

Documentation of grapevine leafroll-associated viruses in wine  
grape varieties and native grape species in Virginia, and  
examination of the movement of grapevine leafroll disease to  
develop management strategies

Taylor Jones

Thesis submitted to the faculty of  
Virginia Polytechnic Institute and State University  
in partial fulfillment of the requirements for the degree of

Master of Science  
In  
Plant Pathology, Physiology, and Weed Science

Mizuho Nita, Chair  
Anton Baudoin  
Sue Tolin  
Elizabeth Bush

December 4, 2012  
Blacksburg, Virginia

Keywords: Grapevine leafroll disease; GLRaV-2; GLRaV-3; Grapevine Fleck Virus;  
Mealybugs; Grapevines; Virginia

# Documentation of Grapevine leafroll-associated viruses in wine grape varieties and native grape species in Virginia, and examination of the movement of the grapevine leafroll disease to develop management strategies

Taylor Jones

## ABSTRACT

Grapevine leafroll-associated virus-2 (GLRaV-2), GLRaV-3, and grapevine fleck virus (GFkV) are widespread in grapes around the world. These viruses can cause significant crop loss and affect wine quality by reducing sugar accumulation and compromising skin color. Mealybugs are vectors of grapevine leafroll-associated viruses (GLRaVs). A statewide survey of commercial and wild grapevines in Virginia was conducted during 2009 through 2011. Also, vector management options were tested in two field studies. GLRaV-2, GLRaV-3, and GFkV were detected in 8%, 25%, and 1%, respectively, of over 1,200 vine samples (41 wine grape varieties) from 77 locations, and 64% of vineyards were positive for at least one of the tested viruses. All 100 wild grapevines tested were free of these three viruses, indicating that they are not alternative hosts. The majority of infected vines from commercial vineyards were planted prior to the 1990's; however, some new plantings were also found to be positive, indicating movement of the viruses among vineyards and also potential infection prior to planting. The high frequency of virus-infected vines emphasizes the importance of clean plant materials, as well as management of vector insects. The insecticide trials resulted in promising vector control with dinotefuran and spirotetramat; however, acetamiprid and pyrethroid resulted in an increase in mealybug population. This study is the first to examine multiple grape viruses in VA. It will aid in developing better strategies aimed at controlling mealybugs to restrict the movement of viral diseases.

## Acknowledgement

I would like to acknowledge my advisor and mentor Dr. Mizuho Nita for his help and aid in project endeavors. I would like to thank former and current members of the Nita lab who worked along with me in data collection and experimentation: Shantal Hover, Sam Lilly, Brandon Millholland, and Kathleen Yablonski. I would also like to thank members of my committee for their guidance and support on the projects outlined. I would like to acknowledge Dr. Naidu Rayapati and members of his lab at Washington State University for their help and support on testing samples and helping me to set up a working lab in Virginia. I would also like to thank my sources of funding: The Virginia Wine Board and the Viticulture Consortium East.

## Table of Contents

List of Figures	vi
List of Tables	viii
Chapter 1: Introduction	
1.1 Introduction	1
1.2 Objectives of this project	3
1.3 References	4
Chapter 2: Literature Review	
2.1 Introduction to the Grape Industry	8
2.1.1 <i>History of the Grape Industry</i>	8
2.1.2 <i>The World Wine Grape Industry</i>	9
2.1.3 <i>The Virginia Wine Industry</i>	9
2.2 Diseases of Grapevines	9
2.2.1 <i>Introduction</i>	10
2.2.2 <i>Diseases caused by Bacteria</i>	10
2.2.3 <i>Diseases caused by Fungi</i>	10
2.2.4 <i>Diseases caused by Nematodes and Insects</i>	11
2.2.5 <i>Diseases caused by Phytoplasmas, Viruses, and Virus-Like agents</i>	11
2.3 Grapevine Leafroll Disease (GLD)	12
2.3.1 <i>History</i>	12
2.3.2 <i>Symptoms</i>	13
2.3.3 <i>Epidemiology and Vectors</i>	13
2.3.4 <i>Management Strategies</i>	14
2.3.5 <i>Detection of GLD and other grapevine viruses</i>	15
2.3.6 <i>Viruses Associated with GLD</i>	15
2.4 Grapevine Leafroll-Associated Virus-2 (GLRaV-2)	16
2.4.1 <i>Taxonomy</i>	16
2.4.2 <i>Morphology</i>	16
2.4.3 <i>Transmission</i>	17
2.5 Grapevine Leafroll-Associated Virus-3 (GLRaV-3)	17
2.5.1 <i>Taxonomy</i>	17
2.5.2 <i>Morphology</i>	17
2.5.3 <i>Transmission</i>	18
2.6 Grapevine Fleck Virus (GFkV)	18
2.6.1 <i>Grapevine Fleck Virus: The disease and symptoms</i>	19
2.6.2 <i>Epidemiology, Transmission, and Vectors</i>	19
2.6.3 <i>Management Strategies</i>	19
2.6.4 <i>Taxonomy</i>	19
2.6.5 <i>Morphology and Virion Properties</i>	20
2.7 Mealybugs and Soft Scale Insects	20
2.7.1 <i>Mealybugs, scale insects, and the order Hemiptera</i>	20
2.7.2 <i>The Family Coccoidea (scale insects)</i>	21
2.7.3 <i>The Family Pseudococcidae (mealybugs)</i>	21
2.7.4 <i>Roles of Coccid and Pseudococcid in GLD</i>	21
2.7.5 <i>Biology and movement of mealybugs on Grape associated with GLD on</i>	22

<i>grape</i>	
2.7.6 <i>Management of mealybugs and soft scales</i>	23
2.8 References	24
Chapter 3: A survey of grapevine leafroll-associated virus-2, grapevine leafroll-associated virus-3, and grapevine fleck virus in wine grape varieties and native grape species in Virginia.	
3.1 Introduction	38
3.2 Materials and Methods	39
3.2.1 <i>Survey of Virginia</i>	39
3.2.2 <i>Intensive sampling and spatial analysis of six vineyards in Virginia</i>	40
3.2.3 <i>Wild Vitis sampling from vineyard and isolated</i>	40
3.2.4 <i>Identification of vineyard mealybugs in Virginia</i>	40
3.2.5 <i>Statistical Methods</i>	40
3.3 Results	41
3.3.1 <i>Survey</i>	41
3.3.2 <i>Intensive Sampling</i>	42
3.3.3 <i>Visible symptoms, spatial association, and the presence of viruses</i>	43
3.3.4 <i>Associations between age of vines and mealybug presence</i>	43
3.3.5 <i>Wild grapevines</i>	43
3.3.6 <i>Identification of mealybug species</i>	44
3.4 Discussion	44
3.5 References	46
Chapter 4: Studies on mealybug biology, roles in GLD spread, and management	
4.1 Introduction	68
4.2 Materials and Methods	69
4.2.1 <i>Field Experiment 1, AREC vineyard</i>	69
4.2.2 <i>Field Experiment 2, Orange vineyard</i>	71
4.2.3 <i>Statistical Analysis</i>	71
4.3 Results	71
4.3.1 <i>AREC vineyard</i>	71
4.3.2 <i>Orange vineyard</i>	72
4.4 Discussion	73
4.5 References	76

## List of Figures

### Chapter 1.

- Figure 1.1:* Disease triangle of GLD showing how the pathogen (the grapevine leafroll-associated viruses), host (grapevines), and environment (insect vectors, planting strategies, insecticide sprays, etc.) are all inter-connected. 6
- Figure 1.2:* Disease cycle of GLD. Infected vines (new nursery materials, in-field vineyard vines, or possibly wild grapevines) are primary sources of GLD. 7

### Chapter 2.

- Figure 2.1:* Symptoms of GLD on a red-fruited variety, Cabernet Sauvignon (A) and on a white-fruited variety, Chardonnay (B). 36
- Figure 2.2:* Genome organization of GLRaV-2, GLRaV-3 and GFkV showing the relative position of the ORFs and their predicted expression products. 37

### Chapter 3:

- Figure 3.1:* Agarose gel showing products from one-tube-one-step RT-PCR for three different viruses. 57
- Figure 3.2:* Within-field spread of GLRaV-3 at the AHS Jr., AREC in Winchester, VA over a three-year time span. 58
- Figure 3.3:* Intensive sampling-plot at commercial vineyard A. 59
- Figure 3.4:* Intensive sampling-plot at commercial vineyard B. 60
- Figure 3.5:* Intensive sampling-plot at commercial vineyard C. 61
- Figure 3.6:* Intensive sampling-plot at commercial vineyard D. 62
- Figure 3.7:* Intensive sampling-plot at commercial vineyard E. 63
- Figure 3.8:* Symptom severity (0=no symptoms, to 4=100% foliar symptoms) by number of vines testing positive for GLRaV-3 at Commercial vineyard A, C, D, and E. 64
- Figure 3.9:* Contingency analysis of GLRaV-3 presence in vines planted both before and after 1990. 65
- Figure 3.10:* Contingency analysis of GLRaV-3 presence in vines with and without mealybugs. 66
- Figure 3.11:* Agarose gel showing products from multiplex PCR for several mealybug species. 67

**Chapter 4:**

*Figure 4.1* Field layout of the AREC plot. Panels, rows, and vines are shown. 84

*Figure 4.2:* Spread of GLRaV-3 within field between 2009 and 2011. 85

*Figure 4.3:* Mealybug (MB) counts on three different vines by treatment in 2011 at the AREC vineyard. 86

*Figure 4.4:* Box plot of mealybug counts on the same treated and untreated Chardonnay vines at a commercial vineyard in 2011 and 2012. 87

## List of Tables

### Chapter 2.

- Table 2.1:* Full Economic Impact of Wine and Wine Grapes on the Virginia Economy—2010: \$747.1 million. 34
- Table 2.2:* Top *V. vinifera*, inter-specific hybrids, and American (*V. labrusca*) grape varieties grown in Virginia in 2010 and 2011 according to the Virginia 2011 Commercial Grape Report. 35

### Chapter 3:

- Table 3.1:* Virus-specific oligonucleotide primers used in the RT-PCR and the size of the RT-PCR product. 48
- Table 3.2:* Species-specific primers for vineyard mealybugs used in the multiplex PCR and the size of the products. 49
- Table 3.3:* Mixed infections that occurred out of 415 total surveyed grapevines. 50
- Table 3.4:* Percentages and numbers of vines testing positive for at least one virus by variety in Virginia. 51
- Table 3.5:* Viruses detected from sampled vines in Maryland, North Carolina, Pennsylvania, and Texas by variety. 52
- Table 3.6:* Locations and years of intensive sampling including sampling grid size and variety tested. 53
- Table 3.7:* Spatial analysis of disease incidence in intensive sampling plots in Virginia. 54
- Table 3.8:* Wild grapevines sampled in Virginia by region and species. 55
- Table 3.9:* Number of vines positive for cases of mixed infections (multiple viruses within the same vine). 56

### Chapter 4:

- Table 4.1:* Virus specific oligonucleotide primers used in the RT-PCR and the sizes of their RT-PCR fragments. 79
- Table 4.2:* Counts of mealybugs on insecticide treated grapevines at AREC vineyard. 80
- Table 4.3:* Effect of treatment and vine location to the number of mealybugs per vine at AREC vineyard in 2010 and 2011 season. 81
- Table 4.4:* ANOVA results of the effect of sampling date and neonicotinoid insecticides at the Orange vineyard in 2011 and 2012. 82



*Table 4.5:* Effects of two in-season foliar applications of a neonicotinoid insecticide on the number of mealybugs at the Orange vineyard in 2011 and 2012. 83

# Chapter 1

## Introduction

### 1.1 Introduction

Virginia's modern wine grape industry started in the late 1970s with just six wineries on 286 acres (VDACS, 2011). Since then, this industry has seen significant growth. By 2007 there were over 130 wineries on nearly 3,000 acres (VDACS, 2011). Currently, there are 190 wineries and continued growth is expected (Zavatto, 2011). In 2007, 5,600 tons of wine grapes and 350,000 cases of wine were produced in Virginia, resulting in a national ranking of 8<sup>th</sup> overall in commercial grape production and bearing acreage (VDACS, 2011). From 2006 to 2007, Virginia bearing wine grape acreage increased 14%, which resulted in total cash receipts of over \$7.5 million (VDACS, 2011). With an estimated \$747.1 million economic impact in 2010 and as many as 4,753 full-time equivalent jobs, the Virginia wine industry is economically significant, and there is potential for more growth (Virginia Wine Board, 2012).

Grapevine leafroll disease (GLD) is a common viral disease found in all grapevine growing regions throughout the world. Approximately ten different viruses are associated with GLD (each named *Grapevine leafroll-associated virus* followed by a number). All GLRaVs belong to the same virus family, Closteroviridae. As with any disease, a complete understanding of the disease triangle depicting the interactions between the host, pathogen, and environment is crucial to comprehending the disease as a whole. In the case of grapevine leafroll disease, the host is grapevines, the pathogen is a group of virus agents, and the environment encompasses weather events, vectors, field management, and any potential interactions between them (Figure 1.1).

Typical GLD symptoms appear on foliage and fruit during the growing season, and become more apparent as the season progresses (Martinson et al., 2008). Symptoms on red-fruited varieties differ somewhat from those on white-fruited varieties (Fuchs, 2007). Red-fruited varieties typically have downward curling leaf margins and interveinal reddening; veins remain green. White-fruited varieties experience the same downward curling; however, there is only a slight chlorosis of the leaf (Fuchs, 2007). Subtle symptoms make this disease difficult to detect by visual assessment alone. Nutrient deficiencies, some trunk and vascular diseases, physical damage to the main trunk, and herbicide injury can also cause symptoms easily confused with GLD symptoms (Rayapati et al., 2008). In addition, symptom expression on many varieties can be very subtle. For example, in GLD surveys of New York's Finger Lakes grape growing regions, 68% of surveyed *Vitis vinifera* and hybrid grapevines (Martinson et al., 2008) and 33% (Wilcox, 1998) of surveyed *Vitis labrusca* grapevines were infected with

GLRaV-3. In their survey, *V. labrusca* vines were asymptomatic. Furthermore, GLRaVs' ability to infect *V. labrusca*-derived grapes, such as 'Concord' (Soule et al., 2006) suggests that other native species present in Virginia such as *V. cordifolia*, *V. rotundifolia*, and *V. riparia* could also be infected, even when asymptomatic.

GLD can significantly reduce both crop yield and grape quality (Kovacs et al., 2001). Both berry color intensity and Brix can be reduced and, thus, negatively affect the wine quality (Martinson et al., 2008; Rayapati et al., 2008; Cabaleiro et al., 1999). In severely infected vineyards with a susceptible variety, crop loss can range from 30% to 50% (Martinson et al., 2008). Loss of vigor can be caused by the infection, which also can make the vines more susceptible to cold injury and other environmental stress factors. In addition, infection by multiple viruses can affect fruit quality. For example, fruit quality parameters of vines infected with both GLRaV-3 and *Grapevine fleck virus* (GFkV, genus *Maculavirus*, family *Tymoviridae*) were inferior to those of healthy vines and vines infected only with GLRaV-3 (Kovacs et al., 2001).

Known means of transmission for the GLRaVs are vegetative propagation, grafting, and through insect vectors (i.e. mealybugs [Pseudococcidae] and soft scales [Coccidae]) (Belli et al., 1994; Cabaleiro & Segura, 1997; Douglas & Krüger, 2008; Peterson & Charles, 1997; Sforza et al., 2003; Tsai et al., 2008). Male mealybug species, unlike females, do not have mouthparts and, therefore, cannot feed nor transmit this disease (Grimes & Cone, 1985; Fuchs, 2007). Also, female mealybugs do not have wings, which limits their movement both on and between vine rows to short-distance crawling unless they are dispersed by wind (e.g. natural or spray induced air flows) (Grimes & Cone, 1985; Fuchs, 2007). Mealybugs can also be moved to neighboring vines by pruners, gloves, spray equipment, and even on the clothing worn by workers in the field. A full disease cycle can be seen in Figure 1.2.

Management of viral diseases is challenging. The only way to 'cure' a field of GLD is to rogue all the infected vines (assuming you can test all the vines) and replant in a few years, neither of which is economically feasible. In some of smaller operations, growers replace vines as symptoms appear. As noted above, visible symptoms are not the best criterion; thus, this practice will likely not eliminate all the infected vines and there is a chance that healthy vines may be removed. Another outcome of removing symptomatic vines is that uneven age structure within a vineyard often cause issues due to uneven fruit ripening of differently aged vines.

Often times, pesticides can be used to target virus vectors of virus agents. However, the effect is only prevention of spread of the disease; it cannot really eliminate the pathogen. However, it is often the only way for growers to reduce the risk of GLD because they may not be able to afford roguing infected vineyards. Therefore, it is important to assess methods for controlling insect vectors.

Prior to 1993, ELISA, PCR, and grafting to indicator hosts were the only techniques used to screen propagation materials by the Foundation Plant Service (FPS, Davis, CA), which was (and still is) the leading supplier of grapevines at that time; however, these methods were not adequately sensitive and robust to detect all virus-infected materials (Golino, 2008). Currently, due to adoption of testing standards from the National Clean Plant Network, virus-free vines can be purchased from FPS and other established grapevine nurseries. However, many vineyards were established prior to the 1990s and older vines, which are still in use, may contain viruses and can serve as a source of inoculum.

GLD has been suspected to be present in Virginia; however, there have been no grapevines tested until recently. A limited number of samples from Virginia vineyards tested

positive for GLRaV-3 in 2008 (M. Fuchs, *unpublished*, 2008). Virginia growers are becoming increasingly concerned about this emerging threat, especially since some white and hybrid varieties (e.g. Vidal blanc) tend to have asymptomatic infections (Kovacs et al., 2001). A recent biennial research needs/priority survey of Virginia Vineyards Association members in 2008 rated leafroll disease as a 3.6 on a 1-5 scale, with 5 being most important.

Current research seems to be trending toward the sequencing and phylogenetic analyses of these viruses; however, due to practical importance in Virginia, we are striving to understand the mechanisms of dispersal and epidemiology of this disease. Many studies that have examined in-field spread of this disease concluded that these viruses spread in a random pattern. This would be consistent with wind dissemination of insect vectors. Cabaleiro et al. (2008) found significant clustering of GLD-infected plants in one of four vineyards studied and significant levels of aggregation that strongly suggest vectorial spread from specific points within the field. The same study found movement of GLRaV-3 across rows and to adjacent vines. The first mealybug instars do not move very far by crawling and rarely reach an adjacent vine in this manner (Grasswitz, 2008). Although mealybugs can be easily wind dispersed, there would be a significant decline in the number of insects landing on a grapevine as the distance increased, thus, the overall range of dispersion for these insects should be very small (Grasswitz, 2008).

In this work, we aim to assess the prevalence of three common grapevine viruses: 1) Grapevine leafroll-associated virus-2 (GLRaV-2), 2) Grapevine leafroll associated virus-3 (GLRaV-3), and 3) Grapevine fleck virus (GFkV). We also aim to study the vectors of these viruses and determine spatial and temporal aspects of GLD and their associated viruses. We also will test management strategies by monitoring the movement of the insect vector and determining the efficacy of various insecticides. Dr. Nita initiated the statewide survey outlined above in 2009.

