Application for Shipley-Skinner Reserve – Riverside County Endowment 2019

Title: Unseen changes over time: quantifying the dynamics of viruses and insect vectors affecting drought tolerant perennial squash.

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Summary:
With prior support from the Shipley Skinner endowment, we characterized insect vector communities and the patterns of infection and co-infection among plant viruses infecting three native perennials (*Cucurbita foetidissima*, *C. palamata* and *Datura wrightii*) each of which is an important drought tolerant, summer-growing species in the Motte Rimrock and Shipley Skinner Multispecies reserves. Our results revealed that infections by multiple crop-associated plant viruses are common in the two *Cucurbita* species, which also experience significant damage from an aphid vector of these pathogens. We will build on these recently published results by (a) determining if these perennial cucurbits retain the most common crop-associated viruses across seasons, (b) quantifying the effects of the aphid vector on plant quality and performance, and (c) testing for host specialization among the aphid populations present on the cucurbit hosts. These results will build a better understanding of the roles that pathogens play in xeric native plant communities by contributing knowledge about long-term associations between viral pathogens and hosts, as well as the population structure of crop pest vectors in native plant communities. The project will also serve as a basis for undergraduate research experiences and outreach about the UC reserve system.

Previous Activities: With the support from the Shipley Skinner endowment in 2017-2018, we used deep sequencing technology to characterize the complement of plant-associated viruses (viromes) of 25 individuals of three native plant species in the Shipley Skinner and Motte Rimrock Reserves (Figure 1). We also initiated monitoring of insect vector activity over the course of the growing season. With our 2017-2018 data, we published a semi-quantitative literature review and case study of our project in the open access journal *Frontiers in Microbiology* (Shates, Sun, Malmstrom, Dominguez, & Mauck, 2018). Our study identified key knowledge gaps in the field of virus ecology, including the need for more dicot study systems and work in Mediterranean climate ecosystems. Our work with the three native species in Figure 1 directly addresses this knowledge gap. In the accompanying case study, we characterized the crop-associated and novel viruses present in these three hosts and determined phylogenetic relationships to known viruses and publicly available sequences of putative virus species. We also developed a more user-friendly bioinformatics workflow for characterizing viromes, which we hope will enable more studies of viruses associated with wild plants.

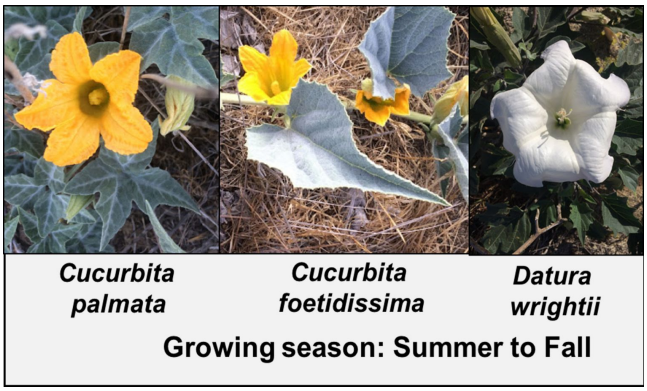

Figure 1: *The three native, perennial target plants under study as models for plant virus ecology. These species were chosen because they are conspicuous and green in the height of summer when other vegetation is senescent or dried out (and thus attractive to vectors). They also re-emerge from their taproots at the beginning of each growing season, which allows us to track virus infections over multiple seasons.*

