1. **Principal Investigator:** Rodrigo P. P. Almeida
Project Title: Mealybug transmission of *Grapevine leafroll-associated virus 3*

2. Summary:

The overarching goal of this research is to obtain information about transmission of *Grapevine leafroll-associated virus 3* (GLRaV-3), the primary virus species associated with spread of the economically damaging Grapevine Leafroll Disease (GLD). Such information is necessary to inform control strategies, it is clear that knowledge-based management of vector-borne diseases requires a robust understanding of how the pathogen spreads in vineyards. Mealybugs are the vectors associated with spread of GLD, but nothing is known about differences in transmission efficiency among mealybug species inhabiting vines. Furthermore, genetically distinct variants of GLRaV-3 exist but nothing is known about differences among these variants in terms of their ability to spread, or what the relevance of that variation is to GLD epidemiology. Lastly, all previous GLRaV-3 transmission studies were done under greenhouse conditions, and it is not known how well the results of such studies predict transmission in vineyards. This research addresses these significant gaps in knowledge.

We have completed proposed single and simultaneous mixed GLRaV-3 variant inoculations in greenhouse trials, using grape and vine mealybugs. Though two GLRaV-3 variants from singly infected source plants did not differ in transmission efficiency, the transmission efficiency of one variant was substantially lower when acquisition occurred from a co-infected source plant, indicating inhibition of transmission by the other variant. This may mean that one variant can be transmitted more efficiently than another and increase its incidence in the landscape (e.g. Napa Valley). It is not known whether some GLRaV-3 variants are more pathogenic than others.

We also set up an experiment in Napa Valley in summer 2011, inoculating 60 mature grapevines with GLRaV-3 using grape mealybugs as vectors. Each vine was inoculated using 10 first instar grape mealybugs, and then treated with insecticide two days later. Three months after inoculation, 20 of 60 plants tested positive for GLRaV-3 from our inoculations. No symptoms appeared in 2011. During the following growing season, GLD symptoms first began to appear in our experimental vines in June. By July, symptoms appeared in 29 of 60 experimental vines, and no other vines became symptomatic in 2012. Berry quality was affected in symptomatic vines compared to asymptomatic vines in the experiment. In 2013 leafroll symptoms first appeared in May, and in June 29 of 60 experimental vines were symptomatic. In 2012 we set up a second field inoculation experiment in Napa to compare transmission of two different GLRaV-3 variants, by grape and vine mealybugs, in both Chardonnay and Pinot Noir. Inoculations were completed in July. Results from the 2012 inoculations are pending. This is the first time it has been shown that GLD symptoms due to mealybug inoculation of GLRaV-3 into established mature vines (~15 years old) in commercial vineyards are expressed in the following growing season. Results also showed that the entire vines were symptomatic in 2012, instead of just the inoculation site. Lastly, transmission success in the field was about 6% per individual mealybug.

3. Project Title:

Mealybug transmission of *Grapevine leafroll-associated virus 3,* OWB Grant #2012-1270.

4. Principal Investigator:

Rodrigo P. P. Almeida, Associate Professor, 130 Mulford Hall, Dept. Environmental Science, Policy & Management, University of California, Berkeley, CA 94720-3114, (510) 642-1603, rodrigoalmeida@berkeley.edu.

Co-Principal Investigators:

Monica L. Cooper, Farm Advisor, UC Cooperative Extension, 1710 Soscol Ave., Suite 4, Napa, CA 94559-1315, (707) 253-4221, mlycooper@ucanr.edu
Kent M. Daane, CE Specialist, 137 Mulford Hall, Dept. Environ. Sci. Policy & Management, UC Berkeley, CA 94720-3114, (510) 643-4019, kdaane@ucanr.edu

5. Objectives:

The objectives in the original proposal were to:
I) compare the transmission efficiency of GLRaV-3 variants by the grape and vine mealybugs
II) determine the outcome of mixed vector inoculations with GLRaV-3 variants
III) determine GLD incubation period in the field after vector transmission

We discuss experiments and results obtained during the first year of this project in Section 6 below.

