

**USDA National Clonal Germplasm Repository for Citrus and Dates,
(NCGRCD), Riverside, CA**

**Annual Report W6
CY 2019**

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Germplasm Holdings

Citrus: As of 2019-12-31, the Repository maintained a total of 1,633 accessions of the genus *Citrus*, related taxa, and citrus pathogens.

Dates: A total of 169 *Phoenix* accessions are maintained, represented by 666 inventory items (individual plants).

Germplasm Backup

Citrus: Citrus germplasm maintained in Riverside exists as both field plantings and as protected plants. The Givaudan Citrus Variety Collection (CVC) at the University of California Riverside maintains most of the Rutaceous genotypes in a traditional field planting. About 40 % of the genotypes exist in the protected, sanitized collection (PSC), which is maintained in a USDA APHIS-approved screenhouse. In 2008, due to the presence of the Asian citrus psyllid (ACP) in Southern California, all CVC accessions not represented in the PSC were propagated with the assistance of the nursey industry, and are maintained as potted plants in several APHIS-inspected greenhouses. Thus, all genotypes without regard to pathogen status are maintained as protected propagations.

Cryopreservation: Approximately 80% of the citrus PSC is now stored in liquid nitrogen at the National Laboratory for Genetic Preservation in Fort Collins, CO. Gayle Volk has demonstrated that citrus germplasm that has gone through therapy to remove pathogens has a greater success rate and/or viability, and therefore only the sanitized accessions are cryopreserved.

Research at the NLGRP has demonstrated that cryopreservation of date pollen is a viable option for long-term preservation in genebanks. In 2019, pollen from 12 male accessions was cryopreserved at NLGRP, and an additional 15 accessions in 2020. Cryopreservation of tissue cultured date palms has proven more challenging due to various factors.

Germplasm Acquisition, Sanitation, and Release

No new germplasm accessions were acquired during CY 2019. However, three new pathogen positives were acquired from John Hartung at the USDA ARS Exotic Pathogens of Citrus Collection (EPCC) to be used as positive controls in the NCGRCD pathogen-testing program. These include: Citrus yellow mosaic virus (CYMV) (1 isolate) and Citrus chlorotic dwarf virus (CCDV) (2 isolates).

Acquisition of new accessions (both citrus and dates) is done under a Plant Controlled Import Permit (PCIP) that requires a sanitation procedure consisting of therapy and pathogen testing. For citrus, 2 therapy methods are used; thermotherapy for heat sensitive pathogens and shoot tip grafting for heat tolerant pathogens.

A plant exploration trip to Vietnam is included in the five year project plan. Numerous contacts with Vietnamese scientists and officials have been established, and some progress in developing the plan has been made. However, the Covid-19 situation will delay the the achievemen of this goal. Discussions with the Australian National Botanic Garden have also not been fruitful to this point.

Germplasm Distributions

The pattern of distributions has changed over the years, for who is requesting material and what form of germplasm. Prior to 2008, international distributions typically accounted for approximately 2/3 of distributions. Since that time, the pattern has reversed and approximately 2/3 of distributions are now domestic. This shift is due to the large

increase in HLB and ACP research in the United States. Furthermore, there has been a significant increase in seed requests for HLB and ACP-associated research projects. Researchers conducting cultivar improvement using molecular technologies such as CRISPR, require seed embryos. Infrequently, seeds of unusual varieties are distributed to individuals that are not available as budwood. Domestic budwood distributions have increased in recent years due to NCGRCD's participation in the National Clean Plant Network - Citrus (NCPN-C). The NCGRCD fills requests for clean budwood from the minor citrus-producing states when a State level program is not in place. The majority of international distributions are also for clean source budwood, as NCGRCD supports clean stock programs in countries that do not have a sanitization program. There are a few international distributions for research in addition. Distributions of nucleic acid are becoming more common, especially as restrictions on movement of citrus leaf tissue have increased; CY 2019 saw the largest distribution of nucleic acid in the history of the unit.

Table 1. Citrus distributions in CY 2019.