Current Progress: With funding from the Shipley-Skinner endowment, in 2018-2019 we continued tracking insect vector activity around wild plants in Motte Rimrock Reserve to obtain two seasons worth of vector dynamics in populations of our target hosts (Fig. 1). Our 2017-2018 activities identified two viruses commonly infecting the sampled wild hosts. As this sampling effort was limited due to costs of next generation sequencing, 2018-2019 activities focused on identifying populations of hosts with different characteristics to determine the prevalence of specific pathogens with targeted diagnostics (mixed - i.e. 2 species present in close proximity, and single - i.e., just one of the three target species present). These pathogens include *Cucurbit aphid-borne yellows virus* [CABYV] and *Cucumber mosaic virus* [CMV], which were detected in most samples at both Shipley Skinner and Motte Rimrock Reserve through next generation sequencing. During 2018-2019 we isolated both pathogens from field tissue for future manipulative studies. CMV was transmitted mechanically to cultivated squash and stored in the freezer, while CABYV was transmitted by aphids from field plants to cultivated melons (Figure 2 inset B). In 2018, we collected 181 samples from Motte Rimrock Reserve [CF=51, CP=44, DW=2] and Shipley Skinner Multispecies Reserve [CF=39, DW=45], approximately 15-20% of each target population. We optimized RNA extraction protocols for chemically defended plants because all three species contain secondary metabolites that can inhibit reverse transcription if not removed (Lacroix et al., 2016). Using this procedure, we extracted RNA and tested for CABYV and CMV using reverse transcription-PCR. Almost all plant samples have been extracted at this point, and we are completing reverse transcription and PCR steps. Our results so far show that *C. palmata* is more likely to be infected than *C. foetidissima*. All *C. palmata* individuals contained one or both of the target pathogens, while some of the *C. foetidissima* tested thus far do not have infections (Fig. 2). Detection of viruses in all processed samples will be completed by the end of June 2019. We will also complete greenhouse experiments to determine negative effects of viruses on cucurbit growth and physiology by the end of Summer 2019.

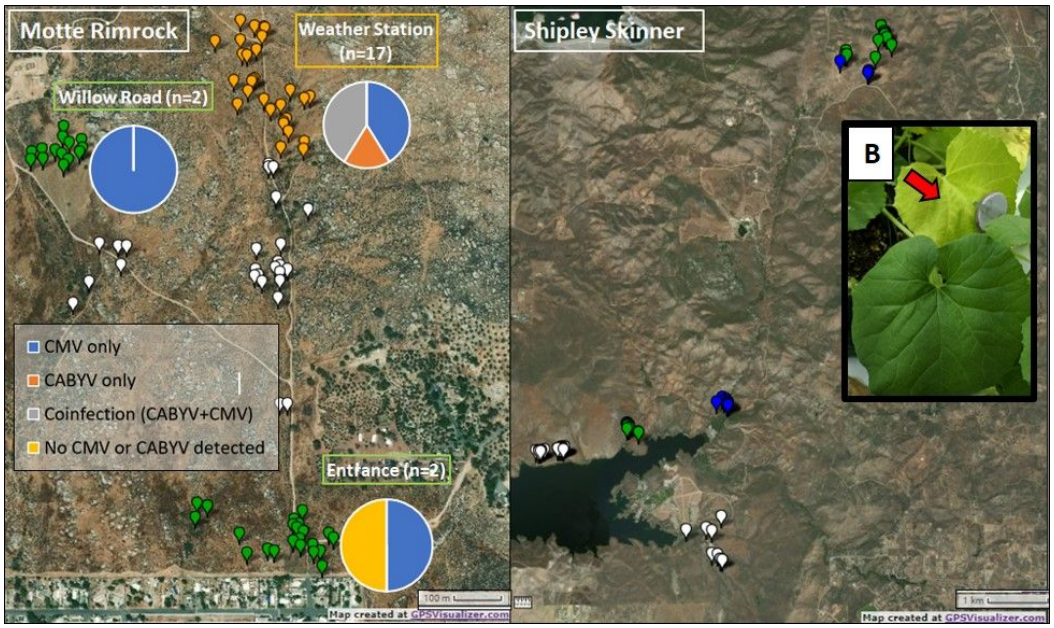

Figure 2. *Current results from virus prevalence studies (in progress for completion by late June, 2019) across plant populations at Motte Rimrock (left) and Shipley Skinner (right). Icons on maps indicate* C. palmata *only (orange),* C. foetidissima *only (green), mixed populations of both cucurbits (white), and populations of* D. wrightii *(blue). For the Weather Station population (comprised only of* C. palmata*) we have 17 samples for which virus screening is completed, and* <u>every plant</u> *was infected with one or both of the target viruses. Remaining samples have undergone RNA extractions and RT-PCR is in progress. We also isolated CABYV from field tissue and transmitted via aphids to cultivated melons. The inset image on the right [B] shows symptoms of CABYV in melons next to a healthy melon leaf.*