6. Summary of Major Research and Accomplishments and Results by Objective:

Objective I. compare the transmission efficiency of GLRaV-3 variants by the grape and vine mealybugs

This Objective was slightly changed in 2012 to focus on field work, incorporating comparisons between grape and vine mealybugs into our field inoculations and a parallel greenhouse inoculation study. We did not rear grape mealybug, thus relying on yearly field collections of females during late Spring. The focus on field work and a parallel greenhouse study was chosen because the number of insects collected would not have allowed for more greenhouse experiments. We will pursue more greenhouse experiments in 2013. The inoculations done will provide information that is more relevant to growers, although the inferences we can make about transmission efficiency will not be as detailed because we used only one acquisition and inoculation access period, expected to be relevant to vineyard conditions.

Instead of the grape mealybug, we completed greenhouse inoculation trials with varied acquisition and inoculation access periods (2, 8, and 24 hours each) using the obscure and vine

mealybugs. We tested four different vector-virus combinations, using the obscure and grape mealybugs and GLRaV-3a and -3e. The obscure mealybug is closely related to the grape mealybug, has a relatively similar life history to that of the grape mealybug, and is found in vineyards in California's Central Coast, North Coast, and Northern Interior winegrape regions. Therefore information obtained about the obscure mealybug is also important. To compare the transmission of GLRaV-3 variants by obscure and vine mealybugs we used a standard transmission experiment, varying vector acquisition and inoculation access periods (AAP and IAP) using groups of five first instar individuals per test plant. Insects were confined to an individual leaf of recipient test plants with small leaf cages. This design was used by Tsai et al. (2008) and will generate data from 2 to 24 hours of AAP and IAP; thus, acquisition and inoculation efficiencies will be determined, for each virus-vector combination as independent linear regressions. Thirty test plants were used per AAP and IAP period combination for each mealybugs species. In total, 600 plants were inoculated in this experiment. Four months after inoculation, three petioles of each test plant were collected for GLRaV-3 infection. The slope of the transmission efficiency regressions will be used to compare virus acquisition and inoculation efficiencies. Because viral populations within plants have been shown to affect transmission efficiency in other systems, we will also quantify GLRaV-3 populations of each virus source plant. These assays will determine the relative transmission efficiency of GLRaV-3 variants by the obscure and vine mealybugs. If differences are detected, the experimental design will determine if variability is due to plant-virus interactions or plant-vector and virus-vector interactions, which can be difficult to dissociate from each other. GLRaV-3 testing for this experiment is in progress.

Objective II. determine the outcome of mixed vector inoculations with GLRaV-3 variants

We have completed single and simultaneous mixed variant inoculations in greenhouse trials, using grape and vine mealybugs. The mixed variant trials were done with virus acquisition from source plants co-infected with two GLRaV-3 variants (vine mealybug), and from two different source plants infected with each one of those variants to one test plant (grape and vine mealybug). In some cases groups of 5 insects per test plant and 24 hour access periods were used, and in some cases groups of 10 insects per test plant and 48 hour access periods were used. Petioles from these inoculation trials were collected four months after inoculations, and some have been tested.

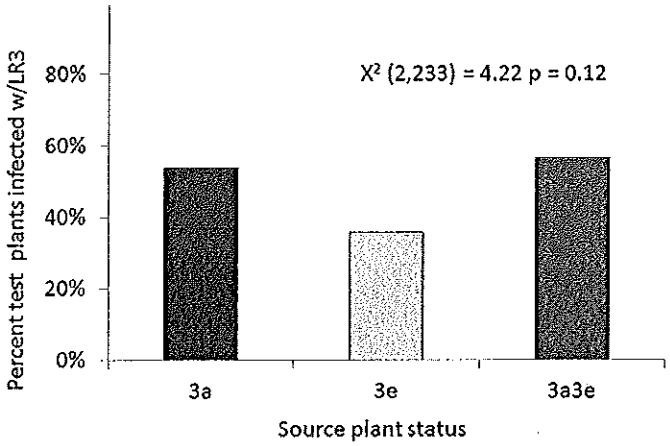

Figure 1. Transmission is the same regardless of GLRaV-3 variant or a mixed infection source plant

Preliminary results are reported here.