## 1.2 Objectives of this project

This project encompasses several objectives and in this thesis, these objectives are discussed in two main chapters:

- **Chapter 3:** A survey of grapevine leafroll-associated virus-2, grapevine leafroll-associated virus-3, and grapevine fleck virus in wine grape varieties and native grape species in Virginia.
  - **Objective 1:** To document the prevalence and spatio-temporal pattern of GLD and associated viruses in Virginia *V. vinifera* and inter-specific hybrids.
  - **Objective 2:** To determine whether native *Vitis* species serve as asymptomatic hosts and, therefore, serve as reservoirs of GLRaVs for newly established and replanted GLD-free vineyards.
  - **Objective 3:** To identify species of mealybugs present within vineyards in Virginia.
- **Chapter 4:** Studies on mealybug biology, roles in GLD spread, and management.
  - **Objective 1:** To develop observational data as to the presence of mealybugs as potential vector.

- **Objective 2:** To determine the movement of GLRaVs from infected vines to a newly planted clean vine within the same row and evaluate the efficacy of insecticides to restrict the movement of mealybugs.

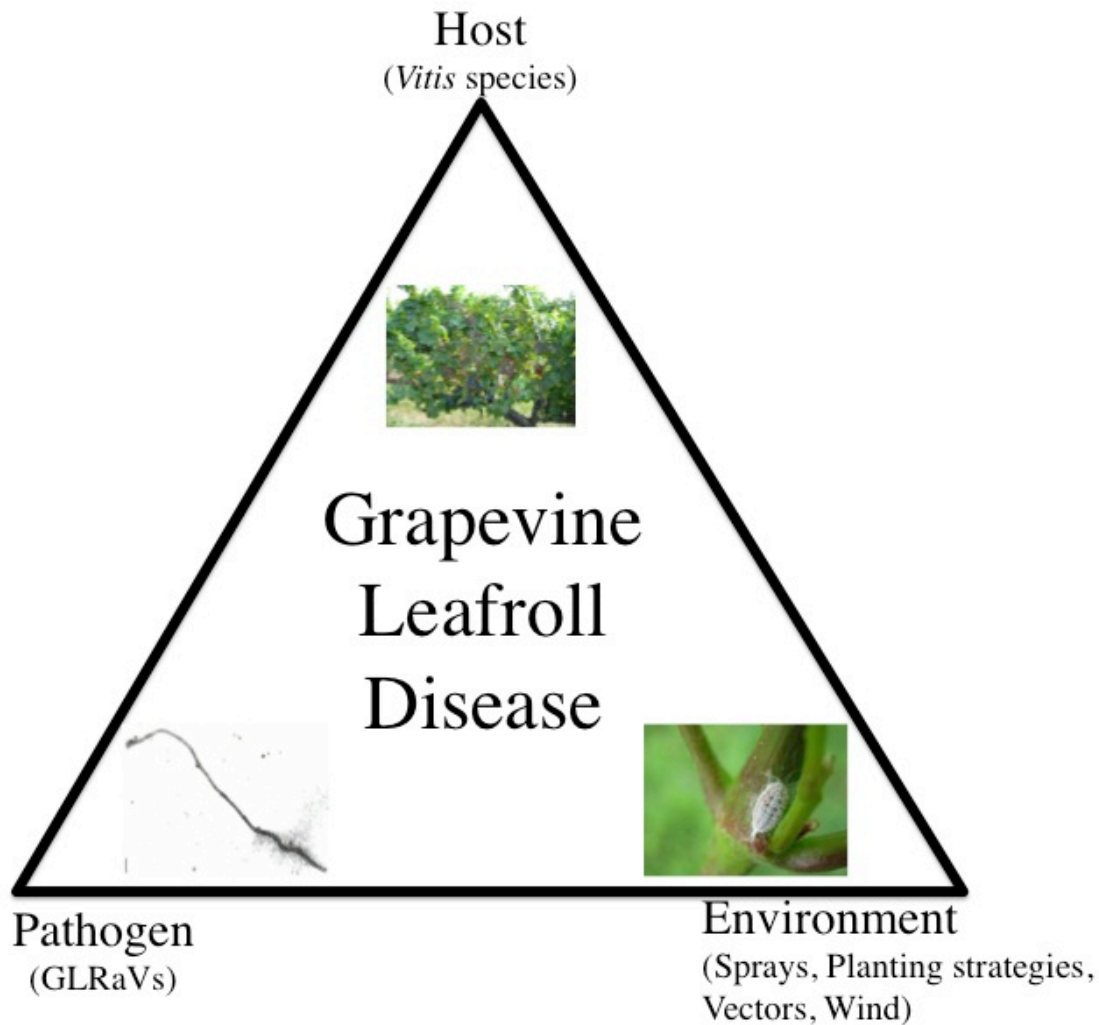
This study is necessary because of the lack of information on viral diseases of grapevines in the state of Virginia. Until now, there have been very few studies on viruses that infect grapevines in Virginia and no work has been attempted on their insect vectors within the state. Obtaining an understanding of GLD in Virginia is pertinent to keeping our wine industry in its current, expanding state. Knowledge of this disease complex will enable growers to make informed decisions about infected vineyards to aid in maximizing production, profit, and economic contributions.

This work will lead to a better understanding of the biology and epidemiology of these virus diseases. Additionally, the results from this study may also be useful for continued improvement to develop guidelines for clean (virus-free) plant material production. Results of this work will also aid growers who face the difficult decision-making process of removing GLD affected vines.

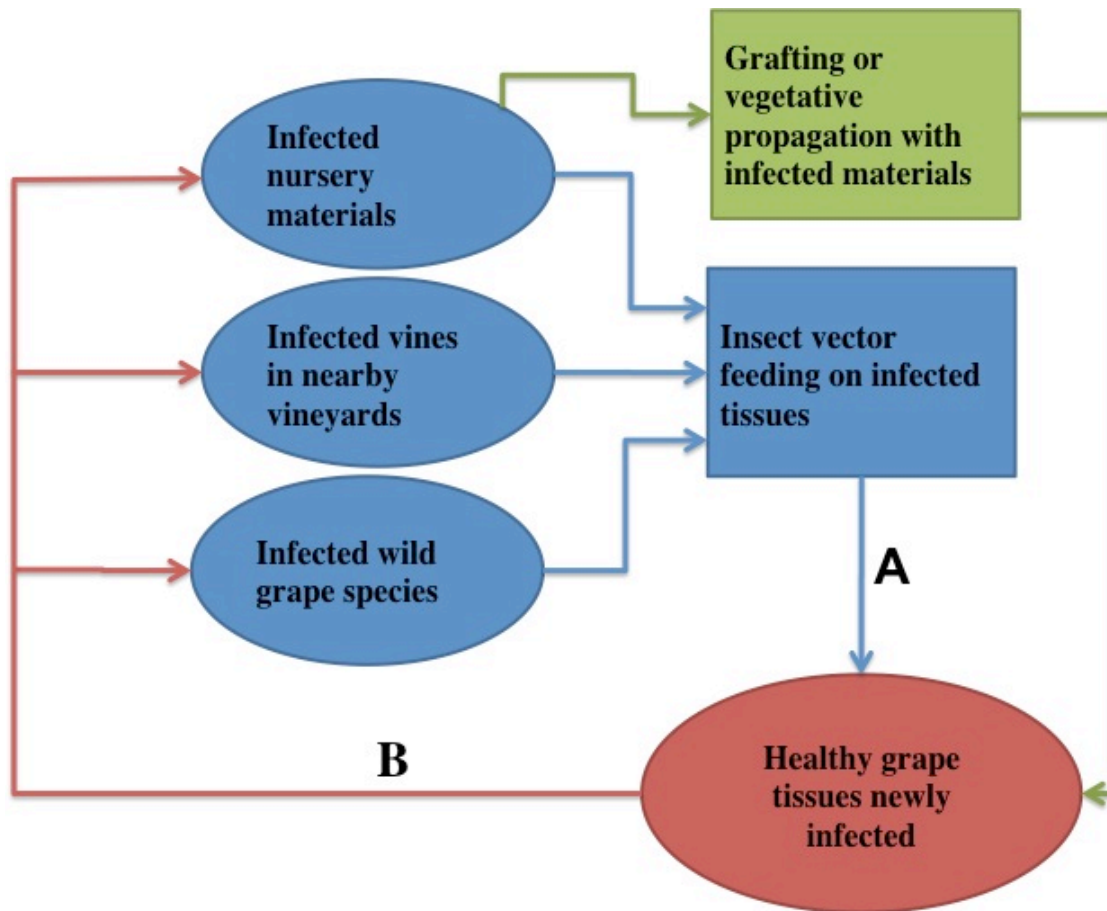
### 1.3 References

- Agrios, G. N. (2005). *Plant Pathology*: Elsevier Academic Press.
- Belli, G., Fortusini, A., Casati, P., Belli, L., Bianco, P. A., & Prati, S. (1994). Transmission of a grapevine leafroll associated closterovirus by the scale insect *Pulvinaria vitis* L. *Rivista di Patologia Vegetale*, 4(3), 105-108.
- Cabaleiro, C., Couceiro, C., Pereira, S., Cid, M., Barrasa, M., & Segura, A. (2008). Spatial analysis of epidemics of Grapevine leafroll associated virus-3. *European Journal of Plant Pathology*, 121(2), 121-130.
- Cabaleiro, C., Segura, A., & García-Berrios, J. J. (1999). Effects of grapevine leafroll-associated virus 3 on the physiology and must of *Vitis vinifera* L. cv. Albariño following contamination in the field. *American Journal of Enology and Viticulture*, 50(1), 40-44.
- Cabaleiro, C., & Segura, S. (1997). Field transmission of grapevine leafroll associated virus 3 (GLRaV-3) by the mealybug *Planococcus citri*. *Plant Disease*, 81(3), 283-287.
- Douglas, N., & Krüger, K. (2008). Transmission efficiency of Grapevine leafroll-associated virus 3 (GLRaV-3) by the mealybugs *Planococcus ficus* and *Pseudococcus longispinus* (Hemiptera: Pseudococcidae). *European Journal of Plant Pathology*, 122(2), 207-212.
- Fuchs, M. (2007). Grape leafroll disease factsheet Retrieved June, 2012, from [http://www.nysipm.cornell.edu/factsheets/grapes/diseases/grape\\_leafroll.pdf](http://www.nysipm.cornell.edu/factsheets/grapes/diseases/grape_leafroll.pdf)
- Fuchs, M., Martinson, T. E., Loeb, G. M., & Hoch, H. C. (2009). Survey for the three major leafroll disease-associated viruses in Finger Lakes vineyards in New York. *Plant Disease*, 93(4), 395-401.
- Gibbs, M. J., Koga, R., Moriyama, H., Pfeiffer, P., & Fukuhara, T. (2000). Phylogenetic analysis of some large double-stranded RNA replicons from plants suggests they evolved from a defective single-stranded RNA virus. *Journal of General Virology*, 81(1), 227-233.
- Golino, D. A., Weber, E., Sim, S., & Rowhani, A. (2008). Leafroll disease is spreading rapidly in a Napa Valley vineyard. *California Agriculture*, 62(4), 156-160.

- Grasswitz, T. R., & James, D. G. (2008). Movement of grape mealybug, *Pseudococcus maritimus*, on and between host plants. *Entomologia Experimentalis et Applicata*, 129(3), 268-275.
- Grimes, E. W., & Cone, W. W. (1985). Life history, sex attraction, mating, and natural enemies of the grape mealybug, *Pseudococcus maritimus* (Homoptera: Pseudococcidae). *Annals of the Entomological Society of America*, 78(4), 554-558.
- Hu, J. S., Gonsalves, D., & Teliz, D. (1990). Characterization of closterovirus-like particles associated with grapevine leafroll disease. *Journal of Phytopathology*, 128(1), 1-14.
- King, A., Leftkowitz, E., Adams, M. J., & Carstens, E. B. (2011). *Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses*: Elsevier.
- Kovacs, L. G., Hanami, H., Fortenberry, M., & Kaps, M. L. (2001). Latent infection by leafroll agent GLRaV-3 is linked to lower fruit quality in French-American hybrid grapevines Vidal blanc and St. Vincent. *American Journal of Enology and Viticulture*, 52(3), 254-259.
- Martelli, G. P., & Boudon-Padieu, E. (2006). *Directory of infectious diseases of grapevines*. (Vol. 55): Options Méditerranéennes, Series B. 55: 11-201.
- Petersen, C. L., & Charles, J. G. (1997). Transmission of grapevine leafroll-associated closteroviruses by *Pseudococcus longispinus* and *P. calceolariae*. *Plant Pathology*, 46(4), 509-515.
- Ravaz, L., & Verge, G. (1924). Le rugeau de la vigne. *Progrès Agricole et Viticole.*, 45, 11-17, 35-38, 86-89, 110-113, 135-141.
- Rayapati, N., O'Neal, S., & Walsh, D. (2008). Grapevine leafroll disease. *Washington State University Extension Bulletin EB2027E* Retrieved 2012, April, from <http://pubs.wsu.edu>
- Scheu, G. (1936). *Mein Winzerbuch*. Berlin: Reichenährstand Verlag.
- Sforza, R., Boudon-Padieu, E., & Greif, C. (2003). New mealybug species vectoring Grapevine leafroll-associated viruses-1 and -3 (GLRaV-1 and -3). *European Journal of Plant Pathology*, 109(9), 975-981.
- Soule, M. J., Eastwell, K. C., & Naidu, R. A. (2006). First Report of Grapevine leafroll associated virus-3 in American *Vitis* spp. Grapevines in Washington State. *Plant Disease*, 90(11), 1461-1461.
- Tsai, C. W., Chau, J., Fernandez, L., Bosco, D., Daane, K. M., & Almeida, R. P. P. (2008). Transmission of Grapevine leafroll-associated virus 3 by the vine mealybug (*Planococcus ficus*). *Phytopathology*, 98(10), 1093-1098.
- VDACS. (2011). Virginia Agriculture--Facts and Figures, from <http://www.vdacs.virginia.gov/agfacts/index.shtml>
- Wilcox, W. F., Jiang, Z. Y., & Gonsalves, D. (1998). Leafroll virus is common in cultivated American grapevines in western New York. *Plant Disease*, 82(9), 1062.
- Zavatto, A. (2011). Declaration of Vindependence. *Imbibe liquid culture magazine* Retrieved March, 2012, from <http://www.imbibemagazine.com/Virginia-s-WIne-Country>



**Figure 1.1:** Disease triangle of GLD showing how the pathogen (the grapevine leafroll-associated viruses), host (grapevines), and environment (insect vectors, planting strategies, insecticide sprays, etc.) are all inter-connected.



**Figure 1.2:** Disease cycle of GLD. Infected vines (new nursery materials, in-field vineyard vines, or possibly wild grapevines) are primary sources of GLD. The virus can be spread by insect vectors or through vegetative propagation or grafting with infectious materials. Vectors (mealybug species and scale insect species) move from infected vines to nearby healthy vines via step (A) through crawling, aerial dispersal, or vineyard workers. With the newly infected vines (B), the cycle can repeat since grapevines, once infected, remain virus-infected and a source of inoculum throughout their lifetime.



# Chapter 2

## Literature Review

### 2.1 INTRODUCTION TO THE GRAPE INDUSTRY

#### 2.1.1 History of the Grape Industry

Approximately 80,000 km<sup>2</sup> of land worldwide is devoted to the production of grapes that are processed into wine or non-alcoholic juices, dried into raisins, distilled into spirits, and consumed as table grapes, making the grape one of the most valuable horticultural crops in the world (Myles, 2011). Archeological records suggest cultivation of this cash crop, specifically the domesticated grape (*Vitis vinifera* subsp. *vinifera*), began about 6-8,000 years ago in the Near East, which is the modern day equivalent of Iraq, Turkey, and Syria (Myles, 2011; McGovern, 2004). It is believed that one species of wild grape, *Vitis vinifera* subsp. *sylvestris*, which can be seen along roadsides, riverbanks, and throughout forests in the Near East and western Mesopotamia, is the ancestor of present cultivars (This, 2006; Zohary, 1995). The *Vitis* genus, within the *Vitaceae* family, consists of around 60 inter-fertile species that are located mostly in the Northern Hemisphere (This, 2006). *Vitis vinifera*, indigenous to Eurasia, is the most extensively used species in the global wine industry and is suggested to have first appeared around 65 million years ago (This, 2006; de Saporta, 1879). Thousands of *Vitis vinifera* cultivars currently exist (Alleweldt, 1994; Galet, 2000; Levadoux, 1956; Viala, 1901-09); however, current wine marketing has led to only a few of these cultivars dominating the global wine market (This, 2006).

The domestication and use of grape as a crop seems to be linked to the discovery of wine (McGovern, 2004). Archeological evidence suggests the earliest evidence of wine production was in the northern Zagros Mountains of Iran at the Hajji Firuz Tepe site around 7400-7000 years ago (McGovern, 2004; McGovern, 1996). It is suggested that grape cultivation spread from the Near East to Egypt and Lower Mesopotamia (5,500-5,000 years ago), quickly followed by the entire Mediterranean area, reaching China by the 2<sup>nd</sup> century (McGovern, 2004; Royer, 1988). The Romans, who were first to give names to the cultivars (Roxas, 1814), spread grapevine cultivation to Europe along main trade routes (This, 2006). During the middle ages, the Roman Catholic Church played an important role in spread of viticulture during the crusades as grapevines could be exchanged and cultivation practices could be shared (Royer, 1988). New names were given to cultivars during the middle ages, some of which are still in use today like Pinot gris, Grenache, and Malvasia (Royer, 1988). During the 16<sup>th</sup> century, *Vitis vinifera* was introduced to New World countries (i.e. North America) by missionaries (This, 2006). Then the

growing regions expanded to South Africa, Australia, and New Zealand by the 19<sup>th</sup> century through cuttings (Royer, 1988).

### *2.1.2 The World Wine Grape Industry*

For years, European wines solely dominated the global wine market since Europe was one of the first successful wine production regions and there was little competition. In the 20<sup>th</sup> century however, new competition has been emerging from the “New World” (i.e. nations like the United States, Australia, and Chile) (Bisson, 2002). This new competition, along with the fact that wine consumption has been declining in the traditional European wine producing countries, has posed new issues to producers in Europe (Bisson, 2002). France, Italy, and Spain together produced more than half of all the world's wine supply in 2001; however, a 30-year trend (1971-2001) of wine consumption in those nations shows that their per capita consumption has fallen 40-50% which has led to an oversupply of wine from these areas (e.g. there is a smaller market for selling thus financial gains are lower) (Bisson, 2002; Protin, 1971; Anonymous, 2000). During the same period, the United States per capita wine consumption has nearly doubled (Bisson, 2002). In the last 20 years the “New World” producers have increased exports from 2% to 15% of the world export market (Bisson, 2002; Aigrain, 2001).

In 2010, the United States had a total of 3,824 km<sup>2</sup> devoted to vineyards, a 0.8% increase since 2007, placing the US in sixth place in terms of world vineyard square kilometers by country (Wine Institute). Similarly, in 2010 the United States was fourth (behind France, Italy, and Spain) in total world wine production, producing 2,653,187 liters that year--a 5.67% increase from 2007 (Wine Institute). Updated statistics can be found on the Wine Institutes website (<http://www.wineinstitute.org/resources/statistics>).