Proposed Extensions: Our results thus far indicate that CABYV and CMV are integral parts of the ecology of the two cucurbit species, both of which are major resources (seeds, flowers) for native mammals and insects throughout the arid summer. We also observed that a key vector of these pathogens, *Aphis gossypii*, can reach very high population sizes on both hosts and even cause premature senescence of plant tissue before fruits are mature. Thus, we hypothesize that most of these plants are living with infections by crop-associated viruses and dealing with aphid vector infestations. However, the consequences of these associations for plant fitness remain unknown. We have made good progress in developing our target species as model systems for addressing this knowledge gap. Based on this, we will pursue three novel directions. First, we will determine if infections are maintained over multiple years. Prior research shows that the negative effects of viruses on fitness of perennial grasses become more severe over multiple seasons of infection (Alexander, et al., 2017), and it is important to know if this could be the case for our dicot species. Second, we will quantify the colonization activities (plants infested and timing of infestation) and population sizes of the major vector herbivore, *Aphis gossypii*, over the course of the entire growing season. This vector is a global crop pest and we observed it causing premature senescence of the two cucurbit hosts. Based on this, we hypothesize that it is a major herbivore in our study system. As we study *A. gossypii* dynamics, we will also sample from populations in the reserve and in surrounding crop and urban habitat to perform an analysis of genetic differences among *A. gossypii* populations on wild cucurbits vs. a related crop (melons) and distantly related hosts (citrus, peppers, and hibiscus). This approach has been used previously to understand ecological specialization by aphid species and will provide important information about potential sources of insect vectors in reserves.

Background and Rationale: Plant-infecting viruses that cause crop losses in agriculture are not limited by the boundaries of human development and can be found infecting wild plants in the natural systems we strive to preserve. These viruses often do not cause apparent symptoms in wild plants, but can potentially change fitness and survival of keystone plant species. Virus infection has been shown to reduce multi-year fitness through effects on plant traits such as root growth and flower production, with implications for changing community composition in natural systems (Alexander et al., 2017; Malmstrom et al., 2005). However, virus infection may be beneficial for some plant hosts in extreme conditions such as drought and frost (Roossinck, 2015; Xu et al., 2008). For example, recent work with genetically identical pepper plants lines with and without a cryptic (asymptomatic), seed-transmitted virus found that infection may confer insect herbivore resistance, and thus resistance to the pathogens they vector (Safari, Ferrari, & Roossinck, 2019). The pepper virus belongs to the family Partitiviridae, of which we found many (four or more) novel species in our target plants in 2017-2018 (Shates et al., 2018). These possibly mutualistic virus associates were co-infecting with common crop-associated viruses, such as CABYV and CMV described above (Shates et al., 2018). It seems clear that chronic infections are a reality faced by our target species, and possibly many other wild plant species. However, the importance of these virus associates for plant ecology remain largely unexplored, especially in dicot perennial plants (Carolyn M. Malmstrom & Alexander, 2016).

Are viruses retained in perennial hosts across multiple seasons? Quantifying virus prevalence across growing seasons is important because multi-year infections can affect lifetime fitness and alter plant population dynamics (Raybould, Maskell, Edwards, Cooper, & Gray, 1999). Not many studies have quantified virus prevalence in wild plant communities, but those that have report varying ranges. Raybould et al., 1999 found prevalence of different viruses in wild *Brassica* between 18 and 60% (with 54% of infected plants being coinfected). Malmstrom et al., 2005 reported an infection prevalence of 37% for Barley/Cereal dwarf viruses infecting native CA bunchgrasses. And a recent multi-region survey of viruses in wild plants across agricultural to wild interfaces in France and South Africa found that prevalence is related to land use patterns, with more infections by crop-associated viruses in wild plants growing in unmanaged landscapes in proximity to agriculture (Bernardo et al., 2018). In contrast, a similar study performed in Finland found that prevalence is not consistent across landscapes, and that even in the same location, prevalence varied between two years (Susi et al., 2019). This latter example demonstrates that multi-year prevalence data are essential for understanding the dynamics and changes of viruses in plants across different seasons. At Motte Rimrock and Shipley Skinner, we found that all 25 plants were infected with at least one virus in 2017. In Summer 2018, we sought to quantify the prevalence of the two most common viruses by sampling at least 20% of plants in each population. Our results so far show that there is a contrast between the two *Cucurbita* species at Motte Rimrock Reserve (Figure 2), with *C. palmata* having 100% infection and supporting more virus co-infections. However, because viruses are so seldom studied in perennials, and even less often studied in roots, it is unknown if these infections are chronic across multiple seasons, or are "cured" from the host during winter senescence of above-ground tissues. To understand virus threats faced by the keystone perennial cucurbits in our native plant communities, and their implications for conservation, we need to determine if the most common viral pathogens are persisting in hosts across seasons. This information is also useful for understanding virus epidemiology and evolution, as prolonged associations between viruses and hosts are seldom studied due to the focus of virology on infections in annual crops.