Findings: Two variants from singly infected source plants did not differ in transmission efficiency by vine mealybugs (Figure 1), and overall GLRaV-3 transmission did not change when source plants had a mixed infection. However, the transmission efficiency of one variant was substantially lower when acquisition occurred on a co-infected source plant, indicating that one variant is transmitted more efficiently than another in the case of mixed infections (i.e. competition between variants exists).

Objective III. determine GLD incubation period in the field after vector transmission

Two field inoculation projects are underway to accomplish this Objective; the second of which relates to the comparison of transmission by the grape and vine mealybug species for Objective I. The first inoculation experiment was started in July 2011. We inoculated Cabernet Franc grapevines, planted in 1994, in a commercial vineyard with GLRaV-3c using grape mealybugs. The experiment included 30 plants inoculated using mesh leaf cages, 30 plants inoculated using no cages, and 30 negative control plants, with these three treatments spatially randomized. Inoculations were done using groups of 10 first instar grape mealybugs, moved from known infected source plants three days after emerging from ovisacs. For each plant inoculated, 10 mealybugs were placed on the underside of one mature leaf. Two days later the experimental area was treated with insecticide, for a 48 hour inoculation access period, and continued to be treated with insecticide per normal commercial practices. Plants were monitored for symptoms every two weeks during 2011 and 2012 growing seasons. Petioles were collected and tested for GLRaV-3 in October 2011. In 2012, berry quality was tested 3 times in August, September, and October. Cuttings were collected from a subset of experimental plants in October 2012, and tested as a source for GLRaV-3, using groups of 10 vine mealybugs per test plant, and a total of 150 test plants from 20 source cuttings. Petioles were collected from them and tested for GLRaV-3 four months after inoculations.

Table 1. Field inoculation experiment started 2011

Treatment	Replicate vines Inoculated July 2011	PCR Positive October 2011	Symptomatic 2012
Caged	30	11	15
Uncaged	30	9	14
Negative Control	30	0	0
Total Plants	90	20	29

Findings: The petioles collected in 2011 were tested using RT-PCR and fragment analysis; twenty of sixty inoculated vineyard plants tested positive for Leafroll 3, and no negative control plants tested positive (Table 1). There was no difference between the caged and uncaged treatments. No symptoms developed in 2011. Symptoms started to appear in June 2012, and progressed over the growing season. Twenty-nine of 60 plants became symptomatic; all plants that became symptomatic in 2012 showed symptoms by July 6, 2012. No negative control plants became symptomatic. Brix, pH, and titratable acidity were all affected in the symptomatic plants when compared to asymptomatic plants (Figure 2). In 2013, leafroll symptoms began to appear in May and the same 29 plants were symptomatic by June.

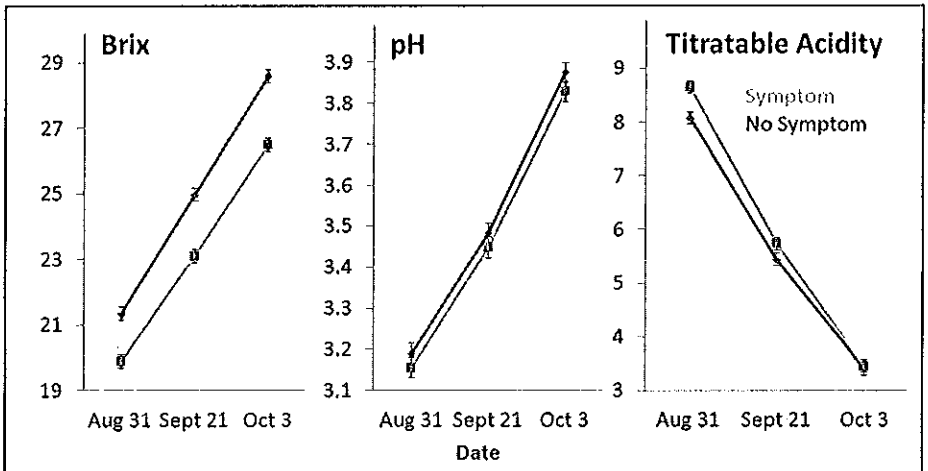

Figure 2. Berry quality 2012. Harvest was October 5.