### *2.1.3 The Virginia Wine Industry*

Virginia's modern wine grape industry started in the late 1970s with just six wineries on 1.16 km<sup>2</sup> VDACS, 2011). Since then, this industry has seen significant growth. A recent study commissioned by the Virginia Wine Board (VWB) in 2010 (Table 2.1) shows that Virginia's booming wine industry contributes \$747.1 million annually to Virginia's economy, an increase of nearly 106% since the last economic study in 2005 (VWB, 2012). In 2010, 193 wineries on 10.93 total growing square kilometers were recorded resulting in a 50% and 35% increase, respectively, since the 2005 study (VWB, 2012). Most importantly 1,591 full-time equivalent jobs in the Virginia wine industry were created from 2005 to 2010 (VWB, 2012). Overall wine production in Virginia increased by 37% in this 5-year time span, producing a little under 3.8 million bottled liters, positioning Virginia at twelfth in the nation in wine production (VWB, 2012). Wine-related tourism in Virginia has also seen an increase since 2005, increasing 62% to account for \$1.6 million in 2010 (VWB, 2012). Wine-related tourism expenditures increased dramatically by 130%, reaching \$131 million in 2010 (VWB, 2012). In 2011, a record high in Virginia wine sales was attained with more than 5.5 million bottles sold, more than an 11% increase over the previous fiscal year (Caldwell, 2012). Currently, Virginia ranks fifth among the nation's largest wine grape producers (Caldwell, 2012). A list of common grape varieties grown in Virginia is outlined in Table 2.2.

## **2.2 Diseases of Grapevines**

### 2.2.1 Introduction

As with many crops, grapevines are susceptible to a significant amount of diseases. Bacteria, fungi, nematodes, insects, phytoplasmas, viruses, and virus-like agents all play important roles in infecting grapevines and affecting fruit production. Plant diseases, when severe, can change the outlook of crop industries. One disease in particular devastated the European grapevine industry in the early 1860s, changing it forever.

As importation of foreign vines to Europe from America became more common, so did the introduction of new diseases. In the early 1860s, European grapevines started to develop small galls on the underside of the leaves (Granett, 2001). These leaves then changed color (from yellow to red) as the season progressed and the vines produced almost no fruit (Granett, 2001). This disease was termed “Phylloxera”, and was soon found to be caused by an aphid-like insect, grape phylloxera (*Daktulosphaira vitifoliae* [Fitch 1855]; family Phylloxeridae) that was imported with American rootstocks that were introduced into Europe (Granett, 2001). These aphids lived on the roots of the grapevines, quickly killing them as they multiplied and fed (Granett, 2001). Since American grapevines are resistant to Phylloxera aphids, American grapevine rootstock was soon used with French varieties creating an effective means of resistance without changing the cultivar (Granett, 2001). In many grape-growing regions, European grapevines are still grafted on American rootstocks in order to prevent these aphids from causing another epidemic.

### 2.2.2 Diseases caused by Bacteria

Crown gall and Pierce’s disease are the two most important bacterial pathogens of grapevines. Crown gall is caused by the bacterium *Agrobacterium vitis*; *Agrobacterium tumefaciens* can be found on many other fruit crops, ornamentals, and trees (Agrios, 2005). This bacterium can invade grapevines that have physical damages to the tissues (weed whacking, mowing, and pruning cause many of these injuries). After bacterial invasion, galls form on the roots and/or base of the trunk of infected grapevines. Vascular tissues will be damaged by the formation of these galls and reduced yield, vine vigor, and vine decline result.

Pierce’s disease is a bacterial disease of grapevines caused by the pathogen *Xylella fastidiosa* (Myers, 2007). This pathogen resides in the xylem tissues within the vine and can be transmitted from plant to plant by several insects in Virginia: the glassy-winged sharpshooter (*Homalodisca vitripennis*), *Oncometopia orbona*, *Graphocephala versuta*, *Draeculacephala constricta* and spittlebug (*Clasirptora* sp.) (Myers, 2007; Schooley et al., 2010). Infected grapevines can decline within a few months to a few years after infection (Agrios, 2005). Early symptoms include marginal leaf scorch, cessation of cluster development and wilt, followed by a decline of the top of the canes and a dieback of the root system (Agrios, 2005). The majority of these symptoms are caused by formation of gum and tyloses (outgrowths of parenchyma cells) due to the grapevine’s reaction to the infection of this pathogen (Agrios, 2005).

### 2.2.3 Diseases caused by Fungi

Many fungal agents can cause disease on grapevines. In Virginia, black rot, downy mildew, powdery mildew, Botrytis gray mold/bunch rot, and Phomopsis cane and leaf spot are

among the most common. Black rot, caused by the fungus *Guignardia bidwellii*, is found mostly in wet climates (such as Ohio and Virginia) and initial symptoms can be seen as small, red necrotic spots on leaves in the spring (Agrios, 2005). Following this leaf spotting, pycnidia will form and infect the berries later in the season (Agrios, 2005). The infected berries will then shrivel and rot. These infected berries are referred to as “mummies” and serve as inoculum for the next season (Agrios, 2005). Black rot can significantly reduce crop yields in severely infected vineyards.

Downy mildew of grape is caused by *Plasmopara viticola*, which is an oomycete. *Plasmopara viticola* infects leaves and fruit from bloom to up to about 3-4 weeks post-bloom, at which point fruit become ontogenically resistant (Ellis, 2008). Infected leaves show yellow-green-translucent spots that later turn a reddish-brown on upper leaf surface, and whitish ‘downy’ growth of sporangia appears on underside of the infected leaf (Ellis, 2008). In addition to direct infection on clusters, this pathogen can cause premature defoliation that can lead to yield loss (Ellis, 2008).

Powdery mildew of grape, caused by *Erysiphe necator*, is commonly found in dry, warm climates (Ellis, 2008) but also in humid climates. Initial infection can be seen as small, white/gray patches of fungal growth on leaves and berries (Ellis, 2008). This fungus produces chasmothecia as sexual fruiting bodies and conidia throughout the season as asexual spores (Ellis, 2008). Overall, this disease results in reduced vine growth, reduced crop yield, poor fruit quality, and reduced winter hardiness (Ellis, 2008).

Botrytis gray mold, or bunch rot, caused by the pathogen *Botrytis cinerea*, infects buds and young fruit in cool, wet environmental conditions (Agrios, 2005). Infected leaves and young shoots have water-soaked patches that become reddish-brown that will lead to shoot death (Travis, 2004). In late summer, infected berries will rot and burst, reducing both crop yield and quality (Travis, 2004).

Another pathogen, *Phomopsis viticola*, causes Phomopsis cane and leaf spot. It infects grapevines early in the season to cause infection that appears as small leaf spots or as necrotic lesions on canes (Agrios, 2005). This pathogen can also cause rachis and fruit infection that can result in premature fruit drop and/or fruit rot (Ellis 2008).

#### 2.2.4 Diseases caused by Nematodes and Insects

Infestation by nematodes can cause a slow decline of grapevines (Pearson, 1988). In addition, some can transmit viruses (ex. *Xiphinema index* can transmit grapevine fanleaf virus) that will aid in the decline of the vine over time and may cause more severe effects.

As noted earlier, Phylloxera is a disease of grapevines caused by the grape root aphid (*Daktyloshpaira vitifolia*) (Agrios, 2005). These aphids feed on grape roots causing small gall formation (Agrios, 2005). A few weeks after gall formation the infected vines leaves turn yellow and/or red and fall off the vine by late July, early August (Agrios, 2005). Affected vines produce little to no fruit and usually the vines die within the next year (Agrios, 2005). This disease no longer causes significant damage due to resistant American rootstocks that can be used for grafting (Agrios, 2005).

#### 2.2.5 Diseases caused by Phytoplasmas, Viruses, and Virus-Like Agents

The most important phytoplasma disease in grapevines is grapevine yellows. There are different types of grapevine yellows: European (caused by either grapevine flavescence dorée phytoplasma or grapevine bois noir phytoplasma) and North American (caused by aster yellows phytoplasma and X-disease *Candidatus* phytoplasma spp.) (Beanland, 2006; Duduk et al., 2006). North American Grapevine yellows is common in Virginia and is spread primarily by leafhopper species (Beanland, 2006). This disease can be lethal to infected vines resulting in yellowing and downward rolling of leaves, die-back of shoot tips, and premature fruit abortion (Beanland, 2006). This fruit abortion can lead to severe monetary losses due to lack of crop.

The number of viruses (> 60) found in grapevines is the most detected in any perennial crop worldwide (Rayapati, 2012). A majority of these viruses are considered as minor threats, in that they are either of less economic significance or are limited in their geographical distribution; however, there are a few that are considered a major threat and very economically important (Rayapati, 2012).

The rugose wood complex is considered a major virus complex that affects grapevines. These viruses, which belong to the family *Flexiviridae*, in the two genera *Foveavirus* and *Vitivirus*, slowly affect the trunks/wood of grapevines by causing pitting, grooving, and severe aberration of the zone underneath the bark (Rosa, 2007). Examples of viruses associated with this complex include grapevine virus A (GVA), grapevine virus B (GVB), and rupestris stem pitting-associated virus (RSPaV). All are quite commonly found in grapevines around the world. In many cases, these viruses cause a slow decline of vines. Another major virus is the grapevine fanleaf virus (GFLV), one of the most devastating viral diseases of grapes worldwide (Andret-Link, 2004). GFLV is a *Nepovirus* transmitted by nematodes (*Xiphinema index*), which can severely reduce crop yield, fruit quality, and grapevine longevity (Andret-Link, 2004; Vuittenez, 1972). Tomato ringspot virus and tobacco ringspot virus are also two somewhat common viruses that infect grapevines in the US. They both are nematode transmissible and can cause severe stunting by the third year of infection (Schilder, 2011). However, American grapes are resistant to this disease (Schilder, 2011). Grapevine leafroll disease, caused by the grapevine leafroll-associated viruses, along with grapevine fleck virus are also major virus pathogens of grapevines worldwide and in Virginia, and will be described in detail in the following sections.

## **2.3 Grapevine Leafroll Disease (GLD)**

### *2.3.1 History*

Grapevine leafroll disease (GLD) is a common disease around the world. Possible accounts of this disease date back to 1906, where Sannino described what was thought to be a physiological disorder in Italian vineyards called “rossore”, characterized by early reddening of grapevine leaves (Martelli et al., 2006). In 1924, the same symptoms were observed in French vineyards by Ravaz and Verge (Ravaz & Verge, 1924). Regardless of when this disease first appeared, there is no question that GLD is now widespread across the world. The first actual survey for GLD was conducted in 1936 by G. Scheu and the disease was found to be widespread throughout German vineyards (Scheu, 1936). Scheu was also the first to demonstrate transmission of GLD to healthy *Vitis vinifera* by grafting (Scheu, 1935). In 1946, a disease known as “white emperor” disease in Europe (name due to the fruit color effects on the Emperor grape) was found to be graft-transmissible and was deemed a viral disease of grapevines (Harmon & Snyder, 1946). By 1958, Goheen et al. determined that “white emperor” disease and

GLD were actually identical (Goheen et al., 1958). In 1971, Mendgen described particles found in diseased grapevines with symptoms of flavescente dorée as closteroviruses associated with leafroll (Mendgen, 1971). A few years later, Namba et al. (1979) found closterovirus-like particles around 1000 nm long in phloem tissue samples from leafroll infected grapevines while healthy vines had none, suggesting that a closterovirus is the causal agent of this disease. Since then, GLD has been found in 33 additional countries around the globe, bringing the total to 36 (Martelli, 2006).

### 2.3.2 Symptoms

Typical GLD symptoms become more apparent as the season progresses (Fuchs et al., 2009). Symptoms on red-fruited varieties differ somewhat from those on white-fruited varieties (Figure 2.1) (Fuchs, 2007). Red-fruited varieties typically have downward curling leaf margins and interveinal reddening whereas veins remain green. White-fruited varieties experience the same downward curling; however, there is only a slight chlorosis of the leaf blade (Fuchs, 2007). Subtle symptoms make this disease difficult to detect by visual assessment alone. Other diseases and disorders, such as trunk diseases and nutrient deficiencies, can also cause symptoms easily confused with typical GLD symptoms. In addition, symptom expression on many varieties can be very subtle. For example, in GLD surveys of New York's Finger Lakes grape growing regions, 68% of surveyed *Vitis vinifera* and hybrid grapevines (Fuchs et al., 2009) and 33% of surveyed *Vitis labrusca* grapevines (Wilcox, 1998) were infected with GLRaV-3. In their survey, *V. labrusca* vines were asymptomatic. Furthermore, the ability of GLRaVs to infect *V. labrusca*-derived grapes, such as 'Concord' (Soule et al., 2006) indicates that other native species present in Virginia such as *V. cordifolia*, *V. rotundifolia*, and *V. riparia* could also be infected, even though these species may be asymptomatic.

GLD can significantly reduce both crop yield and grape quality (Kovacs et al., 2001). Berry color intensity, titratable acidity, pH, and Brix can be reduced and, thus, negatively affect the wine quality (Fuchs et al., 2009; Rayapati et al., 2008; Cabaleiro et al., 1999). Cabaleiro et al., 1999 found that on average, GLRaV-3 infected vines saw a 1g/L decrease in titratable acidity, a 1° decrease in Brix, and a slightly lower pH in all cases. Loss of vigor can be caused by the infection, which also can make the vines more susceptible to cold winter temperatures and other environmental stress factors. In addition, infection by multiple viruses can affect fruit quality. For example, fruit quality parameters of vines infected with both GLRaV-3 and *Grapevine fleck virus* (GFkV, genus *Maculavirus*) were inferior to those of healthy vines and vines infected only with GLRaV-3 (Kovacs et al., 2001). The vines with only GLRaV-3 had a 5% reduction in berry weight and titratable acidity was 5-9% higher (Kovacs et al., 2001). The vines that were infected with both GLRaV-3 and GFkV had a 7% reduction in berry weight and a 14% increase in titratable acidity (Kovacs et al., 2001).

### 2.3.3 Epidemiology and Vectors

Documented means of transmission for the GLRaVs are vegetative propagation, grafting, and through insect vectors (e.g. mealybugs [Pseudococcidae] and soft scales [Coccidae]) (Belli et al., 1994; Cabaleiro & Segura, 1997; Douglas & Krüger, 2008; Peterson & Charles, 1997; Sforza et al., 2003; Tsai et al., 2008). To date, it has been shown that these insects are able to transmit four GLRaVs (GLRaV-1, -3, -5, and -9) (Fuchs et al. 2009; Martelli et al., 2002). Male

mealybug species, unlike females, do not have mouthparts and, therefore, cannot feed nor transmit these viruses (Grimes & Cone, 1985; Fuchs, 2007). Also, female mealybugs do not have wings, which limits both within-vine and between-vine movement to short-distance crawling (Grimes & Cone, 1985; Fuchs, 2007). Thus, long distance (between-vine) dispersal is limited to wind dispersal (e.g. natural or spray induced air flows), and by contact by humans (pruners, gloves, spray equipment, etc.), or potentially other animals in the field.

Recent epidemiological studies of GLD in Spain followed the spatial distribution of GLRaV-3 in four vineyards (Beluso, Meaño, Goián, and Portomarín) from the early 1990s until around 2007 (Cabaleiro et al., 2008). In the Meaño study, GLRaV-3 was found to spread slowly throughout the study, suggesting vectorial transmission and movement; however, they were never able to find the vector (Cabaleiro et al., 2008). In the Goián and Portomarín vineyards, spatial patterns resulted in conclusions that the virus inoculum originated from the planting of infected plant material due to the random distribution of GLD (GLRaV-3) (Cabaleiro et al., 2008). However vector transmission was noted as a possibility when field spread was observed at the Goián plot (Cabaleiro et al., 2008). Golino et al. (2008) recorded a GLD spread rate of 10%/year in a Napa Valley, California vineyard block neighboring a heavily GLD infected block.

Spatial distribution and dynamics of GLD in Western Cape, South Africa that were studied between 2001 and 2005 showed four patterns of spread occurring: 1) primary spread, resulting in random spatial patterns, which is representative of infected materials used at planting; 2) secondary spread (or within field spread) to adjacent vines caused by mealybug that acquired the virus; 3) a correlation between leafroll infected vines in newly established vineyards in locations where infected vines had been removed, which was due to improper removal and/or survival of viruliferous mealybugs; and 4) gradients of leafroll infected vines decrease from edges to the middle to vineyards or across whole vineyards, most likely due to spreading of mealybugs (wind, pruners, birds, etc.) (Pietersen, 2004; Pietersen, 2006; Pietersen 2010).

A recent study by Rayapati et al. (2009) documented the presence of GLRaV-1, -2, -3, -4, -5, -9, *Rupestris stem pitting-associated virus* (RSPaV), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine fanleaf virus* (GFLV), and *Tomato ringspot virus* (ToRSV) in vineyards in the Pacific Northwest of the United States. They found an astounding 69.9% of samples tested positive for one of the six GLRaVs tested, and 30.1% of the positive vines contained more than one GLRaV (Rayapati et al., 2009). GVA, GVB, RSPaV, GFLV, and ToRSV were found in the region as well (Rayapati et al., 2009). Genetic variants of GLRaV-1, GLRaV-2, RSPaV and GFLV in the Pacific Northwest vineyards were also documented (Rayapati et al., 2009).

#### 2.3.4 Management Strategies

Clean plant programs are widely recognized as the first, and most important step to management of viral diseases such as GLD. Many grape producing countries have certification programs for clean plant production to provide clean plants to nurseries or to growers. Meristem tip culture has been used as a primary method for producing clean plants, and it is known as a successful method of eliminating GLRaVs from vines. The first documented successful elimination of leafroll by *in vitro* meristem tip-culture was by Sasahara et al. in 1981 in Japan (Sasahara et al., 1981). GLRaVs infect vines systemically and there are no remedies for plant virus infections in general (Agrios, 2005); thus, management options are limited to roguing of

infected vines, planting of virus-tested certified vines, and insect control (Pietersen, 2010). Since visible symptoms are not a reliable way to diagnose this disease, and most management options are expensive, it is crucial for growers to obtain positive identification of infection before management options are considered.

### 2.3.5 Detection of GLD and other grapevine viruses

Since the discovery of viruses, diverse methods of detection have developed over time. Methods used in the detection of the viruses associated with GLD can be used on other grapevine viruses as well, such as the virus that causes Grapevine Fleck disease. Both serological and molecular methods of detection have been utilized in studying grapevine viruses. In particular, methods such as the conventional enzyme-linked immunosorbent assay (ELISA; a method of antibody detection), reverse transcription-polymerase chain reaction (RT-PCR; amplification of RNA), and quantitative assays, such as real-time PCR (qRT-PCR; quantified polymerase chain reaction for amplified RNA/DNA).

The first polyclonal antibodies produced for detection of GLD using ELISA was by Gugerli et al. in 1984. Gugerli et al. had produced monoclonal antibodies for GLRaV-1 and GLRaV-3 by 1990. ELISA was the most commonly used method of detection for GLRaVs, and it was useful for screening of large sample sizes, as opposed to other methods being used at that time, such as ISEM (immunosorbent electron microscopy) and dsRNA analysis (detecting the double stranded RNA intermediate molecules specific to a virus) (Hu et al., 1991). Around 1994, when Minafra & Hadidi (1994) used PCR as a detection method for viruliferous mealybugs, PCR began to be adopted as a screening method for GLRaVs. Soon, spot-PCR techniques (the use of charged nylon membranes to sample and test using PCR for viruses) (La Notte et al., 1997) and degenerate primer techniques for PCR were developed (Routhe et al., 1998; Saldarelli et al., 1998), and then sequencing of virus genomes was initiated. The first genomic data published was the partial sequence of GLRaV-1 in 2000 by Fazeli & Rezaian. One-tube RT-PCR assays (Nassuth et al., 2000) and multiplex RT-PCR assays (using multiple forward primers but only one reverse primer in the same reaction tube) (Gambino & Gribaudo, 2006) have also been developed. Recently, Engel et al. (2010) developed a microarray for simultaneous detection of a wide range of different grapevine viruses.