Distribution Form	Orders	Order	Count
Budwood	39	272	2,497 budsticks
Seed Orders	38	132	43,474 seeds
Leaf tissue	6	30	25 leaves + 765 g
DNA	2	166	33,000 ul
Fruit	4	9	115 ct + 907 g
Total	89	609	

In CY 2019, date palm germplasm distributions consisted of sending pollen from 14 accessions to Gayle Volk, USDA-ARS National Laboratory of Genetic Resource Preservation, Fort Collins, CO, for cryopreservation research and leaf material from 9 accessions to Michael Purugganan, New York University, Abu Dhabi, for genomics studies.

Databases

NCGRCD maintains accession records in the GRIN-Global database, maintained by the National Plant Germplasm System (NPGS) Database Management Unit at ARS Headquarters in Beltsville, MD. Accession records are thus available to the user community world-wide. NCGRCD also maintains a local database in MS Access. Both databases are up to date regarding accessions. Actual plant inventory is current and up to date in the local database; however, it is currently not maintained in the GRIN database. The local database contains information including: management data used in day-to-day operations; quarantine and pathogen testing data; documentation of propagations; and therapy records.

Citrus Taxonomy

Citrus taxonomy is confusing; in addition to the traditional conflict between the Swingle and Tanaka systems, recent molecular work has called into question some long-held beliefs or concepts in citrus taxonomy. Drastic changes not only affects citrus germplasm conservation, but also has regulatory and economic/trade implications. With this in mind, Dr Melanie Schori, NPGS taxonomist, assembled an ad hoc committee for input concerning the clean up and update of the citrus taxonomy used in the GRIN system. Dr. Schori has been working through this process.

Facilities

The NCGRCD maintains federal facilities on land located on the University of California, Riverside campus and leased from the University of California (UC). A new lease was executed on September 09, 2019 and will expire on September 08, 2029 (10 years). In addition to the land fee, municipal fees for fire and safety are charged based on the square footage of the facilities.

Federal facilities include approximately 16,000 sq. ft. of APHIS-certified screenhouse (used for maintenance of the protected, sanitized collection); 6,050 sq. ft. of greenhouse space (used for propagations, maintenance of pathogen controls, and pathogen testing); 1,372 sq. ft. of headhouse space (work and storage); 850 sq. ft. of lab space; 88 sq. ft. of office/storage space; and 480 sq. ft. office trailer (also housing the PCR equipment). In addition, approximately 7,500 sq. ft. of greenhouse space is rented from the University and is used to maintain accessions that have not been sanitized and tested, and therefore are held under APHIS/CDFA quarantine. In CY 2016-2018, two UCR greenhouses (GH 16-50 and GH 16-46) were renovated using funds from the National Clean Plant Network (NCPN) that were awarded to Georgios Vidalakis specifically for this purpose. Both greenhouses are fully operational. The NCPN funds have been essential to meet the repository's needs since Federal funds cannot be used for the improvement of State facilities and current federal facilities are inadequate in size.

Budget Augmentation

In May 2019, the NCGRCD was appropriated a permanent budget augmentation of \$700,000. A previously identified mission critical need was the expansion of the protective screenhouse which is near capacity. FY 2019 funds have been expended for this project and an agreement is in place with a contractor. The repository Research Leader has been working closely with the UCR Planning and Building Department to obtain required permits and approval for the construction of a 50' X 120' expansion of the SH. In order for approval, the UCR fire marshal requires either the use of fire retardant mesh or a fire sprinkler system; these options are being evaluated.

The FY 2019 funds were also used to acquire a new 24' X 40' modular office unit. This unit will house offices for the Research Leader, secretary, and scientists. In addition, there will be a break room/conference room, and handicap accessible restroom. The repository Research Leader has been working closely with the UCR Planning and Building Department to obtain required permits, acceptable placement, and approval for the construction.

In 2019, a handicap-accessible, combination restroom and shower area was installed in the headhouse.

Critical Issues

Having recently received a significant budget augmentation, the future of the NCGRCD is not so dire. Nonetheless, there are some issues threatening the repository that the Crop Germplasm Committee should be aware of. These include:

- Cooperative field and back up collections with UCR needs rationalization, genetic gaps needs to be identified.
- Impact of HLB Quarantine.
- Personnel Issues: the unit lacks genetic, data management, and tissue culture expertise. This could be alleviated by the hiring of a post-doctoral scientist.
- Term (temporary) position needs to be converted to permanent (request has been submitted)
- Several retirements are anticipated within the next 5 years.