The second inoculation experiment was initiated in 2012. We originally proposed to do three small inoculation studies, each at a different site. Early in the 2012 growing season, two of our identified collaborators decided the risks posed by inoculations with GLRaV-3 in their vineyards were too great, and withdrew their support. Fortunately, the one remaining collaborator allowed us to do a larger experiment at their site. This enabled us to have several well replicated treatments, compare field inoculations by vine and grape mealybugs, with each of two GLRaV-3 variants (a and e), and include both Pinot Noir and Chardonnay. Inoculations were completed in July 2012, following the same uncaged protocol used in 2011. We concurrently set up a parallel greenhouse experiment, identical in treatments and replication, to compare the results of greenhouse inoculation tests to vineyard inoculations.

In each grape variety, we used 30 replicate vines for each of 5 inoculation treatments: (1) grape mealybug GLRaV-3a, (2) grape mealybug GLRaV-3e, (3) vine mealybug GLRaV-3a, (4) vine mealybug GLRaV-3e, and (5) negative control. We also had two treatments of vines inoculated with both GLRaV-3a and 3e together, one group by grape and another by vine mealybugs: each of these virus competition treatments included 20 replicate vines; the replication of this final treatment was smaller due to mealybug availability (Table 2). Twenty replicates are expected to be sufficient for this work, as we originally proposed 15 replicates of mixed inoculation test plants. Field inoculations were completed in July 2012. Petioles of the inoculated leaves of experimental plants were collected in September 2012, just before harvest, to test for GLRaV-3 infection. The experiment will be monitored for multiple growing seasons.

A greenhouse experiment including 30 replicates of the same treatments used in the field inoculation experiment was started at approximately the same time. The goal is to compare results of greenhouse (which are easier to perform) and vineyard transmission experiments, so that work in controlled conditions can be helpful for interpretation of field data and development of management practices. Petioles from this experiment were collected four months after inoculations were completed; results are pending.

Table 2. Field inoculation experiment 2012

Grape Variety	Mealybug Species	Leafroll 3 variant	Replicate Vines
Chardonnay	Vine	A	30
		E	30
		A and E	20
	Grape	A	30
		E	30
		A and E	20
	Negative Control	None	30
Pinot Noir	Vine	A	30
		E	30
		A and E	20
	Grape	A	30
		E	30
		A and E	20
	Negative Control	None	30
Total Vines			380

7. Outside Presentations of Research (2012-2013):

Presentations

By Rodrigo Almeida (PI)

Keynote speaker: Transmission biology of the leafroll viruses. October 2012. 17th Congress of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG). Davis, California.

Distribution of leafroll viruses in California. December 2012. UCCE Workgroup Annual Meeting. UC Davis, Davis, CA.

By Monica Cooper (PI)

Mealybug/Leafroll Update. March, April, May, and August 2012. Monthly meetings of the Yountville-Oakville GLD grower group. Oakville, CA.

By Kent Daane (PI)

Mealybugs in western US vineyards: what is their changing role in grape leafroll disease transmission? June 2012. 5th Annual Viticultural Research Roadshow. Central Valley Winegrowers. Fresno, CA.

Brown marmorated stink bug, spotted wing drosophila, vine mealybug and GLRaVs – the role of invasive species in vineyard IPM. June 2012. Lodi-Woodbridge Grower Breakfast. Lodi, CA.

Mealybugs in western US vineyards: what is their changing role in grape leafroll disease transmission. June 2012. 5th Annual Viticultural Research Roadshow. Easton, CA.

The impact of mealybug controls on grape pest management. June 2012. XIII Symposium Internacional del la Uva. Lima, Peru.

Developing a long-term pesticide program for vineyard mealybugs. September 2012. Fresno-Madera CAPCA. Fresno, CA.

Control of GLRaV's using insecticides targeting the vector. October 2012. 17th International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG). Oakville, CA.