### 2.3.6 Viruses Associated with GLD

GLD can be caused by any one of a group of phloem-limited viruses, referred to as the grapevine leafroll-associated viruses (GLRaVs) (Hu et al., 1990; Fuchs et al., 2009). Currently there are many GLRaVs, each named Grapevine leafroll-associated virus followed by the corresponding number (Fuchs, 2007). For example, the first virion in the group is referred to as Grapevine leafroll-associated virus-1 (GLRaV-1), then GLRaV-2, GLRaV-3, and so on through GLRaV-10. These virus particles vary in length ranging between 1,400 and 2,200 nm and all are flexuous filaments approximately 12nm in width (Fuchs, 2007).

All GLRaVs belong to the virus family *Closteroviridae*. A distinct characteristic of this family is that members code for a homolog of heat shock protein 70 (HSP70) proteins (ubiquitous molecular chaperones) (Dolja et al., 1994). GLRaVs differ in genus classification. GLRaV-2 has been placed in the genus *Closterovirus*, whereas GLRaV-1, -3, -4, -5, -6, -8, -9 have been placed into the recently created genus *Ampelovirus* (Martelli, 2006). Currently, no



virus associated with GLD has been placed into the third genus of the Closteroviridae family, *Crinivirus*. GLRaV-7 and -10 have yet to be assigned to a genus; however, recent sequencing efforts on GLRaV-7 may lead to a new genus. Two recent Greek isolates (GLRaV-Pr and GLRaV-De) are new members and additions assigned to the ampeloviruses (Maliogka et al., 2008).

All Closteroviruses contain positive-stranded ssRNA genomes (King, 2011). Closteroviridae virions are morphologically non-enveloped, filamentous, and generally plant host specific (King, 2011). Taxonomically, the *Capillovirus*, *Trichovirus*, and *Vitivirus* genera have particle morphology (e.g. long flexuous filamentous particles) identical to the Closteroviridae (King, 2011). However, the sequences of their coat proteins, genome sizes, genome organization, and expression strategies differ significantly from the Closteroviridae, thus resulting in their placement in the family Betaflexiviridae (King, 2011). Viruses belonging to the family Closteroviridae are part of the supergroup of alpha-like viruses. In order to be placed into the supergroup of alpha-like viruses, virions must meet three criteria: 1) a positive stranded RNA genome with a 5' cap, 2) production of a subgenomic RNA-encoding virion protein, and 3) homologous RdRp and helicase amino acid sequences (Gibbs, 2000). Similar viruses that are classified into the family Closteroviridae, genus Closterovirus, include *Beet yellow stunt virus* and *Citrus tristeza virus* (King, 2011).

Closteroviridae has three genera distinguished by their genome composition and genome structure; insect vectors also vary among these genera: 1) Closterovirus, type species *Beet yellow virus*, aphid transmitted 2) Ampelovirus, type species *Grapevine leafroll associated virus-3*, mealybug transmitted and 3) Crinivirus, type species *Lettuce infectious yellows virus*, white fly transmitted (Martelli et al., 2002). Of members of the Closteroviridae, GLRaV-2 is the only virus associated with GLD to be placed in the genus Closterovirus (Karasev, 2000; Martelli et al., 2002). Members of the genus Closterovirus include: *Beet yellows virus*, *Beet yellow stunt virus*, *Burdock yellows virus*, *Carnation necrotic fleck virus*, *Carrot yellow leaf virus*, *Citrus tristeza virus*, and *Wheat yellow leaf virus*, along with GLRaV-2 (Martelli et al., 2002).

The three viruses studied here are grapevine leafroll-associated virus-2, -3, and grapevine fleck virus.

## **2.4 Grapevine Leafroll-Associated Virus-2 (GLRaV-2)**

### *2.4.1 Taxonomy*

GLRaV-2 was found by Gugerli et al. (1984) as the second virus with a closterovirus-like particle associated with GLD. Nine years later, Gugerli & Ramel (1993) showed that the previously tested grapevine was actually infected by multiple viruses, which were then designated as GLRaV Ila and GLRaV I Ib. GLRaV I Ib was then shown to be the same as a GLRaV-2 isolate from France; thus, GLRaV I Ib was reclassified into GLRaV-2 and GLRaV I Ia was changed to GLRaV-6 (Boscia et al., 1984).

### *2.4.2 Morphology and Virion Properties*

GLRaV-2, like other members of the family, are flexuous, filamentous particles about 1400-1800nm in length (Gugerli et al., 1984). The full-length sequence of GLRaV-2 was

reported by Meng et al. (2000). GLRaV-2 has a positive sense RNA genome about 16.5kb in size that codes for nine open reading frames (ORFs) (Figure 2.2) (Zhu et al., 1998, Meng et al., 2005).

The nine ORFs are as follows: ORF1a encodes a polyprotein of an L1 and L2 leader proteases, a methyltransferase (MET) and a RNA helicase (HEL); ORF1b encodes the RNA dependent RNA polymerase (RdRp); ORF2 encodes p6, a 6kDa movement protein; ORF3 encodes the HSP70-homologue protein (Ling et al., 1998); ORF4 encodes p63, a 63kD, a protein involved in movement; ORF5 encodes a minor capsid protein (CPm); ORF6 encodes the major capsid protein (CP); ORF7 encodes p19, a 19kDa protein thought to be involved with motility; and ORF8 encodes a 24kDa protein (p24) also thought to be involved in movement (Liu et al., 2009; Meng et al., 2005; Zhu et al., 1998).

### 2.4.3 Transmission

GLRaV-2 is one of the viruses associated with GLD whose vector has not been currently identified. Since other members of the *Closterovirus* genus are transmitted by aphids, it is suspected that this virus may also be aphid transmitted (Karasev, 2000).

GLRaV-2 can be transmitted through vegetative propagation, grafting, and planting of infected vines. An interesting aspect of GLRaV-2 is that some isolates are actually mechanically transmissible (unlike all other GLRaVs) to herbaceous hosts like *Nicotiana benthamiana*; however, mechanical transmission is very inefficient (Goszczyński et al., 1996). GLRaV-2 has been implicated in other grapevine diseases such as graft incompatibility syndrome (Bonfigliolo et al., 2003; Greif et al., 1995), young vine decline (Golino et al., 2000), and rootstock stem lesion disease (Uyemoto et al., 2001). Further investigation into these disorders could uncover the vector(s) of GLRaV-2.

## 2.5 Grapevine Leafroll-Associated Virus-3 (GLRaV-3)

### 2.5.1 Taxonomy

The third closterovirus-like virus found to be associated with GLD was GLRaV-3 (Rosciglione & Gugerli, 1986). The family Closteroviridae was revised in 2002 and included GLRaV-3 as a type member of the Ampelovirus genus in the Closteroviridae family (Martelli et al., 2002). Viruses also belonging to the Ampelovirus genus, and thus closely related to GLRaV-3, are: GLRaV-1, -4, -5, -6, -9, *Little cherry virus-2* (LChV-2), *Pineapple mealybug wilt-associated virus-1* and -2 (PMWaV-1 and -2), and *Plum bark necrosis stem-pitting-associated virus* (PBNPaV) (Martelli et al., 2002).

Analysis of the ampeloviruses by Maliogka et al. (2009), divided the genera into two subgroups based on phylogenetic analysis of the Hsp70h, RdRp, and HEL domains. Subgroup I included GLRaV-4, -5, -6, -9, PMWaV-1, PBNPaV GLRaV-Pr, and GLRaV-De (Maliogka et al. 2009). Subgroup II included GLRaV-1, -3, PMWaV-2, and LChV-2. Further discovery and characterization of other viruses within the family are resulting in placements within new genera as well as reclassification of some.

### 2.5.2 Morphology and Virion Properties

GLRaV-3 is a flexuous virus particle around 1800 nm in length (Jooste, 2005). It is a positive-sense, single stranded RNA virus composed of 17,919 nucleotides (Jooste, 2005). The first full-length genome sequence of GLRaV-3 (NY-1) was obtained in 2004 by Ling et al. They showed that the large genome of GLRaV-3 had 13 open reading frames (ORFs) and demonstrated that the sequence was representative of a monopartite closterovirus (Figure 2.2) (Ling et al., 2004).

ORF1a encodes a polyprotein with four domains: a leader protease (L-Pro) (Ling et al., 2004), a methyltransferase (MET) (Ling et al., 1998), an AlkB domain (Engel et al., 2008), and a helicase (HEL) (Ling et al., 1998). ORF1b encodes an RNA dependent RNA polymerase (RdRp) (Ling et al., 1998). ORF2 encodes a p6 region involved in cell to cell virus movement (Dolja et al., 1994; Ling et al., 1998). ORF3 encodes a hydrophobic transmembrane protein and ORF 4 encodes the HSP70-homologue protein, characteristic of closteroviruses (Ling et al., 1998). ORF5 encodes an HSP90 protein while ORFs6 and 7 both encode the coat protein (ORF7 is just a duplicate copy of ORF6, a unique quality of closteroviruses (Boyko et al., 1992)) (Ling et al., 1998). ORFs 8-12 are suspected to encode virus silencing suppressors and the p20 proteins that are coded by ORF9 and 10 are movement proteins (Dolja et al., 2006).

### 2.5.3 Transmission

GLRaV-3 is transmitted semi-persistently by mealybugs (family *Pseudococcidae*) (Martelli et al., 2002). The first documented report of mealybug transmission of a GLRaV was GLRaV-3 in 1989 by Rosciglione & Gugerli. They confirmed that GLRaV-3 could be transmitted by the mealybug *Planococcus ficus* (vine mealybug) (Rosciglione & Gugerli, 1989). One year later, Engelbrecht and Kasdorf (1990) published work that supported the transmission of GLRaV-3 by *P. ficus* in South Africa. In 1989, Tanne et al. successfully transmitted GLRaV-3 from grapevine to grapevine using the long-tailed mealybug (*Pseudococcus longispinus*) in Israel. GLRaV-3 has now been shown to be transmitted by the following mealybug and soft scale insects: *Heliococcus adenostomae* (McKenzie adenostoma mealybug), *Phenacoccus aceris* (apple mealybug), *Pseudococcus longispinus* (long-tailed mealybug), *Ps. calceolariae* (citrophilus mealybug), *Ps. maritimus* (grape mealybug), *Ps. viburni* (obscure mealybug), *Planococcus citri* (citrus mealybug), *Pl. ficus* (vine mealybug), *Pulvinaria innumerabilis* (cottony maple scale), *Ceroplastes rusci* (fig wax scale), *Pulvinaria vitis* (wooly vine scale), and *Heliococcus bohemicus* (bohemian mealybug) (Belli et al., 1993; Cabaleiro et al., 1997; Petersen & Charles, 1997; Golino et al., 2002; Sforza et al., 2003; Mahfoudi et al., 2009; Tsai et al., 2010). Transmission efficiency studies have shown that one single mealybug nymph of *P. ficus* or *Ps. longispinus* is capable of infecting a healthy grapevine with GLRaV-3 (Douglas & Krüger, 2008). Similarly, first and second instars of *Pl. ficus* are more effective at transmitting the virus when compared to adult females (Mahfoudi et al., 2009). As young mealybugs are more mobile, smaller in size, and lighter in weight, it makes sense that young mealybugs would be more efficient at semi-persistent transmission of a phloem-limited virus.

As with all closteroviruses, GLRaV-3 can also be transmitted through vegetative propagation, grafting with infectious materials, and planting of infected vines. However, there are no reports yet of GLRaV-3 being transmitted mechanically.

## 2.6 Grapevine Fleck Virus (GFkV)

### 2.6.1 Grapevine Fleck Virus: The disease and symptoms

*Grapevine fleck virus* (GFkV) is a widespread viral disease of grapevines worldwide, including Washington State and Missouri in the United States (Martelli, 1993; Naidu et al., 2010; Milkus et al., 1999). GFkV was first documented in 1966 in *Vitis vinifera* from France under the name “grapevine marbrure virus” (Vuittenez et al., 1966) and was later characterized by Boulila et al. in 1990 under the name “grapevine phloem-limited isometric virus”. GFkV causes latent, seemingly symptomless, infections in both *Vitis vinifera*, and American grape rootstocks but induces foliar symptoms of scattered clearing of veinlets and leaf deformation in *Vitis rupestris*, which is the biological indicator for this virus (Hewitt et al., 1972; Brunt et al., 1996). In this indicator vine, GFkV elicits highly characteristic cytopathic structures called multivesiculated bodies (derived from mitochondria), most likely related to symptoms (Castellano & Martelli, 1984).

### 2.6.2 Epidemiology, Transmission, and Vectors

As with many viral diseases, GFkV affects many grape-growing areas around the world, making it important to determine epidemiological factors associated with the causal agent. The agent is limited to the phloem of grapevine tissues, is non-mechanically transmissible, and cannot be transmitted through seed (Boscia et al., 1991; Martelli et al., 2002). Spread of this disease in the field has been documented in South Africa (Engelbrecht & Kasdorf, 1990) and Italy (Fortusini et al., 1996) but to date, the vector of GFkV is still unknown (Walter & Martelli, 1997; Glasa et al., 2011). However, the virus’s ability to be graft transmissible allows the virus to spread rapidly through infectious propagation materials (Glasa et al., 2011).

### 2.6.3 Management Strategies

Since the insect vector is unknown, clean plant programs are probably the most important and only means of management of GFkV. As this disease is latent and usually produces no visible symptoms, molecular or immunoassay-based detection need to be utilized for diagnosing this disease. It is crucial for growers to obtain positive identification of infection before management options are considered. In addition, GFkV can cause severe damage when it is present in a vine with other grapevine viruses (e.g. GLRaV-3) (Walter & Martelli, 1997). Since these mixed infections amplify severity of symptoms, regular testing needs to be implemented for GFkV and other grapevine viruses at nurseries.

### 2.6.4 Taxonomy

*Grapevine fleck virus* belongs to the family *Tymoviridae*, which is comprised of the genera *Tymovirus* (type member *Turnip yellow mosaic virus* [TYMV]), *Marafivirus* (type member *Maize rayado fino virus* [MRFV]), and the newly established *Maculavirus* (type member GFkV) (Martelli et al., 2002). Members of the *Tymoviridae* family share five main characteristics: 1) members are non-enveloped, isometric particles about 30nm in diameter; 2) members are made up of two capsids with and without RNA that form two separate centrifugal components; 3) members possess a positive-sense, single stranded RNA genome with a high

cytidine content; 4) a specific replication strategy; and 5) the presence of unique cytopathic structures in plant infected cells (Martelli et al., 2002).

As a *Maculavirus*, GFkV meets the criteria for separating species. Separation of species requires an overall sequence identity of less than 70%, a capsid protein sequence less than 85%, and serological specificity (Martelli et al., 2002). A recent study by Glasa et al. (2011) suggested that GFkV isolates can be split into two distinct molecular groups and more isolates fell into group one rather than group two, and group one was less variable. Grapevine asteroid mosaic-associated virus (GAMaV) and Grapevine redglobe virus (GRGV), the 47<sup>th</sup> virus to be found in grapevines (Walter & Martelli, 1997) are two viruses that are phylogenetically similar to GFkV (Sabanadzovic et al., 2000).

### 2.6.5 Morphology and Virion Properties

The only complete genome sequence of GFkV is for an Italian isolate (MT48) (Sabanadzovic et al., 2001); a few partial sequences of other isolates have now been completed. As the type member of the maculaviruses, GFkV has the largest genome within the family at 7.5 kb of RNA, encoding four ORFs (Figure 2.2) (Martelli et al., 2002). ORF1 (215 kDa) encodes a polyprotein for replication-associated proteins: a methyltransferase (MET); a helicase (HEL); and an RNA dependent RNA polymerase (RdRp) (Martelli et al., 2002). ORF2 encodes the coat protein (CP) while ORF3 and ORF4 encode for p31 and p16 respectively, both showing a relationship to movement proteins of tymoviruses (Martelli et al., 2002).

## 2.7 Mealybugs and Soft Scale Insects

### 2.7.1 Mealybugs, scale insects, and the order Hemiptera

Mealybugs and scale insects belong to the order Hemiptera, which is a large, diverse group including true bugs, cicadas, hoppers, psyllids, whiteflies, aphids, and scale insects (Johnson and Triplehorn, 2005). Previously, this order was divided into two different orders (Hemiptera and Homoptera) due to the immense diversity within the group (Johnson and Triplehorn, 2005). The key characteristic of this group is a unique piercing/sucking mouthpart (Johnson and Triplehorn, 2005). This intricate mouthpart is used by all insects of this order for sucking both plant sap and blood (Johnson and Triplehorn, 2005).

Members of this order usually undergo a simple metamorphosis (Johnson and Triplehorn, 2005). Mealybugs and scale insects are in the suborder Sternorrhyncha, which are known to have very complex life cycles involving bisexual and parthenogenetic generation, winged and wingless individuals and/or generations, and alternations of food plants. Some species can vector plant or human/animal pathogens (Johnson and Triplehorn, 2005).

Mealybugs and scale insects are further classified into the superfamily Coccoidea (Johnson and Triplehorn, 2005). This superfamily contains 16 different families: Margarodidae (giant coccids, ground pearls), Ortheziidae (ensign coccids), Pseudococcidae (mealybugs), Eriococcidae (felt scales), Cryptococcidae (bark-crevice scales), Kermesidae (gall-like coccids), Dactylopiidae (cochineal insects), Asterolecaniidae (pit scales), Cerococcidae (ornate pit scales), Lecanodiaspididae (false pit scales), Aclerdidae (grass scales), Coccidae (soft scales, wax scales, tortoise scales), Kerriidae (lac scales), Phoenicococcidae (date scales), Conchaspidae (false armored scales), and Diaspididae (armored scales) (Johnson and Triplehorn, 2005).

To date, the only vectors of the GLRaVs are in the families Coccoidea and Pseudococcidae, thus making their biological characteristics more pertinent to understand GDL disease biology.

### 2.7.2 *The Family Coccoidea (scale insects)*

Females are wingless and usually legless and sessile; however, the males have a single pair of wings (they are rarely wingless) (Johnson and Triplehorn, 2005). Visually, males of this superfamily look like small gnats, except mouthparts are absent and they have a style-like process at the end of the abdomen (Johnson and Triplehorn, 2005). Adult males in this superfamily lack mouthparts and do not feed (Johnson and Triplehorn, 2005). The abdomen of males end in one long, style-like process and their hind wings are small, halter-like processes that terminate in a hooked bristle (Johnson and Triplehorn, 2005). Males have two antennae with 10-25 segments; on the other hand, females may have two antennae with up to 11 segments or they may lack antennae (Johnson and Triplehorn, 2005).

Development of members of this superfamily is complex. The first-instar nymphs are very active, having legs and antennae, often referred to as “crawlers” (Johnson and Triplehorn, 2005). Legs and antennae are often lost following the first molt and the insect becomes sessile (Johnson and Triplehorn, 2005). During this period, waxy or scale-like covering is secreted from the insect, covering the body (Johnson and Triplehorn, 2005). Females stay covered under the scale as they mature into adults at which time they produce eggs and live first instars (Johnson and Triplehorn, 2005). Males develop similarly to the females; however, before the final instar stage, wings develop (Johnson and Triplehorn, 2005).