Citrus Research Activities

HLB resistance: An USDA NIFA SREP grant was awarded in late 2016 titled: Selection, molecular and genetic analysis of HLB tolerant/resistant variant citrus plants. Naturally occurring mutant citrus plants or bud sports of commercially grown cultivars will be identified. The nature of the genetic variation will be determined by genome sequencing. Using CRISPR technology, HLB tolerant/resistant cultivars with desirable fruit traits will be delivered to the industry. NCGRCD involvement includes the establishment of field evaluation plots, development of public outreach and extension materials, the organization of grower education and the annual progress report meetings. A

field evaluation block has been assigned and the land has been developed. Several hundred thousand Duncan grapefruit seeds have been sent to Collaborator, Zhanao Deng to complete the CRISPR objective.

Early Detection Technologies: The Citrus Research Board (CRB) has funded several research groups to develop and test early detection technologies (EDTs) to detect CLAs in mature citrus trees prior to symptom development. This project began in February 2017 and is in cooperation with UCR (Mauk, Kahn, Roose, Vidalakis, Ma, Grafton-Cardwell) and ARS (Gottwald & Polek). Teams of HLB detection canines were brought to the UCR Agricultural Research Center to survey the CVC and other citrus research plots. The repository's responsibility is to collect plant tissue from canine-alert trees and adjacent trees, process the samples, conduct qPCR assays, and distribute material to collaborators. In 2019 the canines surveyed both the Agricultural Research Center in Riverside and the UC Lindcove Research and Extension Center in Exeter, CA. No samples tested were positive for CLAs in CY 2019.

A spin-off of this project involves the evaluation of using tissue blots as a replacement for the extraction of nucleic acids. Rather than using costly pre-manufactured extraction kits, blots of plant sap are excised from the nitrocellulose membrane and the nucleic acid is eluted from the blots. A cost savings of 85% has been realized. This method has also been evaluated for the detection of other citrus pathogens including viroids and *Spiroplasma citri*.

Use of CTV as a vector for antimicrobial peptides and RNAi: Polek in cooperation with James Ng, UCR, secured funding from the Citrus Research Board for a project titled, 'High performance, California-derived CTV-based vectors for the control of HLB and other applications'. Similar to the CTV-vector developed in Florida, mild California CTV isolates collected by the Central California Tristeza Eradication Agency are being cloned and transformed by the Ng lab. The NCGRCD provides healthy citrus plants, inoculates them with purified virus produced by the Ng lab, and assays for CTV. The ultimate goal of this research is to have ready a virus to use as a delivery mechanism for therapeutic antimicrobial peptides and RNAi strategies against pathogens such as HLB-associated bacteria and insect pests.

Precision agriculture in citrus production: Krueger is participating in a joint UC-ARS project (A Pourreza, PI, with E Scudiero, J Ferreira, D Corwin) that is developing or adapting precision agriculture techniques to citrus production. A mandarin and a navel orchard in Tulare County are being monitored by hyperspectral measurements and the results compared with leaf mineral nutrient levels and fruit quality measurements with the goal of developing predictive models. Preliminary data analysis indicates that soil type influenced crop output and that high-resolution virtual orchard (VO) imagery captured leaf status variability across scales, from a single tree to the whole orchard. Fruit quantity and quality data has not been analyzed yet.

Evaluation of Pigmented Australian Finger Lime Selections: This is a cooperative project between NCGRCD (R Krueger, B Moreland, V Newman) and CVC (T Kahn, T Siebert, K Trunnelle). Three lots of open pollinated Australian finger lime (AFL) seed from pigmented selections were received from Australia in 2012 by NCGRCD. Budded trees were propagated and planted in the field in 2016. They are being evaluated for morphological traits of interest (shape, pigmentation, taste). Several are of potential interest to the industry. We have received some input from the citrus nursery industry at the UCR Citrus Day in January, 2020 and intend to solicit more input at the California Citrus Nursery Society Meeting in Riverside in the Fall of 2020. We hope to release several varieties via the USDA cultivar release program in the next year and send several accessions through CCPP in order to make them available to the industry.