How do vector populations change over time and affect plant health? Of the crop-associated viruses we found in the target plants in summer 2017, and detected again in 2018, five are transmitted by aphids (Shates et al., 2018). We observed that the aphid, *Aphis gossypii,* reaches extremely high populations on both cucurbit species. These herbivorous vectors may be having strong effects on host fitness, as we

observed premature senescence of hosts due to severity of aphid infestations. Even though these minute insects are economically important in agricultural systems, there is little regional information about their roles and dynamics in wild systems. Our initial sampling of virus communities suggests *A. gossypii* is integral to the ecology of these hosts, but population data across the season are not known for any non-agricultural hosts in this region. Moreover, we also do not know the extent of mixing between agricultural and natural populations of this very generalist aphid species. More information on this aspect would provide insight into the potential for pathogen transmission across agroecological interfaces and crop-associated pathogen exposure risks for native plant communities.

Research Plan: With support of the Shipley-Skinner endowment and access to the UCANR natural reserve lands, we have made good progress in developing two keystone perennial cucurbits as wild study systems for virus ecology. To expand on this success, we will pursue three objectives:

1) Quantify the multi-year prevalence of *Cucumber mosaic virus* and *Cucurbit aphid-borne yellows virus* at Motte Rimrock Reserve and Shipley Skinner Multispecies Reserve.
2) Track the arrival, proliferation, and impacts of *Aphis gossypii* on plant health over the course of the growing season.
3) Determine the level of ecological specialization of *A. gossypii* on wild cucurbits.

These objectives directly address the priorities of this RFA, including *evaluation of the status and quality of ecosystems* (by determining the importance of major viral pathogens and their vectors within and across plant populations over multiple seasons and quantifying impacts of a major vector herbivore on plant health) and *developing techniques to predict mechanisms causing ecosystem, community, and population fluctuations* (by exploring the population structure and potential origins of *Aphis gossypii* on a regional scale). Our ongoing activities to quantify virus impacts on plant performance and fitness in the greenhouse (to be completed by end of Summer 2019) are complementary to these objectives, as they address the third goal to *conduct experiments to address questions of conservation concern*.

Methods: *Objective 1: Quantify multi-year prevalence of CMV and CABYV*: We will re-sample plants that we previously sampled in 2018 to determine if infections are retained from one season to the next, and if so, at what frequency this occurs. If plant-regrowth is not consistent, we will sample the same number of plants in the population and compare proportions of infections. Using the methods that we optimized in 2018-2019, we will extract total RNA using the RiboZol (VWR) protocol, perform reverse transcription to cDNA using random hexamers, and then use PCR and unique coat-protein primers to screen for the presence of each virus. Overall prevalence, spatial associations among viruses, and within-host virus associations (co-infections) will be compared to 2018 summer prevalence and analyzed according to the methods in Susi et al. 2019, which also quantified virus prevalence over two years. We predict that prevalence will fluctuate across years and that some previously infected hosts will not have detectable virus infections. This objective will provide information about virus persistence (and potential impacts on host fitness) over multiple seasons.

Objective 2: Track the arrival, proliferation, and impacts of Aphis gossypii on plant health. Starting in late spring when plants first send out new shoots, we will monitor leaves for the presence of winged and non-winged *Aphis gossypii*. Sampling will occur in the populations identified in Figure 2. From each population, we will remove one leaf from each of 15 plants, store it in a ziplock bag on ice for transport back to the lab. We have developed a protocol for automating counting of small, largely sessile Hemipterans on cucurbit leaves by photographing the leaves while back-lit with light box, then using ImageJ software to quantify insects present. *Aphis gossypii* is not easily disturbed from feeding positions on leaves so most insects will remain on the plant tissue while chilled. We will count any insects

dislodged into the Ziplock bag. Plant health will be recorded every 2 weeks using a rating scale (scores from 1 to 9) that was developed for assessing disease and damage severity in vining cucurbits (McCreight & Wintermantel, 2011). We will also document the types of damage observed (using photos) and number of flowers present when blooming commences. Tracking vectors in connection with plant health will aid our understanding of how changes in *A. gossypii* populations may affect fitness and host phenology.