### 2.7.3 *The Family Pseudococcidae (mealybugs)*

The name mealybug derived from the waxy secretions that cover their body (Johnson and Triplehorn, 2005). The body of a female is elongate-oval, segmented, and has three legs (Johnson and Triplehorn, 2005). Some species lay eggs, which are placed in a cottony wax, while others give birth to live 1<sup>st</sup> instars (Johnson and Triplehorn, 2005). There are over 240 different species of mealybugs in North America alone, many of which are problematic when it comes to crops (either by vectoring disease or by feeding on crops) (Johnson and Triplehorn, 2005). Members of this family excrete honeydew (Johnson and Triplehorn, 2005), which is a major attractant to ants who “herd” mealybugs to utilize this sugar source. Honeydew also promotes the growth of sooty mold by providing nutrients to microbes that live on plant surfaces.

### 2.7.4 *Roles of Coccid and Pseudococcid in GLD*

In the early 1960s, a few mealybugs were suspected of having the ability to transmit GLD (Rosciiglione et al., 1983); however, there was no actual proof of GLD’s ability to spread naturally in the field until 1973 (Dimitrijevic, 1973). Following that discovery, Rosciiglione & Gugerli (1989) became the first to document the successful transmission of a closterovirus associated with GLD to a healthy grapevine in a laboratory by the mealybug *Pl. ficus*. Tanne et al. (1989) and Engelbrecht & Kasdorf (1990) confirmed *Pl. ficus* was able to transmit GLD to healthy vines.

Transmission of GLD by these vectors is considered semi-persistent, since acquisition of the virus takes approximately 0.25-12 h and the virus can be retained within the vector for 12 h-5days (Charles et al., 2006). Charles et al. (2006) also found that transmission efficiency among mealybugs is variable at around 15-25%. Another study has shown that the first instars of mealybugs are the most efficient vectors (Petersen & Charles, 1997). However, the vector's ability to transmit the virus after transfer to a new plant is reduced significantly, as found by Cabaleiro & Segura (1997). According to their study, the GLRaV-3 infected vines remained latent and undetectable by ELISA for 13 months (Cabaleiro & Segura, 1997). A recent study in the Finger Lakes region (NY, United States) by Marc Fuchs et al. (2009) showed that individual mealybugs could acquire GLRaV-1 and GLRaV-3 simultaneously through direct feeding on host plants.

It is known that some whitefly and aphid species may contain endosymbiotic bacteria that produce proteins that play a role in virus transmission (van den Heuvel et al., 1994; Morin et al., 1999). No such work has been done yet on mealybugs, even though they also contain these endosymbionts.

### 2.7.5 *Biology and movement of mealybugs associated with GLD on grape*

Of all the stages in mealybug development, first instars are the most mobile since mealybugs tend to settle as soon as they encounter dense areas of trichomes or cracks in the shoot of vines (Cornwell, 1958; Furness, 1976; Grasswitz & James, 2008). Adult females do move later in their lives seeking sheltered areas underneath the bark of vines where they lay their eggs (Grasswitz & James, 2008).

The life cycle of the grape mealybug (*P. maritimus*), which is a common species in the north-west coast of the United States, is not very complicated. The grape mealybug overwinters as eggs, first instars, or even second instar nymphs underneath loose bark in cottony sacs (Varela, 2005). As spring approaches, the young nymphs move to the base of spurs, eventually reaching new shoots in late May and June (Valera, 2005). Throughout June, the adult females move back to the old wood and lay eggs in the loose bark, and these eggs hatch by mid-June to early July (Valera, 2005). The newly hatched nymphs quickly move to infest fruit and foliage in late June and July (Valera, 2005). Some females have been observed to lay eggs around August in the fruit clusters as well (Valera, 2005). Finally, by late August the adult females return to the old wood to lay eggs that will overwintering (Valera, 2005). A graphic representation of this and the life cycle of the vine mealybug can be found at: <http://cesonoma.ucdavis.edu/files/27229.pdf>

In California, the three main mealybug pests are the grape mealybug, the obscure mealybug, and the vine mealybug. Each has their own seasonal activities and lifecycles. While the grape mealybug only has two generations per year (summer and winter), the obscure mealybug has 2-3 overlapping generations and the vine mealybug can have up to 6 overlapping generations (Valera & Smith, 2009). As temperatures warm in the spring, young nymphs move up the trunk and go toward the base of spurs or onto new shoots (Valera & Smith, 2009). For grape mealybugs, females return to the old wood in the summer to lay eggs (Valera & Smith, 2009). For the obscure and vine mealybugs, the summer is a time of population boom and all stages of mealybug development can be seen on the same vine (particularly for the vine mealybug) (Valera & Smith, 2009). The vine mealybug is the only one of the three that actually lays eggs on any aboveground part of the grapevine, including leaves higher than the fruiting zone (Valera & Smith, 2009). In the fall, population decline can be seen in all species, as the

females and nymphs return to the old wood to lay eggs and feed (Valera & Smith, 2009). In the case of the vine mealybug, these colder temperatures drive them to the lower trunk and even the roots of the grapevine (Valera & Smith, 2009).

Environment plays an important role in mealybug activities. Cornwell (1958) found a strong correlation between motility of mealybugs and temperature, where higher temperatures correlated with a greater population and a higher mobility rate. Grasswitz and James (2008) also found that second generation mealybugs moved further and took longer to become sessile than the first generation, most likely due to differences in shoot development and temperature.

A recent study on the grape mealybug (*P. maritimus*) by Grasswitz & James (2008) looked at movement of mealybugs over a three-year time span. During the three years, they found mealybugs are capable of walking on vines in the field up to 90 cm. from their original point of release; however, most were found walking considerably shorter distances. Aerial dispersal of mealybugs was observed, proving that wind dispersal of these tiny insects between rows and vineyards is possible (Grasswitz & James, 2008). A few studies have looked into aerial dispersal of mealybugs. The maximum distance recorded for aerial dispersal was 103 m, but the majority of studies have shown dispersal of a little over 10 m (Strickland, 1950; Cornwell, 1960; Barrass et al., 1994). In addition, ants are known to utilize the honeydew produced by mealybugs. This causes ants to actually herd and protect mealybugs, increasing mobility of these insects, as well as promoting high mealybug populations (Geiger & Daane, 2001).

#### 2.7.6 Management of mealybugs and soft scales

Combinations of chemical control, biological control, and cultivation methods can be implemented to aid in management of mealybugs and scale insects. The first step in mealybug/softscale management is avoidance: do not plant in mealybug-infested locations. Also, use of cultivars with genetic resistance can be considered. There is evidence that some cultivars are more sensitive to mealybug infestations than others (e.g. Chardonnay, Cabernet Sauvignon, Merlot (Walton, 2001). Cultural practice such as removal of a reservoir area from vineyards can be used. Mealybugs can survive and breed in weeds (on the roots or on above ground tissues); therefore weed management is an option for reducing the mealybug population. Additionally, management of ant populations with chemical sprays has also been considered (Daane et al., 2004). In addition, it is important to protect/promote natural enemies/beneficial insects (parasitic wasps, spiders, green lacewings, transverse ladybirds, etc.) (Grimes & Cone, 1985) that will reduce the mealybug population.

Chemical control is another option. A dormant or delayed dormant oil spray can provide control of scale insects and mealybugs if populations are not too high in vineyards by lowering emergence rates due to suffocation (Smith et al., 2009; Varela et al., 2012). Also, in-season spray program, starting from delayed dormant stage, has been practiced (Schooley et al., 2010). Insect growth regulators, such as buprofezin (Applaud), can be applied, as well as neonicotinoids like acetamiprid (Assail), dinotefuran (Venom), and imidacloprid (Provado, Admire) (Schooley et al., 2010). Organophosphates (phosmet [Imidan]) have shown efficiency at control, but are impractical for most growers due to its PHI of 14 days (Schooley et al., 2010). Pyrethroids such as cyfluthrin (Baythroid) can also be used as a postharvest control of mealybugs (Schooley et al., 2010). Spirotetramat (Movento) is a tetracyclic acid derivative (ketoenone) chemical spray that can also be utilized to kill off mealybugs in-season (Schooley et al., 2010). Chemical management



can also play a key role in the dispersal of mealybugs. Spraying with high power sprayers can spread mealybugs throughout the field with wind force.

Mealybug parasitoids have also been investigated as a possible control strategy on grapevines in California and on other crops elsewhere. Parasitic wasps, green and brown lacewings, and coccinellid beetles have all been listed as potential biological controls for mealybugs (Daane et al., 2006). The most effective agent is the parasitic wasp *Anagyrus pseudococci*, which acts as an internal parasitoid. This wasp was shown in one study to parasitize almost 90% of the sampled mealybug population (Daane, Malakar-Kuenen et al., 2004). A similar study showed that mealybug populations over an entire season was significantly lowered where *Anagyrus pseudococci* was released than at the untreated area (however this population decline could not be attributed to the wasp alone since other parasitoids and weather events may have contributed to the decline) (Daane et al., 2006). One potential issue associated with these parasitic wasps is that they prefer larger mealybugs (Daane et al., 2006). This preference may hinder their ability to control the population early in the season when only crawlers are present. Therefore, the efficacy of this beneficial may be best when the release of this wasp occurs after an early season application of insecticide is made.

Some research has shown that pesticides can actually reduce predators of mealybugs. The active ingredients fipronil, a-cypermethrin, and chlorpyrifos-methyl can cause significant acute toxicity to some parasitoids, whereas other chemicals like buprofezin, mancozeb, spirotetramat, and Prev-Am have no toxic effects (Mgocheki & Addison, 2009; Mansour et al., 2011).

If there are significant numbers of ants within a vineyard, protection of mealybugs by ants (Daane, Sime et al., 2004) may hinder the ability of beneficial insects to naturally reduce the mealybug populations. Therefore, if there is a significant presence of ants within a vineyard, the efficacy of a biological agent may suffer. Thus, controlling the ant populations may benefit naturally-occurring parasitoids. Moreover, ants are known to assist movement of mealybugs (Daane et al., 2003). Phillips and Sherk, 1991 found that an increase of the obscure mealybug was associated with Argentine ant activity. By controlling the ant population, the level of mealybug infestation was significantly reduced. They also found that the timing of ant control was critical and should be applied as soon as the ants start foraging for food in spring prior to re-colonization. In their study an organophosphate (Lorsban 4E, Dow AgroSciences) provided the best results when applied in the early spring to the base of the grapevine trunks.

A few studies (Daane et al., 2006; Walton et al., 2006) have shown that pheromone-based mating disruption for the vine mealybug seems to significantly lower mealybug population levels and egg production when applied to vineyards using a sprayable, microencapsulated formula. This provides another possible option that can be utilized by growers in combination with ant control and pesticide use.

It has also been shown that vines that are cane-pruned have fewer mealybugs present than vines that are spur-pruned due to the increased amount of bark left with spur-pruned vines (Geiger et al., 2001). This suggests that spur-pruning can reduce mealybug populations. The same study found that mealybug locations on vines constantly changed throughout the season, and their location was directly related to the insect's stages of development. The same group also found that mealybugs within infested vineyards were found in clumped patches in uneven distributions, due to their movement limitations.

## 2.8 References

- Agrios, G. N. (2005). *Plant Pathology*: Elsevier Academic Press.
- Aigrain, P. (2001). Conjuncture vitiviniculture mondiale. *Office Int Vigne Vin Bull*, 74, 209-225.
- Alleweldt, G., & Dettweiler, E. (1994). *The Genetic Resources of Vitis: World List of Grapevine Collections. (2nd edition)*: Geilweilerhof.
- Andret-Link, P., Laporte, C., Valat, L., Ritzenthaler, C., Demangeat, G., Vigne, E., . . . Fuchs, M. (2004). Grapevine fanleaf virus: still a major threat to the grapevine industry. *Journal of Plant Pathology*, 86(3), 183-195.
- Anonymous. (2000). The state of viticulture in the world and the statistical information for 1999. *Office Int Vigne Vin Bull*, (suppl.) 20-30.
- Barrass, I. C., Jerie, P., & Ward, S. A. (1994). Aerial dispersal of first- and second-instar longtailed mealybug, *Pseudococcus longispinus* (Targioni Tozzetti) (Pseudococcidae: Hemiptera). *Australian Journal of Experimental Agriculture*, 34(8), 1205-1208.
- Beanland, L., Noble, R., & Wolf, T. K. (2006). Spatial and temporal distribution of North American grapevine yellows disease and of potential vectors of the causal phytoplasmas in Virginia. *Environmental Entomology*, 35(2), 332-344.
- Belli, G., Fortusini, A., Casati, P., Belli, L., Bianco, P. A., & Prati, S. (1994). Transmission of a grapevine leafroll associated closterovirus by the scale insect *Pulvinaria vitis* L. *Rivista di Patologia Vegetale*, 4(3), 105-108.
- Belli, G., Fortusini, A., & Prati, S. (1993). Natural spread of grapevine leafroll disease in a vineyard of Northern Italy. Paper presented at the Extended Abstracts 11th meeting of ICVG, 1993, Montreaux.
- Bisson, L. F., Waterhouse, A. L., Ebeler, S. E., Walker, M. A., & Lapsley, J. T. (2002). The present and future of the international wine industry. [10.1038/nature01018]. *Nature*, 418(6898), 696-699.
- Bonfiglioli, R., Edwards, F., Hoskins, N., & Pantaleo, A. (2003). Graft incompatibility syndrome in New Zealand Merlot vines involves another possible variant of GLRaV-2. *Australian & New Zealand Grapegrower & Winemaker* (476), 50-54.
- Boscia, D., Greif, C., Gugerli, P., Martelli, G. P., Walter, B., & Gonsalves, D. (1995). Nomenclature of grapevine leafroll-associated putative closteroviruses. *Vitis*, 34(3), 171-175.
- Boscia, D., Martelli, G. P., Savino, V., & Castellano, M. A. (1991). Identification of the agent of grapevine fleck disease. *Vitis*, 30(2), 97-105.
- Boulila, M., Boscia, D., Terlizzi, B. d., Castellano, M. A., Minafra, A., Savino, V., & Martelli, G. P. (1990). Some properties of a phloem-limited non mechanically-transmissible grapevine virus. *Journal of Phytopathology*, 129(2), 151-158.
- Boyko, V. P., Karasev, A. V., Agranovsky, A. A., Koonin, E. V., & Dolja, V. V. (1992). Coat protein gene duplication in a filamentous RNA virus of plants. *Proceedings of the National Academy of Sciences of the United States of America*, 89(19), 9156-9160.
- Brunt, A. A., Crabtree, K., Dallwitz, M. J., Gibbs, A. J., Watson, L., & Zurcher, E. J. e. (1996). Plant Viruses Online: Descriptions and Lists from the VIDE Database. Version: 20th, August 1996, from <http://biology.anu.edu.au/Groups/MES/vide/>
- Cabaleiro, C., Couceiro, C., Pereira, S., Cid, M., Barrasa, M., & Segura, A. (2008). Spatial analysis of epidemics of Grapevine leafroll associated virus-3. *European Journal of Plant Pathology*, 121(2), 121-130.

- Cabaleiro, C., Segura, A., & García-Berrios, J. J. (1999). Effects of grapevine leafroll-associated virus 3 on the physiology and must of *Vitis vinifera* L. cv. Albariño following contamination in the field. *American Journal of Enology and Viticulture*, 50(1), 40-44.
- Cabaleiro, C., & Segura, S. (1997). Field transmission of grapevine leafroll associated virus 3 (GLRaV-3) by the mealybug *Planococcus citri*. *Plant Disease*, 81(3), 283-287.
- Caldwell, J. (2012). Virginia wine industry jobs grow by 50%; Economic impact doubles, new study finds, from <http://www.governor.virginia.gov/News/viewRelease.cfm?id=1114>
- Castellano, M. A., & Martelli, G. P. (1984). Ultrastructure and nature of vesiculated bodies associated with isometric virus-like particles in diseased grapevines. *Journal of Ultrastructure Research*, 89(1), 56-64.
- Charles, J. G., Cohen, D., Walker, J. T. S., Forgie, S. A., Bell, V. A., & Breen, K. C. (2006). A review of the ecology of grapevine leafroll associated virus type 3 (GLRaV-3). *New Zealand Plant Protection*, 59, 330-337.
- Cornwell, P. B. (1958). Movements of the vectors of virus diseases of cacao in Ghana. I. Canopy movement in and between trees. *Bulletin of Entomological Research*, 49, 613-630.
- Cornwell, P. B. (1960). Movements of the vectors of virus diseases of cacao in Ghana, II. Wind movements and aerial dispersal. *Bulletin of Entomological Research*, 51, 175-201.
- Daane, K. M., Bentley, W. J., Walton, V. M., Malakar-Kuenen, R., Millar, J. G., Ingels, C. A., . . . Gispert, C. (2006). New controls investigated for vine mealybug. *California Agriculture*, 60(1), 31-38.
- Daane, K. M., Malakar-Kuenen, R., Guillén, M., Bentley, W. J., Bianchi, M., & González, D. (2003). Abiotic and biotic pest refuges hamper biological control of mealybugs in California vineyards (pp. 389-398). Washington: United States Department of Agriculture, Forest Service.
- Daane, K. M., Malakar-Kuenen, R. D., & Walton, V. M. (2004). Temperature-dependent development of *Anagyrus pseudococci* (Hymenoptera: Encyrtidae) as a parasitoid of the vine mealybug, *Planococcus ficus* (Homoptera: Pseudococcidae). *Biological Control*, 31(2), 123-132.
- Daane, K. M., Sime, K. R., Cooper, M. L., & Battany, M. C. (2004). Ants in your vineyard? *UC Plant Protection Quarterly*, 11(2), 3.
- de Saporta, G. (1879). *Le monde des plantes avant l'apparition de l'homme*.: G. Masson.
- Dimitrijevic, B. (1973). Some observations on natural spread of grapevine leafroll disease in Yugoslavia. *Rivista di Patologia Vegetale*, (S IV) 9 (suppl.), 114-119
- Dolja, V. V., Karasev, A. V., & Koonin, E. V. (1994). Molecular biology and evolution of closteroviruses: sophisticated build-up of large RNA genomes. *Annual Review of Phytopathology*, 32, 261-285.
- Dolja, V. V., Kreuze, J. F., & Valkonen, J. P. T. (2006). Comparative and functional genomics of closteroviruses. *Virus Research*, 117(1), 38-51.
- Douglas, N., & Krüger, K. (2008). Transmission efficiency of Grapevine leafroll-associated virus 3 (GLRaV-3) by the mealybugs *Planococcus ficus* and *Pseudococcus longispinus* (Hemiptera: Pseudococcidae). *European Journal of Plant Pathology*, 122(2), 207-212.
- Duduk, B., & Ivanovic, M. (2006). Grapevine yellows. *Biljni Lekar (Plant Doctor)*, 34(2), 105-111.
- Ellis, M. A. (2008a). Downy Mildew of Grape: Fact sheet HYG-3013. *The Ohio State University Agriculture and Natural Resources*. Retrieved September, 2010, from [http://ohioline.osu.edu/hyg-fact/3000/pdf/HYG\\_3013\\_08.pdf](http://ohioline.osu.edu/hyg-fact/3000/pdf/HYG_3013_08.pdf)