Regeneration of Cryopreserved Citrus Accessions: In cooperation with Gayle Volk, NLGRP accessions that have been cryopreserved and maintained in liquid nitrogen for 5 or more years are regenerated in agar medium and sent back to the NCGRCD. Explants are adapted to soil medium within controlled greenhouse conditions. Plants will be propagated and planted into field plots on the UC Riverside Agricultural Research Center. Plants will be evaluated for trueness to type (Is the fruit quality the same as prior to cryopreservation).

Development of an array for simultaneous detection of 15 RNA viruses and viroids: A novel array was developed for simultaneous detection of two targets each from 15 different citrus RNA pathogens in addition to two reference gene targets. The 96 well assay plate can be used for assaying two plant samples (RNA extractions) along

with one pooled positive control RNA sample in about 75 minutes. The array consists of 32 sets of primers and probes along with stabilizers and reporters placed in individual wells, lyophilized and stored frozen. The platform was used to detect actual pathogen status (with regard to 15 RNA pathogens) of about 50 accessions in our positive inventory. This method is currently being validated by the laboratory personnel in independent tests.

Evaluation of RNA-pathogen inventory using full length genome sequences: Transcriptomic libraries of selected plants in the pathogen inventory were constructed using a Zymo library kit and sequenced using Illumina HiSeq platform. Using the CLC Genomics workbench, sequences specific to genomic sequences of Clementine mandarin and Valencia sweet orange along with mitochondrial and chloroplast sequences were removed, and the remainder of the sequences were aligned against a local library of citrus pathogens. A novel picorna-like virus was detected from most isolates classified as psorosis and concave gum and a real time PCR assay was developed. Conventional PCR followed by Sanger sequencing and Nanopore technology is in progress to further evaluate and describe this virus.

Date Research Activities

Puffy Skin in Date Palms: Production of soft cultivars of dates (fruit consistency) suffers from a problem involving skin separation, called “puffy skin” by the growers. This is a fruit quality defect in which excessive portions of the skin of the date pull away from the flesh, causing a blistered or puffed appearance. This decreases the market value of the date. This problem appears to be influenced by temperature and humidity during the khalal period of date fruit maturation. A cooperative project with Dr. Thomas Perring, Entomology, UC Riverside is investigating bunch and irrigation management as tools to mitigate this problem. Initial results indicate that date palms can grow with substantially less water applied than standard industry practice. This research is supported by a CDC grant to Dr. Perring.

Date Palm Water Use: To supplement the above experiment, an SCRI grant was received in late 2018. The PI on the proposal is Ali Montazar, UCCE Farm Advisor for Irrigation in Riverside and Imperial Counties. R Krueger is a co-PI along with other UCCE personnel. Experimental plots were established in Coachella and Bard Valleys, actual irrigation application measured, and measurements of stress, yield, and fruit quality made. Initial results support the water requirements estimated in the puffy skin experiments.

Date Palm Decline: The Sky Valley area is experiencing a large amount of date palm decline and death. This problem is under investigation by several scientists and extension personnel. Initial symptoms appear in the fruit bunches, then lower leaves necrose, and finally the terminal bud dies and the tree with it. In many cases, the root system is weak or nearly non-existent. Initial sampling was inconclusive, with several usually non-pathogenic fungi being isolated. In addition, declines exhibiting slightly different symptoms have been observed in the Coachella and Imperial Valleys. Whereas these declines are not extensive throughout the Valley, the incidence appears to be increasing. Systematic surveys have not been performed; palms are observed and sampled when growers contact cooperating personnel. The most consistently isolated species is *Fusarium proliferatum*, which has been reported as a pathogen in other countries and possibly in Florida. Pathogenicity tests have thus far been negative. However, a personal communication from Professor Monica Elliott, retired, University of Florida, indicates that this species does not usually produce a pathogenic response under experimental conditions even when consistently associated with declining palms. Chief personnel are D Hodel, UCCE, Los Angeles County (retired); P Mauk, University of California, Riverside; and R Krueger. Assistance in diagnoses were formerly provided by Dr Akif Eskalen, however, he has moved to UC Davis. Dr. Philippe Rolshausen, UC Riverside, now provides diagnoses.