Objective 3: Determine the level of ecological specialization of A. gossypii on wild cucurbits. For this objective, we will use previously developed and validated microsatellite markers for *A. gossypii* to determine the level of host specialization for aphids present in wild cucurbit populations (Vanlerberghe-Masutti, Chavigny, & Fuller, 1999). During Objective 2 activities, we will collect *Aphis gossypii* from cucurbit hosts in target populations at the Motte Rimrock Reserve and Shipley Skinner Multispecies Reserve at early, middle, and late time points in the season. At each of the three time points, we will collect 10 aphids from each cucurbit species within each population in which it occurs (Figure 2) for a total of 270 aphids from our sites. Aphids will be stored in individual micro-tubes in ethanol at -80 degrees Celsius until use. To determine if the populations in the reserve exhibit host specialization on the two wild *Cucurbita* species, we will also collect aphids from crop and urban/suburban habitats having cucurbit hosts (melons), Solanaceous hosts (peppers), Rutaceae (citrus) and hosts in the Malvaceae (hibiscus) (target: 30-50 aphids per host). Sites will be located with the help of the County Agricultural Commissioner (through pesticide spray applications), ground scouting, and use of UCR agricultural operations facilities (which maintains plantings of these hosts). We will extract DNA from individual insects using an inexpensive Chelex extraction procedure that is already used in the lab for work with whiteflies, and then perform multiplex PCR reactions to amplify microsatellite markers and analyze them using the UCR Core facility Advanced Analytics Fragment Analyzer (3bp difference resolution) (Charaabi et al., 2008; Vanlerberghe-Masutti et al., 1999). We will determine the multilocus genotypes (MLGs) present and test for genetic structure due to host plant and collection period using a hierarchical analysis of molecular variance model. If the aphids on our target hosts have specialized on these wild plants, we expect to see MLGs that are distinct from those present for other hosts, and especially for *A. gossypii* sampled from melons (another cucurbit).

Conservation implications and rationale for continued work in Riverside County Reserves: Our work in 2017 and 2018 provides evidence that both endemic and crop-associated viruses are common in long-lived perennial squash in Southern CA reserves. By monitoring insect vectors, we have established that a globally important crop pest, *Aphis gossypii*, is a major herbivore and vector of xeric cucurbits. These efforts establish that viruses and vectors from crops are a part of the conservation challenges facing the habitats in which these keystone perennials reside. Although this finding is important, we do not yet have data on how the dynamics of virus infection change over seasons, and how insect vectors can impact plant health beyond their capacity to spread viral pathogens. Continued work at Motte Rimrock and Shipley-Skinner reserves over multiple seasons will address this knowledge gap. Both reserves are ideal for this work because they contain native chaparral and grassland habitat in proximity to human development, which is increasingly the reality for conserved natural areas. Broader impacts to the community: The graduate student PI conceived and initiated this project as her primary dissertation research. She has presented work on this research at two Entomological Society of America national conferences and two Pacific Branch ESA Conferences, as well as the Wildland Urban Interface Symposium in 2019. Photos and records of our activities at the reserves have been used as components of outreach materials showcasing research in the UCR Department of Entomology and natural reserves. Additionally, the project also served as the basis for an undergraduate student project on vertical transmission of plant viruses (June 2018-present) under the mentorship of the graduate student PI.

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Budget & Budget Narrative:

Objective/Type	Budget item	Cost
Objective 1 (virus prevalence)	Ribozol (181 samples)	$325
	Reverse transcription (Superscript, RiboLock, Random hexamers, dNTPs – 181 samples)	$1,610
	Gels (10) and Sanger sequencing	$326
Objective 1+3 (prevalence and insect populations)	PCR: Phusion DNA polymerase enzyme (~800 reactions)	$437
Objective 2+3 (insect populations)	Use of fragment analyzer for characterizing allele combinations (400 samples at $49/12 samples)	$1,634
Travel (all objectives)	$0.58/mile. Estimated mileage 584 miles (both sites)	$339
Fall 2019 Graduate PI support	Stipend ($6184) and tuition ($5398)	$11,582
		TOTAL: $16,253