- Ellis, M. A. (2008b). Powdery Mildew of Grape: Fact sheet HYG-3018-08. *The Ohio State University Agriculture and Natural Resource Extension* Retrieved October, 2010, from [http://ohioline.osu.edu/hyg-fact/3000/pdf/HYG\\_3018\\_08.pdf](http://ohioline.osu.edu/hyg-fact/3000/pdf/HYG_3018_08.pdf)
- Engel, E. A., Escobar, P. F., Rojas, L. A., Rivera, P. A., Fiore, N., & Valenzuela, P. D. T. (2010). A diagnostic oligonucleotide microarray for simultaneous detection of grapevine viruses. *Journal of Virological Methods*, 163(2), 445-451.
- Engel, E. A., Girardi, C., Escobar, P. F., Arredondo, V., Domínguez, C., Pérez-Acle, T., & Valenzuela, P. D. T. (2008). Genome analysis and detection of a Chilean isolate of Grapevine leafroll-associated virus-3. *Virus Genes*, 37(1), 110-118.
- Engelbrecht, D. J., & Kasdorf, G. G. F. (1990). Transmission of grapevine leafroll disease and associated closteroviruses by the vine mealybug, *Planococcus ficus*. *Phytophylactica*, 22(3), 341-346.
- Fazeli, C. F., & Rezaian, M. A. (2000). Nucleotide sequence and organization of ten open reading frames in the genome of Grapevine leafroll-associated virus 1 and identification of three subgenomic RNAs. *Journal of General Virology*, 81(3), 605-615.
- Fitch, A. (1855). The first report on the noxious, beneficial, and other insects of the state of New York. *Transactions of the New York State Agricultural Society*(14), 705-880.
- Fortusini, A., Scattini, G., Cinquanta, S., & Prati, S. (1996). Natural spread of grapevine leafroll virus 1 (GLRV-1), grapevine leafroll virus 3 (GLRV-3) and grapevine fleck virus (GFkV). *Informatore Fitopatologico*, 46(12), 39-43.
- Fuchs, M. (2007). Grape leafroll disease factsheet Retrieved June, 2012, from [http://www.nysipm.cornell.edu/factsheets/grapes/diseases/grape\\_leafroll.pdf](http://www.nysipm.cornell.edu/factsheets/grapes/diseases/grape_leafroll.pdf)
- Fuchs, M., Marsella-Herrick, P., Loeb, G. M., Martinson, T. E., & Hoch, H. C. (2009). Diversity of ampeloviruses in mealybug and soft scale vectors and in grapevine hosts from leafroll-affected vineyards. *Phytopathology*, 99(10), 1177-1184.
- Fuchs, M., Martinson, T. E., Loeb, G. M., & Hoch, H. C. (2009). Survey for the three major leafroll disease-associated viruses in Finger Lakes vineyards in New York. *Plant Disease*, 93(4), 395-401.
- Furness, G. O. (1976). The dispersal, age-structure and natural enemies of the long-tailed mealybug, *Pseudococcus longispinus* (Targioni-Tozzetti), in relation to sampling and control. *Australian Journal of Zoology*, 24(2), 237-247.
- Galet, P. (2000). *Dictionnaire Encyclopedique des Cepages*.: Hachette.
- Gambino, G., & Gribaudo, I. (2006). Simultaneous detection of nine grapevine viruses by multiplex reverse transcription-polymerase chain reaction with coamplification of a plant RNA as internal control. *Phytopathology*, 96(11), 1223-1229.
- Geiger, C. A., & Daane, K. M. (2001). Seasonal movement and distribution of the grape mealybug (Homoptera: Pseudococcidae): developing a sampling program for San Joaquin Valley vineyards. *Journal of Economic Entomology*, 94(1), 291-301.
- Geiger, C. A., Daane, K. M., Bentley, W. J., Yokota, G. Y., & Martin, L. A. (2001). Sampling program for grape mealybugs improves pest management. *California Agriculture*, 55(3), 19-27.
- Gibbs, M. J., Koga, R., Moriyama, H., Pfeiffer, P., & Fukuhara, T. (2000). Phylogenetic analysis of some large double-stranded RNA replicons from plants suggests they evolved from a defective single-stranded RNA virus. *Journal of General Virology*, 81(1), 227-233.
- Glasa, M., Predajná, L., & Komínek, P. (2011). Grapevine fleck virus isolates split into two distinct molecular groups. *Journal of Phytopathology*, 159(11/12), 805-807.

- Goheen, A. C., Harmon, P. N., & Weinberger, J. H. (1958). Leaf roll (White Emperor disease) of Grapes in California. *Phytopathology*, 48(1), 51-54 pp.
- Golino, D., Sim, S., & Rowhani, A. (2000). *Identification of the latent viruses associated with young vine decline in California*. Extended Abstracts of the 13th meeting of ICVG, Adelaide, 2000, 85-86.
- Golino, D. A., Sim, S. T., Gill, R., & Rowhani, A. (2002). California mealybugs can spread grapevine leafroll disease. *California Agriculture*, 56(6), 196-201.
- Golino, D. A., Weber, E., Sim, S., & Rowhani, A. (2008). Leafroll disease is spreading rapidly in a Napa Valley vineyard. *California Agriculture*, 62(4), 156-160.
- Goszczynski, D. E., Kasdorf, G. G. F., Pietersen, G., Tonder, H. v. (1996). Detection of two strains of grapevine leafroll-associated virus 2. *Vitis*, 35(3), 133-135.
- Granett, J., Walker, M. A., Kocsis, L., & Omer, A. D. (2001). Biology and management of grape phylloxera. *Annual Review of Entomology*, 46, 387-412.
- Grasswitz, T. R., & James, D. G. (2008). Movement of grape mealybug, *Pseudococcus maritimus*, on and between host plants. *Entomologia Experimentalis et Applicata*, 129(3), 268-275.
- Greif, C., Garau, R., Boscia, D., Prota, V. A., Fiori, M., Bass, P., . . . Prota, U. (1995). The relationship of grapevine leafroll-associated closterovirus 2 with a graft incompatibility condition of grapevines. *Phytopathologia Mediterranea*, 34(3), 167-173.
- Grimes, E. W., & Cone, W. W. (1985). Life history, sex attraction, mating, and natural enemies of the grape mealybug, *Pseudococcus maritimus* (Homoptera: Pseudococcidae). *Annals of the Entomological Society of America*, 78(4), 554-558.
- Gubler, W. D., Smith, R. J., Varela, L. G., Vasquez, S., Stapleton, J. J., & Purcell, A. H. (2009). Grape: Botrytis Bunch Rot. *University of California Integrated Pest Management Program*. Retrieved 2010, October, from <http://www.ipm.ucdavis.edu/PMG/r302100111.html>
- Gugerli, P., Brugger, J. J., & Bovey, R. (1984). L'enroulement de la vigne: mise en evidence de particules virales et développement d'une methode immuno-enzymatique pour le diagnostic rapide. *Revue Suisse de Viticulture Arboriculture et Horticulture*, 16, 299-304.
- Gugerli, P., & Ramel, M. E. (1993). *Grapevine leafroll associated virus II analyzed by monoclonal antibodies*. Paper presented at the Extended Abstracts 11th Meeting ICVG, Montreux.
- Harmon, F. N., & Snyder, E. (1946). Investigations on the occurrence, transmission, spread and effect of " white " fruit color in the Emperor [*Vitis vinifera*] grape. *Proceedings. American Society for Horticultural Science*, 1946, 47, 190-194.
- Heuvel, J. F. J. M. v. d., Verbeek, M., & Wilk, F. v. d. (1994). Endosymbiotic bacteria associated with circulative transmission of potato leafroll virus by *Myzus persicae*. *Journal of General Virology*, 75(10), 2259-2565.
- Hewitt, W. B., Goheen, A. C., Cory, L., & Luhn, C. (1972). Grapevine fleck disease, latent in many varieties, is transmitted by graft inoculation. *Annales de Phytopathologie*, 43-47.
- Hu, J. S., Gonsalves, D., Boscia, D., Maixner, M., & Golino, D. (1991). Comparison of rapid detection assays for grapevine leafroll disease associated closteroviruses. *Vitis*, 30(2), 87-95.
- Hu, J. S., Gonsalves, D., & Teliz, D. (1990). Characterization of closterovirus-like particles associated with grapevine leafroll disease. *Journal of Phytopathology*, 128(1), 1-14.

- Institute, W. (2012). California, U.S., and World wine statistics Retrieved May, 2012, from <http://www.wineinstitute.org/resources/statistics>
- Jarugula, S., Gowda, S., Dawson, W., & Naidu, R. (2010). 3'-coterminal subgenomic RNAs and putative cis-acting elements of Grapevine leafroll-associated virus 3 reveals 'unique' features of gene expression strategy in the genus Ampelovirus. *Virology Journal*, 7(1), 180.
- Jooste, A. E. C., & Goszczynski, D. E. (2005). Single-strand conformation polymorphism (SSCP), cloning and sequencing reveals two major groups of divergent molecular variants of grapevine leafroll-associated virus 3 (GLRaV-3). *Vitis*, 44(1), 39-43.
- Karasev, A. V. (2000). Genetic diversity and evolution of closteroviruses. *Annual Review of Phytopathology*, 38, 293-324.
- King, A., Leftkowitz, E., Adams, M. J., & Carstens, E. B. (2011). *Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses*: Elsevier.
- Kovacs, L. G., Hanami, H., Fortenberry, M., & Kaps, M. L. (2001). Latent infection by leafroll agent GLRaV-3 is linked to lower fruit quality in French-American hybrid grapevines Vidal blanc and St. Vincent. *American Journal of Enology and Viticulture*, 52(3), 254-259.
- Levadoux, L. (1956). Les populations sauvages et cultivees de *Vitis vinifera* L.. *Ann. Amelior. Plantes*, 6, 59-117.
- Ling, K. S., Zhu, H. Y., Drong, R. F., Slightom, J. L., McFerson, J. R., & Gonsalves, D. (1998). Nucleotide sequence of the 3' -terminal two-thirds of the grapevine leafroll-associated virus-3 genome reveals a typical monopartite closterovirus. *Journal of General Virology*, 79(5), 1299-1307.
- Ling, K. S., Zhu, H. Y., & Gonsalves, D. (2004). Complete nucleotide sequence and genome organization of Grapevine leafroll-associated virus 3, type member of the genus Ampelovirus. *Journal of General Virology*, 85(7), 2099-2102.
- Liu, Y. P., Peremyslov, V. V., Medina, V., & Dolja, V. V. (2009). Tandem leader proteases of Grapevine leafroll-associated virus-2: host-specific functions in the infection cycle. *Virology*, 383(2), 291-299.
- Mahfoudhi, N., Digiario, M., & Dhouibi, M. H. (2009). Transmission of grapevine leafroll viruses by *Planococcus ficus* (Hemiptera: Pseudococcidae) and *Ceroplastes rusci* (Hemiptera: Coccidae). *Plant Disease*, 93(10), 999-1002.
- Maliogka, V. I., Dovas, C. I., & Katis, N. I. (2008). Evolutionary relationships of virus species belonging to a distinct lineage within the Ampelovirus genus. *Virus Research*, 135(1), 125-135.
- Maliogka, V. I., Dovas, C. I., Lotos, L., Efthimiou, K., & Katis, N. I. (2009). Complete genome analysis and immunodetection of a member of a novel virus species belonging to the genus Ampelovirus. *Archives of Virology*, 154(2), 209-218.
- Mansour, R., Suma, P., Mazzeo, G., Grissa Lebdi, K., & Russo, A. (2011). Evaluating side effects of newer insecticides on the vine mealybug parasitoid *Anagyrus* sp. pseudococci , with implications for integrated pest management in vineyards. *Phytoparasitica*, 39(4), 369-376.
- Martelli, G. P. (1993). *Graft-transmissible diseases of grapevines: handbook for detection and diagnosis*. Rome: Food and Agriculture Organization (FAO).

- Martelli, G. P., Agranovsky, A. A., Bar-Joseph, M., Boscia, D., Candresse, T., Coutts, R. H. A., . . . Yoshikawa, N. (2002). The family Closteroviridae revised. *Archives of Virology*, *147*(10), 2039-2044.
- Martelli, G. P., & Boudon-Padiou, E. (2006). *Directory of infectious diseases of grapevines*. (Vol. 55): Options Méditerranéennes, Series B. 55: 11-201.
- Martelli, G. P., Sabanadzovic, S., Abou Ghanem-Sabanadzovic, N., & Saldarelli, P. (2002). Maculavirus, a new genus of plant viruses. *Archives of Virology*, *147*(9), 1847-1853.
- Martelli, G. P., Sabanadzovic, S., Abou-Ghanem Sabanadzovic, N., Edwards, M. C., & Dreher, T. (2002). The family Tymoviridae. *Archives of Virology*, *147*(9), 1837-1846.
- McGovern, P. E. (2003). *Ancient Wine The Search for the Origins of Viniculture*. Princeton: Princeton University Press.
- McGovern, P. E., Glusker, D. L., Exner, L. J., & Voigt, M. M. (1996). Neolithic resinated wine. [10.1038/381480a0]. *Nature*, *381*(6582), 480-481.
- Mendgen, K. (1971). Studies on a yellows disease of vines along the Rhein, Mosel and Saar. *Weinberg und Keller*, *18*(8/9), 345-431.
- Meng, B., Goszczynski, D. E., Zhu, H. Y., Ling, K. S., & Gonsalves, D. (2000). *The 5' sequence of grapevine leafroll associated closterovirus 2 genome*. Paper presented at the Extended Abstracts of the 13th meeting of ICVG, Adelaide, 2000. pp. 28.
- Meng, B., Li, C. H., Goszczynski, D. E., & Gonsalves, D. (2005). Genome sequences and structures of two biologically distinct strains of Grapevine leafroll-associated virus 2 and sequence analysis. *Virus Genes*, *31*(1), 31-41.
- Mgocheki, N., & Addison, P. (2009). Effect of contact pesticides on vine mealybug parasitoids, *Anagyrus* sp. near pseudococci (Girault) and *Coccidoxenoides perminutus* (Timberlake) (Hymenoptera: Encyrtidae). *South African Journal of Entology and Viticulture*, *30*(2), 110-116.
- Milkus, B. N., & Goodman, R. N. (1999). A survey of Missouri vineyards for the presence of five grape viruses. *American Journal of Entology and Viticulture*, *50*(1), 133-134.
- Minafra, A., & Hadidi, A. (1994). Sensitive detection of grapevine virus A, B, or leafroll-associated III from viruliferous mealybugs and infected tissue by cDNA amplification. *Journal of Virological Methods*, *47*(1-2), 175-187.
- Morin, S., Ghanim, M., Zeidan, M., Czosnek, H., Verbeek, M., & Heuvel, J. F. J. M. v. d. (1999). A GroEL homologue from endosymbiotic bacteria of the whitefly *Bemisia tabaci* is implicated in the circulative transmission of tomato yellow leaf curl virus. *Virology (New York)*, *256*(1), 75-84.
- Myers, A. L., Sutton, T. B., Abad, J. A., & Kennedy, G. G. (2007). Pierce's disease of grapevines: identification of the primary vectors in North Carolina. *Phytopathology*, *97*(11), 1440-1450.
- Myles, S., Boyko, A. R., Owens, C. L., Brown, P. J., Grassi, F., Aradhya, M. K., . . . Buckler, E. S. (2011). Genetic structure and domestication history of the grape. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(9), 3530-3535.
- Naidu, R. A. (2012). Virus Diseases. *WSU Viticulture & Entology: Research & Extension* Retrieved June, 2012, from <http://wine.wsu.edu/research-extension/grape-growing/plant-health/virology/virus-diseases/>
- Naidu, R. A., & Mekuria, T. A. (2010). First report of Grapevine fleck virus from Washington vineyards. *Plant Disease*, *94*(6), 784.

- Namba, S., Yamashita, S., Doi, Y., Yora, K., Terai, Y., & Yano, R. (1979). Grapevine leafroll virus, a possible member of closteroviruses. *Annals of the Phytopathological Society of Japan*, 45(4), 497-502.
- Nassuth, A., Pollari, E., Helmecky, K., Stewart, S., & Kofalvi, S. A. (2000). Improved RNA extraction and one-tube RT-PCR assay for simultaneous detection of control plant RNA plus several viruses in plant extracts. *Journal of Virological Methods*, 90(1), 37-49.
- Notte, P. I., Minafra, A., & Saldarelli, P. (1997). A spot-PCR technique for the detection of phloem-limited grapevine viruses. *Journal of Virological Methods*, 66(1), 103-108.
- Pearson, R. C., & Goheen, A. C. (1988). *Compendium of grape diseases*: APS Press.
- Petersen, C. L., & Charles, J. G. (1997). Transmission of grapevine leafroll-associated closteroviruses by *Pseudococcus longispinus* and *P. calceolariae*. *Plant Pathology*, 46(4), 509-515.
- Phillips, P. A., & Sherk, C. J. (1991). To control mealybugs, stop honeydew-seeking ants. *California Agriculture*, 45(2), 26-28.
- Pietersen, G. (2004). Spread of Grapevine Leafroll Disease in South Africa - a difficult, but not insurmountable problem - A technical guide for wine producers. Retrieved May, 2012, from [http://www.wineland.co.za/index.php?option=com\\_zine&view=article&id=504%3Aspread-of-grapevine-leafroll-disease-in-south-africa-a-difficult-but-not-insurmountable-problem&Itemid=5](http://www.wineland.co.za/index.php?option=com_zine&view=article&id=504%3Aspread-of-grapevine-leafroll-disease-in-south-africa-a-difficult-but-not-insurmountable-problem&Itemid=5)
- Pietersen, G. (2006). *Spatio temporal distribution dynamics of grapevine leafroll disease in western cape vineyards*. Paper presented at the Extended Abstracts 15th Meeting of ICVG, Stellenbosch, South Africa, April 2006, pp 126-127.
- Pietersen, G. (2010). Grapevine leafroll disease control in South Africa. Wynboer. Retrieved May, 2012, from [https://41.203.19.179/index.php?option=com\\_zine&view=article&id=548%3AGrapevine-leafroll-disease-control-in-south-africa&Itemid=10](https://41.203.19.179/index.php?option=com_zine&view=article&id=548%3AGrapevine-leafroll-disease-control-in-south-africa&Itemid=10)
- Protin, R. (1971). Situation de la viticulture dans le monde en 1970. *Office Int Vigne Vin Bull*, 44(489), 1010-1057.
- Ravaz, L., & Verge, G. (1924). Le rugeau de la vigne. *Progrés Agricole et Viticole.*, 45, 11-17, 35-38, 86-89, 110-113, 135-141.
- Rayapati, N. (2012). Virus Diseases Retrieved November, 2012, from <http://wine.wsu.edu/research-extension/plant-health/virology/virus-diseases/>
- Rayapati, N., O'Neal, S., & Walsh, D. (2008). Grapevine leafroll disease. *Washington State University Extension Bulletin EB2027E* Retrieved 2012, April, from <http://pubs.wsu.edu>
- Rayapati, N., Tefera, M. A., Olufemi, A. J., Sridhar, J., Gandhi, K., Gutha, L. R., & Martin, R. R. (2009). Current status of grapevine viruses in the Pacific Northwest vineyards of the United States. In: *Extended Abstracts, 16th meeting of the ICVG, 2009. Dijon, France.*, 108-109.
- Rosa, C. (2007). *Etiology of "rugose wood complex" and functional analysis of Grapevine virus D*: University of California, Davis.
- Rosciglione, B., Castellano, M. A., Martelli, G. P., Savino, V., & Cannizzaro, G. (1983). Mealybug Transmission of grapevine virus A. *Vitis*, 22, 331-347.
- Rosciglione, B., & Gugerli, P. (1986). Maladies de l'enroulement et du bois strié de la vigne: analyse microscopique et sérologique. *Revue Suisse de Viticulture, Arboriculture, Horticulture*, 18, 207-211.