Date palm mineral nutrition: The declines described above in some cases appear similar to problems associated with mineral deficiencies. We have sampled the declining palms and analyzed the mineral levels which appear to be like those in apparently healthy palms, however, nutritional relationships in date palms are not well understood. Nutrient levels in US-grown date palms are generally lower than in date palms grown in other countries, but the significance of this is unknown. Chloride ion levels in leaves are generally very high, but this is common in healthy-appearing palms as well. We would like to get a better handle on nutritional aspects of date production and sought funding from the California Department of Food and Agriculture Fertilizer Research and Education Program

(FREP) by Mauk and Krueger. However, the proposal was not funded primarily because date palms were not a priority crop for the FREP. We hope to pursue additional work in this area in the future.

Alternatives to glyphosate for weed control in date palms: There is increasing societal pressure to reduced use of glyphosate. In addition, resistance to glyphosate is appearing in some weed populations. Thus, there is a need to identify alternatives to glyphosate for various crops, including date palms. Two proposals were submitted to address this question by Sonia Rios, UCCE, Riverside County, with R Krueger as one of the PI's. One proposal was not funded; the decision on the second is pending.

Genomics research: The date palm germplasm collection has proven valuable in date palm genomics studies. Our two main collaborations are with the Weil Medical School Qatar (J Malek, PI) and New York University Abu Dhabi (M Purugganan, PI). Over the years, genomics studies in which NCGRCD has participated have suggested multiple sites of domestication with main east and west gene pools; an X-Y type system of sex expression in this dioecious crop; and genetic bases for fruit color and other important traits. Collaboration in this area is ongoing.

Cryopreservation: Collaborative work done at NLGRP by Dr. Gayle Volk and a visiting scholar, Annie Oliveira, has demonstrated that cryopreservation of date pollen is possible. After reducing moisture content by equilibration, pollen was placed into liquid nitrogen vapor (LNV). Viability was assessed periodically for up to 9 months by rehydrating, plating onto a medium, and counting germinated pollen grains after 18 hr. Germination rates of up to 47 % were achieved, demonstrating that long-term storage of date pollen in LNV can be used for long-term preservation in secure genebanks.

Grants CY 2019

National Clean Plant Network, FY 2019: PI G Vidalakis, ARS PI M Polek, R Krueger. NCGRCD received \$61,459 for the purchase of shoot tip grafting and cryoprocessing supplies, laboratory supplies for diagnostic tests, 25% salary & benefits for Biology Lab Assistant (Rawstern) and student salaries (greenhouse care and lab support).

Multi-Agency Coordinated Response, FY 2016 - 2017: G Vidalakis, PI, ARS PI M Polek, R Krueger. NCGRCD will receive approximately USD \$70 K for 2 years to fund a position supporting greenhouse operations (see Personnel section above). Expires in 2019.

Citrus Research Board, FY 2017-2020: "High performance California-derived CTV-based vectors for the control of HLB and other applications". PI J Ng, UCR, ARS PI M Polek. NCGRCD will receive \$6,547 for plant propagation, maintenance, inoculation, and assay. Project will develop clones of CTV to be used as a delivery system for antimicrobial peptides and RNAi systems.

Citrus Research Board, FY 2017-2020: "Ensuring Security and Integrity of Valuable Breeding, Research, and Germplasm Collections". PI P Mauk, UCR ARS PI M Polek. NCGRCD will receive \$35,360 to collect and process plant tissue samples, conduct qPCR assays, and assist with other Early Detection Technologies.

USDA NIFA SREP 2016 to 2022: Selection, molecular and genetic analysis of HLB tolerant/resistant variant citrus plants. F Luo, F Gmitter, Y Duan, M Polek, Z Deng, L Cano. NCGRCD will receive \$8,000 for evaluation of developed germplasm in California, public/extension outreach and the organization of the annual progress report meetings.

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