). Some

specific peach by P. davidiana crosses have resulted in progeny with exceptional levels of vigor; being comparable to that of peach by almond hybrids. Even when crown gall occurs in these more vigorous hybrids, it seems to be contained in its ability to do damage (Figure 4). Following additional field evaluations, the most promising individuals from these preliminary evaluations will be forward to the Kluepfel lab for more precise inoculation/evaluation. A complex almond-peach-P. tangutica cross in 2020 yielded 98 seed which, when grown out, will be evaluated for drought and salinity tolerance as well as possible crown gall resistance.



Resistance to Flooded Soils. Almond has been found to be very susceptible to root asphyxiation in saturated soils with peach being more tolerant. Plum rootstocks have shown the best tolerance both in field trials as well as controlled studies. In preliminary 2020 flood-tolerance evaluations, we subjected seedlings from 30 plum, peach, almond and interspecies crosses to fully saturated soils for a period of 30 days. As expected, plums, and in particular P. cerasifera types showed good tolerance, followed by peach by plum hybrids. Interestingly, a few of the almond accessions tested, demonstrated surprising levels of tolerance, though most almond quickly perished (Figures 5 and 6). Populations of *P. americana* not only tolerated fully saturated soils, but seem to prosper (Figure 6). In spring, 2020 we made a large number of crosses to almond and to a lesser extent peach using P. americana combined with P. cerasifera pollen targeting tolerance to ORF, saturated soils and possibly phytophthora. (Because of the difficulty in making almond by plum crosses, including the unknown cross-compatibility of different plum species, we typically bulk pollen from several desired species to protect against incompatible combinations and so ensure at least some seed-set. A total of 71 seed were recovered but the determination of their exact parentage will occur after the seedling stage based on plant morphology and molecular marker composition).

Oak root fungus (ORF). Plums remain the only proven source of resistance to ORF with peach, almond and their hybrids showing high susceptibility (Figure 7). Most traditional plum and plum hybrid rootstocks suffer from susceptibility to nematodes and boron toxicity as well as the risk of graft incompatibility with major cultivars such as Nonpareil (see publication 4). Earlier, 2018/19 collaborations between Davis and Clemson University identified two P. cerasifera USDA/ARS accessions *(2101 and 2314)*, as having very promising levels of resistance based on controlled *in-vitro* inoculation and disease screenings (Figure 8). In 2019



we were able to recover 12 seedlings from crosses of Guardian peach (which has resistance to ring, lesion and RKN nematodes as well as bacterial cancer) to accession *2101* as well as seed from controlled crosses between different resistant cherry species. In 2020 we were able to increase the number to 53 seed from P. cerasifera-2101 by almond and 18 seed from P. cerasifera-2101 by peach. In addition we were able to recover a number of seed from crosses between almond and bulked pollen from P. cerasifera-2101, and 2314 plus P. americana, though, as previously described, seed have yet to be germinated and individual parentage verified. Finally, in 2020 a multistate consortium, headed by Dr. Gasic at Clemson University and including our breeding program along with several other public Prunus breeding programs were successful in securing multi-year SCRI funding to be used towards developing improved rootstocks with ORF resistance.


Fig. 8.

D. Outreach Activities

Table 2. Test sites, traits evaluated and cooperators evaluating UCD rootstock selections.

Location	Trait	Cooperator	Material under evaluation	Species evaluated
KAC,Parlier	Ring, and Root-knot nematodes	Andreas Westphal	46 clones	a, b, d, dv, m, p, pl, t, w
UCR,Riverside	Salinity tolerance	Devinder Sandhu	30 clones	a, ar, d, f, m, p, s, t, w
UCD, Davis	Asphyxia	Bruce Lampinen	32 clones/pop.	a, d, dv, m, p, pl, t, w
KAC,Parlier	Phytophthora	Greg Browne	25 clones	a, ar, d, dv, p, pl, w
UF, Gainesville	Botryophaeria resistance	Jose Chaparro (UF)	40 clones, 108 F2 seedlings	a, b, f, m, pd, p, pl sp, t, tr, w
UCD, Winters Clemson, NC	Armillaria	Ksenija Gasic (Clemson)	45 clones	a, d, dv, m, p, pl, t, w
UCD, Davis	Crown gall	Dan Kluepfel	~200 seedlings, ~400 seed	a, d, m, p, t, w
Firebaugh	Effect on scion architecture	Wonderful Orchards	7 clones	a, dv, p
Esparto	Dryland culture	Andrew Langford	Almond seedlings	d, p
UCD, Winters	General architecture for high density plantings	Ian Thorpe	20 clones and ~400 seedlings	d, , f, m, p, w
Multiple	Replant decline	Various nurseries	20 clones & ~1000 seed	a, dv, m, p, s, t, w

Almond {P.dulcis} (d), Peach {P.persica} (p), P.argentea (ar), P.fenzliana (f), P.mira (m), P.webbii (w), P.bucharica (b), P.pedunculata (pd), Plum spp. (pl), P.tangutica (t), P.triloba (tr), P.davidiana (dv), P.scoparia (s).