- Rosciglione, B., & Gugerli, P. (1989). *Transmission of grapevine leafroll disease and an associated closterovirus to healthy grapevine by the mealybug Planococcus ficus Signoret*. Paper presented at the Proceedings 9th Meeting of ICVG, Kiryat Anavim.
- Routh, G., Zhang, Y., Saldarelli, P., & Rowhani, A. (1998). Use of degenerate primers for partial sequencing and RT-PCR-based assays of grapevine leafroll-associated viruses 4 and 5. *Phytopathology*, 88(11), 1238-1243.
- Roxas, C. (1814). *Essai Sur Les Variétés de la Vigne Qui Végètent en Andalousie*. Paris: Imprimerie Poulet.
- Royer, C. (1988). Movement historiques de la vigne dans le monde. *La Vigne et la Vin* (pp. 15-25): Graficas.
- Sabanadzovic, S., Ghanem-Sabanadzovic, N. A., Saldarelli, P., & Martelli, G. P. (2001). Complete nucleotide sequence and genome organization of Grapevine fleck virus. *Journal of General Virology*, 82(8), 2009-2015.
- Saldarelli, P., Rowhani, A., Routh, G., Minafra, A., & Digiario, M. (1998). Use of degenerate primers in a RT-PCR assay for the identification and analysis of some filamentous viruses, with special reference to clostero- and vitiviruses of the grapevine. *European Journal of Plant Pathology*, 104(9), 945-950.
- Sannino, F. A. (1906). [English title not available]. *Rivista di Patologia Vegetale.*, 1, 162-163.
- Sasahara, H., Tada, K., Iri, M., Takezawa, T., & Tazaki, M. (1981). Regeneration of plantlets by meristem tip culture for virus-free grapevine. *Journal of the Japanese Society for Horticultural Science*, 50(2), 169-175.
- Scheu, G. (1935). Die Rollkrankheit des Rebstockes. *Der Deutsche Weinbau.*, 14, 222-223, 345-346, 356-358.
- Scheu, G. (1936). *Mein Winzerbuch*. Berlin: Reichnährstand Verlag.
- Schilder, A. (2011). Tomato/tobacco ringspot virus decline Retrieved November, 2012, from [grapes.msu.edu/ringspot.htm](http://grapes.msu.edu/ringspot.htm)
- Schooley, N., Baudoin, A. B., Derr, J. F., Pfeiffer, D. G., Weaver, M. J., & Wolf, T. K. (April 2010). Crop profile for grapes in Virginia, from <http://www.ipmcenters.org/cropprofiles/docs/vagrapes.pdf>
- Sforza, R., Boudon-Padiou, E., & Greif, C. (2003). New mealybug species vectoring Grapevine leafroll-associated viruses-1 and -3 (GLRaV-1 and -3). *European Journal of Plant Pathology*, 109(9), 975-981.
- Smith, H., & Cowles, R. (2009). Scale insect pests of Connecticut trees and ornamentals Retrieved November, 2012, from [http://www.hort.uconn.edu/ipm/nursery/caes\\_fs/caes\\_fs\\_scalepests.htm](http://www.hort.uconn.edu/ipm/nursery/caes_fs/caes_fs_scalepests.htm)
- Soule, M. J., Eastwell, K. C., & Naidu, R. A. (2006). First Report of Grapevine leafroll associated virus-3 in American Vitis spp. Grapevines in Washington State. *Plant Disease*, 90(11), 1461-1461.
- Strickland, A. H. (1950). The dispersal of Pseudococcidae (Hemiptera-Homoptera) by air currents in the Gold Coast. *Proceedings of the Royal Entomological Society, London.*, 25, 1-9.
- Tanne, E., Ben-Dov, Y., & Raccach, B. (1989). Transmission of closterolike particles by mealybugs (Pseudococcidae) in Israel. *Phytoparasitica*, 17(64).
- This, P., Lacombe, T., & Thomas, M. R. (2006). Historical origins and genetic diversity of wine grapes. *Trends in Genetics*, 22(9), 511-519.

- Triplehorn, C. A., Johnson, N. F., & Borror, D. J. (2005). *Borror and DeLong's Introduction to the Study of Insects*. Belmont, CA: Thompson Brooks/Cole.
- Tsai, C., Rowhani, A., Golino, D. A., Daane, K. M., & Almeida, R. P. P. (2010). Mealybug transmission of grapevine leafroll viruses: an analysis of virus-vector specificity. *Phytopathology*, 100(8), 830-834.
- Tsai, C. W., Chau, J., Fernandez, L., Bosco, D., Daane, K. M., & Almeida, R. P. P. (2008). Transmission of Grapevine leafroll-associated virus 3 by the vine mealybug (*Planococcus ficus*). *Phytopathology*, 98(10), 1093-1098.
- Uyemoto, J. K., Rowhani, A., Luvisi, D., & Krag, C. R. (2001). New closterovirus in 'Redglobe' grape causes decline of grafted plants. *California Agriculture*, 55(4), 28-31.
- Varela, L. G. (May 2005). Grape and Vine Mealybug Life Cycles in the North Coast, from <http://cesonoma.ucdavis.edu/files/27229.pdf>
- Varela, L. G., Elkins, R. B., Van Steenwyk, R. A., & Ingels, C. A. (2012). Pear Mealybugs Retrieved 2012, November, from <http://www.ipm.ucdavis.edu/PMG/r603301211.html>
- Varela, L. G., & Smith, R. J. (2009). Mealybugs Retrieved November, 2012, from <http://ucanr.org/sites/SoCo/files/27649.pdf>
- VDACS. (2011). Virginia Agriculture--Facts and Figures, from <http://www.vdacs.virginia.gov/agfacts/index.shtml>
- Viala, P., & Vermorel, V. (1901-09). *Ampélographie. Traité général de viticulture*. (Vol. Vol. 1-7): Vol. 1-7. Masson.
- Vuittenez, A., Legin, R., & Kuszala, J. (1966). Observations sur une mosaïque de la vigne, probablement indépendante du virus du court-noué. *Ann. Epiphyt.*, 17(No hors serie), 67-73.
- Vuittenez, A., Legin, R., Kuszala, J., & Cardin-Munck, M. C. (1972). 'NEPO' viruses of grapevine and their nematode vectors. *Annales de Phytopathologie*, 4(4), 373-392.
- VWB. (2012). The Economic Impact of Wine and Wine Grapes on the State of Virginia--2010. St. Helena, CA: A Frank, Rimerman + Co. LLP.
- Walter, B., & Martelli, G. P. (1997). Clonal and sanitary selection of the grapevine. In: Walter B (ed) Sanitary selection of the grapevine. Protocols for detection of viruses and virus-like diseases. *Les Colloques*(86), 43-95. INRA Editions, Paris.
- Walton, V. M. (2001). Mealybug: Biology and control strategies, from <http://www.wynboer.co.za/recentarticles/0301mealybug.php3>
- Walton, V. M., Daane, K. M., Bentley, W. J., Millar, J. G., Larsen, T. E., & Malakar-Kuenen, R. (2006). Pheromone-based mating disruption of *Planococcus ficus* (Hemiptera: Pseudococcidae) in California vineyards. *Journal of Economic Entomology*, 99(4), 1280-1290.
- Wilcox, W. F., Jiang, Z. Y., & Gonsalves, D. (1998). Leafroll virus is common in cultivated American grapevines in western New York. *Plant Disease*, 82(9), 1062.
- Zhu, H. Y., Ling, K. S., Goszczynski, D. E., McFerson, J. R., & Gonsalves, D. (1998). Nucleotide sequence and genome organization of grapevine leafroll-associated virus-2 are similar to beet yellows virus, the closterovirus type member. *Journal of General Virology*, 79(5), 1289-1298.
- Zohary, D. (1995). Domestication of the Grapevine *Vitis vinifera* L. in the Near East. In P. E. McGovern (Ed.), *The origins and Ancient History of Wine* (pp. 23-30): Gordon and Breach.

**Table 2.1:** Full Economic Impact of Wine and Wine Grapes on the Virginia Economy—2010: \$747.1 million. (VWB, 2012)

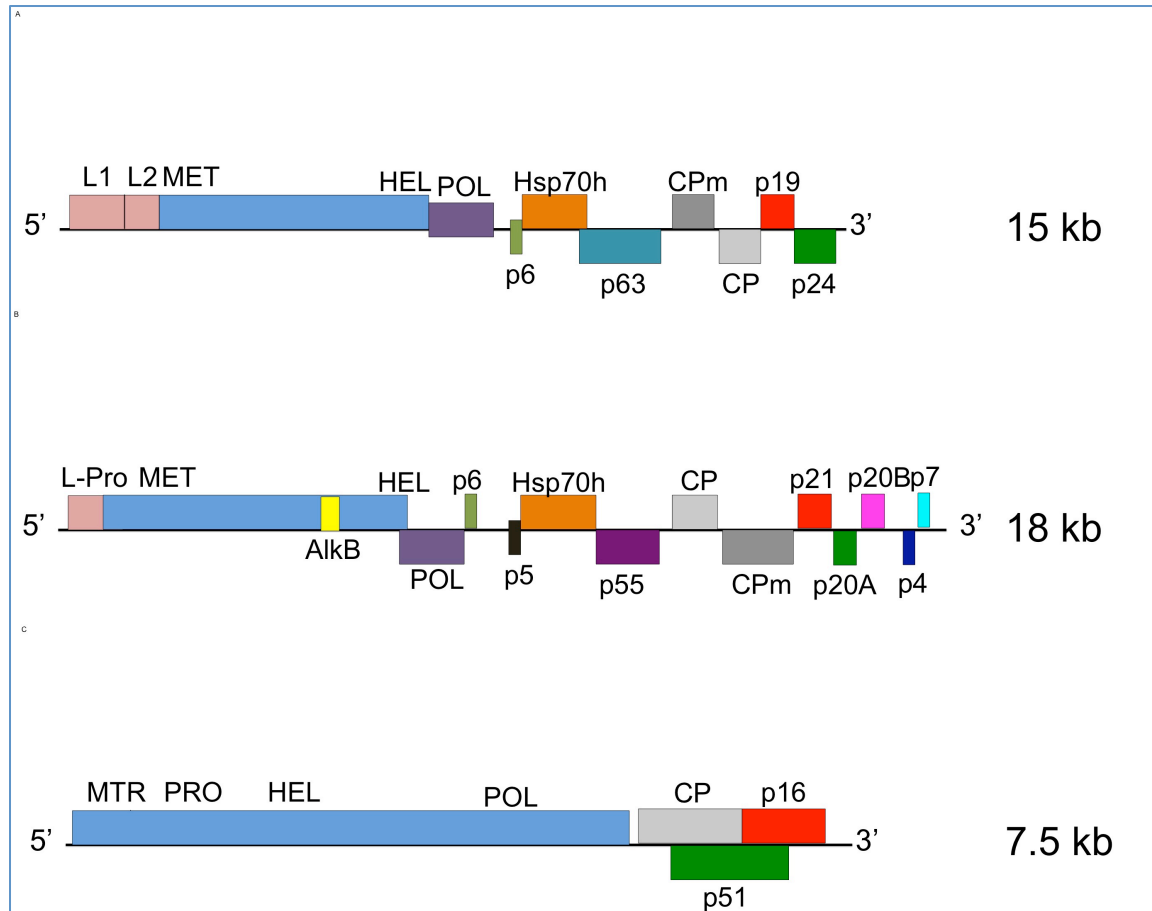
<b>Virginia Wine, Wine Grapes and Vineyards</b>	<b>2005 Economic Impact</b>	<b>2010 Economic Impact</b>
Full-time Equivalent Jobs	3,162	4,753
Wages Paid	\$84 million	\$156 million
Wine Produced (cases)	320,200	439,500
Retail Value of Virginia Wine Sold	\$45 million	\$73 million
Vineyard Revenue	\$8 million	\$11 million
Number of Wineries	129	193
Number of Grape Growers	262	386
Grape-Bearing Square Kilometers	8.09	10.93
Wine-Related Tourism Expenditures	\$57 million	\$131 million
Number of Wine-Related Tourists	1,000,000	1,618,000
Taxes Paid: Federal/State and Local	\$15 million / \$21 million	\$42 million / \$43 million

**Table 2.2:** Top *V. vinifera*, inter-specific hybrids, and American (*V. labrusca*) grape varieties grown in Virginia in 2010 and 2011 according to the Virginia 2011 Commercial Grape Report. <sup>x</sup> denotes *V. vinifera*; <sup>y</sup> denotes inter-specific hybrid; and <sup>z</sup> denotes *V. labrusca*

<b>Red Varieties</b>	<b>White Varieties</b>
Cabernet franc <sup>x</sup>	Albarino <sup>x</sup>
Cabernet Sauvignon <sup>x</sup>	Chardonnay <sup>x</sup>
Merlot <sup>x</sup>	Gewurztraminer <sup>x</sup>
Petit Verdot <sup>x</sup>	Petit Manseng <sup>x</sup>
Pinot noir <sup>x</sup>	Pinot gris <sup>x</sup>
Syrah <sup>x</sup>	Riesling <sup>x</sup>
Tannat <sup>x</sup>	Sauvignon blanc <sup>x</sup>
Chambourcin <sup>y</sup>	Viognier <sup>x</sup>
Concord <sup>z</sup>	Seyval blanc <sup>y</sup>
Norton <sup>y</sup>	Traminette <sup>y</sup>
	Vidal blanc <sup>y</sup>
	Niagara <sup>z</sup>



**Figure 2.1:** Symptoms of GLD on a red-fruited variety, Cabernet Sauvignon (A) and on a white-fruited variety, Chardonnay (B).



**Figure 2.2:** Genome organization of GLRaV-2, GLRaV-3 and GFkV showing the relative position of the ORFs and their predicted expression products. (Zhu et al., 1998; Martelli et al., 2002)

# Chapter 3

## **A survey of grapevine leafroll-associated virus-2, grapevine leafroll-associated virus-3, and grapevine fleck virus in wine grape varieties and native grape species in Virginia.**

### **3.1 Introduction**

Grapevine leafroll disease (GLD) is a common disease found in all grapevine growing areas of the world. The first possible accounts of this disease date back to 1906 (Martelli, 2006; Sannino, 1906). Since then, GLD has been found in 33 additional countries around the globe, bringing the total to 36 (Martelli, 2006). GLD can be caused by any one of at least ten known phloem-limited viruses, referred to as the grapevine leafroll associated viruses (GLRaVs) (Fuchs, 2007; Fuchs et al., 2009). The first virus in the group is referred to as Grapevine leafroll-associated virus-1 (GLRaV-1), then GLRaV-2, GLRaV-3, and so on through GLRaV-10. These virus particles vary in length ranging between 1,400 and 2,200 nm and all are flexuous filaments approximately 12nm in width (Fuchs, 2007). All GLRaVs belong to one of two genera in the family *Closteroviridae*; however, they differ by genus classification. GLRaV-2 has been placed in the genus *Closterovirus*, whereas GLRaV-1, -3, -4, -5, -6, -8, -9 have been placed into the recently created genus *Ampelovirus* (Martelli, 2006).

GLD can significantly reduce both crop yield and grape quality (Kovacs et al., 2001). Both berry color intensity and Brix can be reduced, which negatively affects the wine quality (Cabaleiro et al., 1999; Fuchs et al., 2009; Rayapati et al., 2008). In severely infected vineyards with a susceptible variety, crop loss can range from 30% to 50% (Fuchs et al., 2009). The disease can cause loss of vigor, which also can make the vines more susceptible to winter injury and other environmental stress factors. In addition, infection by multiple viruses can negatively affect fruit quality more substantially than single infection scenarios. For example, fruit quality parameters of vines infected with both GLRaV-3 and *Grapevine fleck virus* (GFkV, genus *Maculavirus*, family *Tymoviridae*, order *Tymovirales*) were inferior to those of healthy vines and vines infected only with GLRaV-3 (Kovacs et al., 2001).

Known means of transmission for the GLRaVs are vegetative propagation, grafting, and through insect vectors (i.e. mealybugs [*Pseudococcidae*] and soft scales [*Coccidae*]) (Fuchs et al., 2009; Rayapati et al., 2008; Charles et al., 2006). It was recently found that a wild grapevine in California (*Vitis californica*) could be infected with GLRaV-2 and GLRaV-3 (Klaassen et al., 2011). There may be other wild hosts that can harbor these viruses and, if the vectors are present, they could possibly transmit viruses into nearby fields.

The current status of viruses in grapevines within vineyards in Virginia is unknown. With the recent expansion and growing popularity of the Virginia wine industry, which resulted in the economic contribution of over \$740 million annually (Virginia Wine Board, 2012), Virginia growers are becoming increasingly concerned about this emerging threat. A recent biennial research needs/priority survey of Virginia Vineyards Association members rated leafroll disease as a 3.6 on a 1-5 scale, with 5 being most important.

The objectives of this study are to: 1) determine the prevalence of GLD and associated virus (GFkV) in Virginia commercial vineyards; 2) examine spatio-temporal pattern of GLD; 3) determine whether native *Vitis* species serve as asymptomatic hosts in Virginia; and 4) determine what species of mealybugs (vectors of GLD) are present in the state.

## 3.2 Materials and Methods

### 3.2.1 Survey of Virginia

In order to identify potential regional differences, growers were selected randomly from each of five major grape growing regions of Virginia. At each grower's vineyard, one to three blocks (i.e., different varieties) were selected, three consecutive vines were randomly selected from each block, and seven petioles per vine were sampled (= 21 petioles/sample). Due to the uneven distribution of virus in the plant (Charles et al., 2006), petiole samples were collected from different locations on a vine (e.g. petioles from random shoots on the vine all over the canopy, including the top, middle, bottom and edges) and pooled for testing. The petiole samples were sealed in a plastic bag and stored in a cooler containing ice. In the lab, 0.25 g of each sample was placed into grinding bags (BIOREBA, Switzerland) containing 5 ml of a filter-sterilized grapevine extraction buffer (1.59 g/liter Na<sub>2</sub>CO<sub>3</sub>, 2.93 g/liter NaHCO<sub>3</sub>, 2% Polyvinylpyrrolidone-40, 0.2% Bovine Serum Albumin, and 0.05% Tween 20) (Sigma-Aldrich Co. LLC, St. Louis, MO), and ground using a mechanical grinder (BIOREBA, Switzerland, Homex 6 [115V]). Crude extracts were then transferred into 1.5-ml microcentrifuge tubes and stored at -80°C until the next step.

Crude extract was then used in a one-tube, one-step RT-PCR protocol that was developed by Dr. Rayapati (Washington State University) for the detection of different GLRaVs (Rowhani et al., 2000; Naidu et al., 2006; Rayapati et al., 2008). For each sample, 2  $\mu$ l of crude extract was transferred to a 0.5-ml microcentrifuge tube containing 25  $\mu$ l of 1X GES (0.1 M glycine, pH 9.0; 50 mM NaCl; 1 mM EDTA; 0.5% Triton X-100) and 1%  $\beta$ -mercaptoethanol was added to the 25  $\mu$ l volume prior to addition of crude extract (Sigma-Aldrich Co. LLC, St. Louis, MO). Tubes containing the crude extract mixture were subjected to a denaturing temperature (95°C) for 10 min, then placed immediately on ice for 5 min. Two  $\mu$ l of each sample's denatured extract was transferred to a 200- $\mu$ l PCR tube containing the following reaction mixture: 13.4  $\mu$ l nuclease-free H<sub>2</sub>O, 2.5  $\mu$ l 10X PCR buffer containing 15 mM Mg (Roche, Indianapolis, IN), 2.5  $\mu$ l sucrose/cresol red (20% w/v sucrose, 1 mM cresol red) (Sigma-Aldrich Co. LLC, St. Louis, MO), 1.25  $\mu$ l forward primer (20  $\mu$ M) 1.25  $\mu$ l reverse primer (20  $\mu$ M), 1.25  $\mu$ l 100mM dithiothreitol (Roche), 0.5  $\mu$ l dNTPs (10 mM) (Invitrogen, Grand Island, NY), 0.1  $\mu$ l RnaseOUT (40 U/ $\mu$ l) (Invitrogen), 0.035  $\mu$ l Superscript III RTase (200 U/ $\mu$ l) (Invitrogen), and 0.25  $\mu$ l Taq DNA polymerase (5 U/ $\mu$ l) (Roche). The reactions were placed in a thermal cycler and subjected to the following cycles: 52°C for 1 h, 35 cycles of (94°C for 30 s, 54°C for 45 s, 72°C for 1 min) with a final extension at 72°C for 2 min. Ten  $\mu$ l of each reaction was run on a 1% TBE agarose



gel for 1 h at 120 V and products were visualized with Gel Red™ (Phenix Research, Candler, NC). Specific primers used for RT-PCR detection of different GLRaVs and other major viruses of grape are shown in Table 3.1. Positive (confirmed virus infected samples) and negative (virus-free samples) controls were used to verify the test results. Sequencing of a few selected sampled viruses is currently underway.