Are areawide controls for Mealybugs & GLD a feasibility or fantasy? December 2012. University of California Grape Workgroup. Davis, CA.

By Kai Blaisdell (post-doctoral researcher)

Experimental vineyard inoculations with GLRaV-3. October 2012. 17th Meeting of the International Council of Viruses of Grapes, Post-Conference Tour. Oakville, CA.

Current knowledge and research of leafroll transmission by mealybugs. November 2012. Current Issues in Vineyard Health Annual Workshop. Davis, CA.

Current knowledge and research in leafroll transmission by mealybugs. December 2012. UCCE Workgroup Annual Meeting. Davis, CA.

Field Inoculations. January 2013. Oakville Neighbors Mealybug/Leafroll Workgroup Meeting. Oakville, CA.

By Christina Wistrom (staff research associate in Daane Lab)

Grape mealybug ecology and its role in grape leafroll associated virus epidemiology. June 2012. Pacific Division meeting, American Phytopathological Society. Sacramento, CA.

Publications

Almeida, R.P.P., Daane, K.M., Bell, V.A., Blaisdell, G.K., Cooper, M.L., Herrbach, E. and Pietersen, G. 2013. Ecology and management of grapevine leafroll disease. Frontiers in Microbiology 4:94.

Blaisdell, G.K., S. Zhang, K. Daane, and R. P.P. Almeida. 2012. Patterns of virus transmission from hosts with mixed infections. 17th Meeting of the International Council of Viruses of Grapes.

Blaisdell, G.K., S. Zhang, K. Daane, M. Cooper, and R. P.P. Almeida. 2012. Patterns of virus transmission from hosts with mixed infections. 2012. 5th Annual Plant Virus Ecology Network Workshop.

Daane, K. M., R. P. P. Almeida, V. A. Bell, M. Botton, M. Fallahzadeh, M. Mani, J. L. Miano, R. Sforza, V. M. Walton, and T. Zaveizo. 2012. Biology and management of mealybugs in vineyards, pp. 271-308. In N. J. Bostanian, R. Isaacs, and C. Vincent (eds.) Arthropod Management in Vineyards. Springer, the Netherlands.

Maree, H.J., Almeida, R.P.P., Bester, R., Chooi, K.M., Cohen, D., Dolja, V.V., Fuchs, M.F., Golino, D.A., Jooste, A.E.C., Martelli, G.P., Naidu, R.A., Rowhani, A., Saldarelli, P. and Burger, J. 2013. *Grapevine leafroll-associated virus 3*. Frontiers in Microbiology 4:82.

Communication strategy

We are using a multi-layered approach to reach an audience as broad as possible. All three PIs and the post-doctoral researcher have given talks to (and met with) growers, stakeholders, and scientists in the private and academia. Those have ranged from the 'Current issues in Vineyard Health Annual Workshop' to the 'UCCE Workgroup Annual Meeting' and the 'Congress of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine'. We hope to start communicating the results of some of this work in a written form (i.e. research papers) this year, which would include results from the first year of the project. Those should include a review article that discusses the ecology and management of leafroll disease in various regions of the world.

8. Research Success Statements:

This project is generating much needed information on how GLRaV-3 is transmitted by mealybug vectors. First, it provides general information on the transmission efficiency of different virus variants by mealybug vector species, showing that it is possible that one variant may spread faster. The immediate relevance and impact of this work is self-evident in the results of our field experiments. We showed, for the first time, that mealybugs efficiently transmit GLRaV-3 to established commercial vines in the field, that infections take one year to develop into symptoms, and that symptoms in that year were present throughout the plant and not localized near the inoculation site. We also demonstrated that berry quality was significantly reduced one year after infection. These results will inform ecological studies aimed at developing sustainable management practices (lead by Daane in our group) and provide support to epidemiological work on rogueing by others and economic studies. More importantly, it tells stakeholders that plants 'get sick' within one year if inoculated by mealybugs in the field, and that fruit quality will be impacted in the first season during which symptoms are observed. The continuation of this work will expand on these findings and clarify vector-virus-plant relationships being studied.