E. Materials and methods

Genetic material. A diverse germplasm, including heirloom varieties, and related Prunus species and inter-species hybrids and introgression lines is being developed at the UCD almond breeding program as detailed in 2018 and 2019 annual reports. Hybridizations, introgression and general breeding methods. Breeding strategies,
including standard and modified intra-and interspecific hybridization methods as well as marker assisted breeding are routinely employed as detailed in 2018 and 2019 annual reports.
Nematode resistance. See methods in Andreas Westphal's annual report. Test plantings were also made with nursery cooperators in known root knot, ring and lesion nematode hotspots.
Salinity tolerance. See methods in D. Sandhu 2020 proposal.
Root asphyxia from saturated soils. My lab has used very rudimentary studies involving soil saturation where containerized plants would be submerged for 30 days (when Nonpareil seedlings used as susceptible controls began to show damage). At this point, containers were drained and 10 days later plants rated for damaged. Selections showing promise from this initial study will be more accurately evaluated by the Phytophthora and asphyxia screening facilities of Greg Browne at KAC, Parlier. See Browne proposal for methods.
Armillaria and Crown gall. Test plantings were made at known disease hotspots in Winters and Davis, respectively. Controlled laboratory inoculations/evaluation is also occurring at the Gasic lab at Clemson University for Armillaria.

F. Publications that emerged from this work

1. Johnson, E.P., Preece, J.E., Aradhya, M., Gradziel, T. Rooting response of Prunus wild relative semi-hardwood cuttings to indole-3-butyric acid potassium salt (KIBA). Scientia Horticulturae, Volume 263, 15. https://doi.org/10.1016/j.scienta.2019.109144.
2. Angela S. Prudencio, Raquel Sánchez-Pérez, TM Gradziel, Pedro J. Martínez-García, Federico Dicenta, Thomas M. Gradziel and Pedro Martinez Gomez.. 2020. Genomic Designing for New Climate-Resilient Almond Varieties . In: Chittaranjan Kole (Ed.) Genomic Designing of Climate-Smart Fruit Crops. ISHS Jhlpd68505c015976.
3. Felipe Pérez de los Cobos, Pedro J Martínez-García , Agustí Romero , Xavier Miarnau, Iban Eduardo , Werner Howad, Mourad Mnejja, Federico Dicenta, Rafel Socias i Company, Maria J Rubio-Cabetas, Thomas M Gradziel, Michelle Wirthensohn , Henri Duval, Doron Holland, Pere Arús , Francisco J Vargas and Ignasi Batlle. 2021. Pedigree analysis of 220 almond genotypes reveals two world mainstream breeding lines based ononly three different cultivars. Horticulture Research (2021) 8:11. https://doi.org/10.1038/s41438-020-00444-4.

4. Kourosh Vahdati, Saadat Sarikhani, Neus Aletà, Charles A. Leslie, Abhaya M. Dandekar, Mohamad Mehdi Arab, Beatriz Bielsa,, Thomas M. Gradziel, Álvaro Montesinos,, María J. Rubio-Cabetas,, Gina M. Sideli, Ümit Serdar, Burak Akyüz, Gabriele Loris Beccaro, Dario Donno, Mercè Rovira, Louise Ferguson, Mohammad Akbari, Abdollatif Sheikhi0, Mahmoud Reza Roozban, LJ Grauke, Keith, Kubenka, Warren Chatwin, Amandeep Kaur, Srijana Panta, Lu Zhang, Shawn A. Mehlenbacher, Xinwang Wang. (in-press). Physiological and molecular aspects of nut crops rootstock-scion interactions: current and future. CABI
5. Gradziel, T.M. 2020. Redomesticating Almond to Meet Emerging Food Safety Needs Frontiers in Plant Science, Volume 11, 12 June 2020. 89/fpls.2020.00778. https://doi.org/10.33
6. Gina Sideli, Ted DeJong, and Sebastian Saa. 2020. Almond Variety Program; The Continuum of Variety Development, Screening, and Evaluation. ABC Special Technical Report. 54 pages.
7. Katherine M. D'Amico-Willman, Chad E. Niederhuth, Matthew R. Willman, Thomas M. Gradziel, Wilberforce Z. Ouma, Tea Meulia, and Jonathan Fresnedo-Ramírez. (in Press) DNA methylation status is associated with divergent exhibition of non-infectious bud failure, an age-related disorder, in twin almonds. The Plant Journal.
8. Gradziel, Thomas M. and Jonathan Fresnedo-Ramírez. (2019). Noninfectious Bud-failure As a Model for Studying Age Related Genetic Disorders in Long-Lived Perennial Plants. Journal of the American Pomological Society 73(4): 240-253 2019
9. Gradziel T, B. Lampinen and J.E. Preece. (2019). Propagation from Basal Epicormic Meristems Remediates an Aging-Related Disorder in Almond Clones. Horticulturae 2019, 5(2), 28; https://doi.org/10.3390/horticulturae5020028