The amount requested will support project activities from June 1, 2019-June 30, 2020. The budget includes one quarter stipend and benefits for graduate student PI (Tessa Shates) who will complete the bulk of analysis and laboratory work in the academic year and therefore will require support that does not involve additional teaching assistant duties. The graduate student PI is experienced in DNA and RNA extractions, library preparation and plant and insect identification. The other parts of the budget are separated by objectives (Prevalence [Obj. 1] and Insect Populations [Obj. 2/3]) except for travel, which includes trips for all three objectives (estimated 4 trips to Shipley Skinner Multispecies Reserve and 8 trips to Motte Rimrock Reserve). The Objective 1 (prevalence of viruses) budget includes reagents for reverse transcription of the RNA extraction to cDNA (SuperScriptIV Reverse Transcriptase, RiboLock, dNTPs and random hexamers) sufficient for the 181 samples. It also includes costs for PCR reagents and Sanger Sequencing to confirm virus identities. The Objective 3 (insect populations) activities will use some reagents purchased for Objective 1 (Phusion Taq polymerase, dNTPs) as well as reagents already in the lab (Chelex, consumables). The primary expense for Objective 3 is fragment analysis to determine allele combinations present in individual insects. Rather than use gels, this procedure will be done using parallel capillary electrophoresis performed on an Advanced Analytics Fragment Analyzer (cost $49 per 12 samples) and the user-friendly ProSize data analysis software that accompanies this instrument.

Data Management Plan

1) Types of data to be collected: Objective 1- GPS coordinates and photographs will be taken of plant individuals from which tissue is collected. Samples and RNA extracted from them will be stored in -80 degrees Celsius. cDNA from reverse transcription and PCR products will be stored in -20 degrees Celsius in the Mauck laboratory. Detection results will be recorded as gel photos (.jpg) and in tabular format as .csv files and .xlsx files. We will also deposit voucher specimens in the UCR Herbarium.
Objective 2 - Photographs (.tif format) will be taken of the leaves with aphid colonies and counts of aphids entered in tabular formats (.csv and .xlsx). Recordings of plant health metrics will be collected and stored as physical data (lab notebooks) and digital data (.csv, .xlsx).
Objective 3- Approximately 400 aphids will be kept in undiluted ethanol (in micro-tubes) at -80 degrees Celsius. DNA extractions and PCR products from these samples will be stored at -20 degrees C and long-term at -80 degrees C. From PCR products we will generate files documenting detected fragments (ProSize files, .csv, and .xlsx). A subset of samples from each location will be slide-mounted and submitted as voucher specimens to the Entomology Research Museum.

2) Standards for data types anticipated: The format standards for individual data types are listed in section (1) above where each type is mentioned. Meta-data will include: dates of collections, GPS coordinates, weather conditions, names of personnel involved in carrying out sample collection and processing, sources of reagents and batch numbers, make and calibration of equipment, core facility personnel involved in equipment use, images of plant condition ratings, and information on local crop production. Metadata will be stored as .tif, .txt, and .docx formats electronically and in lab notebooks.

3) Roles and Responsibilities of all parties: Graduate student Tessa Shates is responsible for collecting physical data (leaves and insects) and placing in relevant storage conditions, sample processing, recording data and submitting voucher specimens. Tessa Shates will ensure that data storage adheres to the policies described in this document. Tessa will write up preliminary and final reports and papers for publication. Assistant Professor Kerry Mauck will host the cloud-based backup system and ensure that data standards are being followed. Kerry Mauck will also provide feedback on products for dissemination of data (4).

4) Data and methods will be made available through the interim report and final report, through posters and talks at conference presentations (during and after the project period), and through outreach materials showcasing research at the UC reserves. Conferences will include the Entomological Society of America's national and Pacific branch meetings as well as local events. Data and metadata will be made available publicly following manuscript acceptance in peer reviewed journals.

5) All data and metadata that are in digital formats (computer accessible) will be stored on a laboratory computer, external hard drive, and a cloud-based automated backup system provided to UCR faculty. Handwritten data will be stored as hard copies in laboratory notebooks. Sequencing data will be deposited in NCBI genbank. When submitted for publication, all materials will also be uploaded to a data management system called eScholarship. The materials will be tagged uniformly with metadata about the source and nature of the data. Any electronic file format (e.g., text, spreadsheets, images) can be accepted. A curator from the College reviews all submissions before they are accepted.

6) Physical samples (leaf and insect material) will be stored in -80 Celsius for up to 2 years. After this point, only a subset of material (extracted and preserved nucleic acids) will be kept for future work. RNA and DNA extracted from samples will be kept in -80 Celsius until publication of results, or longer if useful for future projects. All data will be publicly available following publication. Instructions for access to these data will be provided in supplementary material of publications in which the data appear.