### 3.2.2 Intensive sampling and spatial analysis of six vineyards in Virginia

In six selected vineyards, a grid of vines was sampled (Table 3.6). The grid was either 10 consecutive vines by 10 consecutive rows (10 x 10), 5 consecutive vines by 20 consecutive rows (5 x 20), or 20 consecutive vines by 5 consecutive rows (20 x 5). At each location, distance between vines within a row was about 5 feet, and distance between rows was about 10-12 feet. At least one of the vines in a grid had visible symptoms and/or a positive identification of virus-infection, previously detected by RT-PCR. On each vine, 20 petioles were randomly selected for RT-PCR, as described earlier.

### 3.2.3 Wild *Vitis* sampling near vineyards and isolated

The surrounding habitat of each vineyard, whether it was the roadside into a vineyard or woodland areas around the vineyard, were examined for presence of wild grape species. *Vitis aestivalis*, *V. cinerea*, and *V. rupestris* were found most commonly in Virginia. A sample of 20 leaves per vine was taken. In addition, wild grapes grown in locations remote from existing vineyards were examined with the expectation that they would not harbor any GLRaVs. Wild grapes found were identified by visual examination of collected tissues, using a grape identifier on the USDA website (plants.usda.gov). In addition, a PCR-based method was used to identify species. These wild grape samples were examined using the RT-PCR testing as described above. If any wild vines are found to be positive for a virus, the sample will be sequenced to further validate identification of the *Vitis* species.

### 3.2.4 Identification of vineyard mealybugs in Virginia

Along with the survey of Virginia for viruses, mealybug identification was also performed on multiple samples from commercial vineyards as well as vines at the AHS Jr. AREC (Winchester, VA). A multiplex PCR protocol from Daane et al. (2011) was used to identify species of vineyard mealybugs.

Mealybugs were collected from commercial grapevines. Each mealybug sample was placed in a 1.5-ml microcentrifuge tube containing 95% ethanol and stored at -20°C. Individual mealybugs were ground in liquid nitrogen, and the powdered tissue was used for total DNA extraction using the QIAGEN DNeasy Blood and Tissue kit following manufacturer's protocol. The mealybug multiplex PCR was performed using the QIAGEN Multiplex PCR kit and species-specific primers. Primer design and amplification protocol used were identical to those in Daane et al., 2011 (Table 3.2). PCR products were then purified using the QIAquick PCR Purification kit, following the QIAquick standard protocol.

### 3.2.5 Statistical Methods

JMP 10 (SAS institute, Cary, NC) was used for calculating basic statistics and conducting contingency analysis and chi-square analysis. Spatial and spatio-temporal analyses on intensive sampling data were done using Spatial Analysis by Distance IndicEs, or SADIE (Perry et al., 1996; Perry, 1998; Perry et al., 1999). SADIE uses a distance to regularity ( $D_a$ ) method that, in this case, utilizes coordinates of sampled grapevines and counts of virus infected samples in order to determine the total distance individuals have to move before the pattern becomes uniform (Nita et al., 2011). The average distance ( $E_a$ ) moved can then be used to calculate the index of aggregation ( $I_a$ ) where  $I_a = D_a/E_a$  (values  $>1.5$  indicate an aggregated pattern) (Nita et al., 2011). Indices of clustering can also be calculated in SADIE, and spatial associations between two viruses, or spatio-temporal associations of one virus over two years can be examined from this cluster analysis. Two types of clusters, patch and gap, can be inferred depending on the dataset (Perry, 1999; Madden et al., 2007). When an index of clustering for patches is  $>1.5$ , or for gaps is  $<-1.5$ , then a significance in association between clusters in years can be concluded (Perry et al., 1999; Winder et al., 2001).

### 3.3 Results

#### 3.3.1 Survey

A total of 415 samples were collected from vineyards across Virginia while 6, 6, 10, and 19 samples were collected from Maryland, North Carolina, Pennsylvania, and Texas, respectively. Intensive sampling of some vineyard blocks was also done in 6 vineyards in Virginia, resulting in an additional 800 grapevine samples. A total of 100 wild grapevines were sampled from areas throughout Virginia. These vineyard and wild grapevine samples were tested for GLRaV-2, GLRaV-3, and GFkV.

In Virginia, 77 vineyards were sampled and tested for the three viruses; of these, 64% were positive for at least one of the three viruses, 18% of the vineyards were positive for at least 2 viruses, and only 3% of the vineyards were positive for all three viruses. Among the tested samples, 8%, 25%, and 1% of vines tested positive for GLRaV-2, GLRaV-3, and GFkV respectively in Virginia. Figure 3.1 displays an agarose gel showing the RT-PCR products that confirm virus infections of the three viruses. There were only five isolated cases of grapevine fleck virus (in Albarino, Chancellor, Chardonnay, Pinot gris, and Vidal blanc varieties) found in the state.

A total of 41 varieties were tested (Table 3.4). Cabernet Sauvignon (81 samples), Cabernet franc (54 samples), Chardonnay (36 samples), Petit Verdot (36 samples), Merlot (33 samples), and Viognier (27 samples) were the most commonly sampled varieties since they are commonly planted in VA. Percentage of samples positive for at least one virus varied from 0 to 100% (Table 3.4). Cabernet Sauvignon, the most frequently sampled variety, had a 31% infection rate. Cabernet franc, the second most frequently sampled variety, and a variety that is also frequently used as an indicator for GLD, had a 15% infection rate.

Virus co-infection was found in 4.6% of vines. Of these, the combination of GLRaV-2 and GLRaV-3 was much more common than any other combination (Table 3.3). Table 3.9 shows specific cases of mixed infections found within different varieties. There were no cases of all three viruses being found infecting the same vine.

Table 3.5 shows a data summary of samples collected from the other states. Among 6 vines from Maryland, two vines were positive for GLRaV-3, and none for GLRaV-2 or GFkV.

In North Carolina and Pennsylvania, all samples were negative for the viruses tested. For Texas, five cases of GLRaV-2 and one case of GLRaV-3 were found in the Lenoir variety.

### 3.3.2 Intensive sampling

The first intensive sampling plot was at the AHS Jr. AREC in Winchester, VA. Vines were 2 years old when the first sample was taken in 2010, and were monitored for the three years. This 10x10 matrix had 8 confirmed GLRaV-3- positive vines in 2010. In 2011 GLRaV-3 had spread to 22 additional vines. By the end of the growing season in 2012, GLRaV-3 was present in 37 out of the 100 vines, and increase in seven additional infections over that year. Pattern of within-field spread can be seen in Figure 3.2. Spatial analysis shows three different significant aggregation values of GLRaV-3 within this plot over the three years (Table 3.7). The index of aggregation values increased as time progressed. Similarly, SADIE was used to evaluate associations between GLRaV-3 presence from one year to the next. For the 2010-2011 season, SADIE's overall index of association ( $\chi_i$ ) was 0.7283 ( $P<0.0001$ ), indicating that the 2010 GLRaV-3 positive vines and the 2011 GLRaV-3 positive vines were spatially associated. Similarly, SADIE's overall index of association in the 2011-2012 season was 0.9176 ( $P<0.0001$ ), indicating that the 2011 GLRaV-3 positive vines and the 2012 GLRaV-3 positive vines were, also, spatially associated.

In addition to the AHS-AREC plot, 5 commercial vineyards were selected for intensive sampling. At commercial vineyard A, samples from a 10x10 grid of Chardonnay were collected in 2009. The vineyard manager had previously rogued one vine out of this matrix, and all 99 remaining vines were positive for GLRaV-3 (Figure 3.3). The index of aggregation was insignificant due to the high disease incidence (Table 3.7). This grid was next to a Cabernet Sauvignon vineyard, greater than 20 years old, which exhibited characteristic symptoms of GLD.

Commercial Vineyard B was planted in 2007 and consists of varieties Merlot and Viognier. A 10x10 grid was sampled; however, samples were pooled during grinding (every 5 vines/row were pooled and tested, resulting in a 10x2 matrix for analysis). SADIE's index of aggregation showed a significant ( $P<0.05$ ) level of aggregation of diseased vines (Table 3.7). GLRaV-3 was the only virus present in this field and was found only at the southern end of the plot (Figure 3.4).

At Commercial Vineyard C, Cabernet Sauvignon was sampled. Samples from a 10x10 grid were collected in 2009 and tested for GLRaV-2, GLRaV-3, and GFkV. All but 8 of the infected vines had a mixed infection of GLRaV-2 and -3 (Figure 3.5). This plot had 99% disease incidence, and thus SADIE's index of aggregation was not significant. However, when spatial association of these two viruses was examined, SADIE's overall index of association ( $\chi_i$ ) was 0.5143 ( $P<0.0001$ ), indicating that both viruses were spatially associated with one another.

Pinot gris and Gewurztraminer were sampled at commercial Vineyard D (Figure 3.6). This vineyard had approximately 50% disease incidence, which was a result of GLRaV-3 and GFkV infection. Four vines were infected with both GLRaV-3 and GFkV. Regardless, the randomness of infected vines throughout the field resulted in a non-significant index of aggregation by SADIE (Table 3.7).

Commercial Vineyard E consisted of a young planting (vines between 1-5 years old) of Chardonnay at the time of sampling in 2009 (Figure 3.7). SADIE's index of aggregation was not significant, indicating no within-field aggregation within the 5x20 matrix that was sampled

(Table 3.7). In all, 64% of the vines were infected. Except for one vine with a mixed infection of GLRaV-2 and GLRaV-3, only GLRaV-3 was found.

### *3.3.3 Visible symptoms, spatial association, and the presence of viruses*

At four of the intensively-sampled vineyard plots (Figures 3.3 through 3.7), foliar symptoms were assessed visually, and symptom severity was recorded on a 1-5 scale (1=no symptoms, 2=0-25%, 3=26%-50%, 4=51%-75%, 5=75%-100% disease severity). Then symptom data were compared with RT-PCR results. Chi-square analysis was performed on the presence of GLRaV-3 in all vineyards, and SADIE's overall index of association was calculated between visible symptoms and RT-PCR results.

Symptoms, or lack thereof, are not a reliable indicator of infection by the virus being tested. There were vines that had no symptoms and were positive for GLRaV-3 while on the opposite end of the spectrum there were vines that had foliar symptoms consistent with GLD but that tested negative. For example, in Figure 3.8, 35 of 63 samples rated 5 (=75-100% of canopy is showing symptoms) were indeed positive with GLRaV-3. On the other hand, 16 out of 40 samples rated 1 (= no symptoms) tested positive. Chi square analysis confirmed these results: The association between symptom presence and virus-positive PCR was insignificant for both GLRaV-2 ( $P=0.71$ ) and GLRaV-3 ( $P=0.50$ ). When spatial association was examined between visible symptoms and virus-positive vines, SADIE's overall index of association ( $\chi_i$ ) was -0.0396 ( $P<0.6445$ ) at commercial vineyard E, meaning the visible symptoms and GLRaV-3 positive vines were not spatially associated. I.e., locations of symptomatic vines were not associated with locations of GLRaV-3 positive vines.

### *3.3.4 Associations between age of vines and mealybug presence*

Age of vines recorded at time of sampling was used in a contingency table and chi square analysis to determine the relationship between the age of vines and infection with GLRaV-2 or GLRaV-3 (Figure 3.9). Chi-square analysis confirmed that vines planted prior to 1990 had a significantly higher chance of being infected with GLRaV-3 ( $P<0.05$ ). The same test was run on data for GLRaV-2, and resulted in the same outcome as GLRaV-3: Vines planted prior to 1990 had a significantly higher ( $P<0.05$ ) probability of being infected with GLRaV-2 than vines planted after 1990. Therefore age of vines is a significant consideration when sampling for viruses in Virginia, as older vines are more likely to be infected.

The presence of mealybugs on vines was also noted during survey sample collections. To determine the relationship between infected vines and detected mealybug presence, GLRaV-2, GLRaV-3 and GFkV infected vines were compared with vines that had mealybugs (Figure 3.10). Chi-square analysis confirmed that vines infected with GLRaV-3 had a significantly higher chance of having mealybug infestations than those vines that had no GLRaV-3 ( $P<0.05$ ). For GLRaV-2 and GFkV, Chi-square results showed that the probability of the presence of these viruses were not significantly high ( $P<0.05$ ) with the presence of mealybugs.

### *3.3.5 Wild grapevines*

All wild grapevines sampled between 2009 and 2012 were negative for all three viruses. Species and number of wild grapevines found in Virginia and tested that were identified visually

were: 10 *Vitis aestivalis*, 9 *V. cinerea*, 2 *V. labrusca*, 13 *V. riparia*, 27 *V. rotundifolia*, 19 *V. rupestris*, and 20 *V. vulpina*. Numbers of species by location is listed in Table 3.8.

### 3.3.6 Identification of mealybug species

Mealybugs were collected from 5 different vineyards in Virginia. No scale insects were found. Multiplex PCR using mealybug species-specific primers resulted in two bands (Figure 3.11). One band at 150 bp (the expected product size for *Ferrisia gilli* [Gill's mealybug]) and the other at approximately 425 bp. This product is between two possible mealybug species band sizes (400 bp for *Ps. maritimus* and 450 bp for *Pl. ficus*). Sequencing of these products is underway to conclusively identify the mealybug species found.

## 3.4 Discussion

This is the first report of grapevine leafroll associated virus-2, -3, and grapevine fleck virus in Virginia. Similarly, it is the first report of GLRaV-3 in Maryland, and GLRaV-2 and -3 in Texas. With 64% of vineyards positive for at least one virus, it is now clear that Virginia has a viral epidemic. Similar to observations around the world (Rayapati et al., 2008), GLRaV-3 is the most common virus in Virginia. Although GLRaV-2 and GFkV are present in Virginia, they do not pose as large a risk as GLRaV-3, since their effect on vine-decline is not as severe (Rayapati et al., 2008). It is also not surprising that GLRaV-3 was more commonly found, since many mealybugs have the capability of vectoring this virus (as opposed to GLRaV-2 or GFkV, which have no known vectors) (Tsai, 2010), and the presence of these mealybugs is now confirmed in VA. Ongoing sequencing efforts may lead to a better understanding of which strains of GLRaV-2, -3, and GFkV are present in Virginia and provide a possible clue as to where these viruses originated.

Due to the uneven distribution of grape varieties among sample plots, conclusions cannot be made regarding variety susceptibility to these viruses. However, some results are interesting to note. Norton is a hybrid species with a strong background of *V. aestivalis* that has high disease resistance in general, yet 54.55% of the vines sampled were positive for at least one of the three viruses. Also, despite the small sample size, most of the American grapes (*V. labrusca*, such as Concord and Niagara) sampled were positive for GLRaV-3 (Table 3.4). In agreement with a study done by Wilcox (1998), these vines were asymptomatic at the time of sampling.

Our intensive-sampling results are consistent with other research across the globe, indicating that infection within fields is usually either random or sometimes aggregated where vectors are present (Cabaleiro et al., 2008; Golino et al., 2008). Of the six locations, only two fields had significant aggregation (or clustering) of the positive vine locations. The other four had a more random dispersion or were just completely infected, yielding no significant trends. Since our sample dimension was relatively small (10 x 10 vines), it was not surprising to see vineyards that were completely infected. Both aggregated and random results can be explained by movement patterns of the vectors of these viruses. Within the field, mealybugs usually walk very slowly between adjacent vines (which could cause a more clustered pattern). However, a random pattern could also develop with aerial, wind driven movement of this vector. These factors make it difficult to predict which vines in a field are most likely to become infected from a nearby source.

Results from the AHS-AREC plot, which was observed over three years, suggest that there is a strong spatio-temporal association with infected vines from one year to the next. This result was expected since once the vine is infected with GLRaV-3, the virus becomes systemic and will remain for the rest of the vine's life. Thus, when GLRaV-3 is introduced to a newly established vineyard, one could initially observe the clustering due to limited movement of mealybugs, and possibly predict subsequent infection locations and focus management tactics in those areas. However, the movement of GLD can be very rapid. For example, the 2010 data from the AHS-AREC plot showed a disease incidence of 22 more infected vines than the previous year. A 375% increase in infected vines is a very high number. Golino et al., (2008) found that, over a five-year period, leafroll spread throughout a vineyard in California by an average rate of over 10% per year. There have been reports that plant viruses can actually modify the vector behavior, causing them to either feed more (Stafford et al., 2011) or prefer to feed on healthy plants if they are harboring the virus and feed on infected plants if they are not harboring the virus (Ingwell et al., 2012).

As has been demonstrated in other work (Cabaleiro et al., 2008; Naidu et al., 2009), we found that symptoms are not a reliable indicator of the presence or lack of presence of these viruses. Molecular diagnostic techniques are necessary for testing candidate vines. This is crucial information for growers since the only "remedy" to these viral diseases is rouging of infected vines. Relatively easy diagnostic kit, such as the magnetic strip immunoassay kit from BIOREBA (BIOREBA, 2011), could be a very useful tool for growers.

Results from this survey indicate that older vines have a much higher probability of virus-infection. This may be attributed to the cut-off dates that were used (pre-1990 and post-1990) in the analysis. Prior to 1990, although clean plant programs were in existence, they were not commonly used, and sophisticated molecular detection tools were not available. In addition, it was not uncommon for growers to obtain vines from un-tested sources. If more dates were used (e.g. pre-1990, 1990-1995, 1995-2000, 2000-present) in the analysis there is a possibility that we could track back to the point where the effect of clean plants program began influencing the VA wine grape industry.

This study also suggested that there is a strong correlation between GLRaV-3 infected vines and mealybug infestations. This is not a surprise since many species of mealybugs can transmit GLRaV-3 (Tsai et al., 2010) and plant viruses are generally associated with their vectors. With older vines harboring the viruses, it is likely that younger vines are becoming infected by these older vines through within-field vector transmission. As expected, neither GLRaV-2 nor GFkV were associated with the presence of mealybugs, since they are not vectored by mealybugs.

Since all sampled wild grapevines tested negative for the viruses, the likelihood of wild grapevines providing inoculum source appears low. Some of these vines were collected right next to a commercial vineyard, and others were collected from mountain areas. However, with only 100 wild vines sampled, more work needs to be done to determine whether or not wild grapevines may serve as alternative virus hosts. Recently, in California, viruses were found within wild grapevines (Klaassen et al., 2011), which means that however improbable this scenario may be in Virginia, it is still possible.

The species-specific multiplex PCR for mealybugs detected the presence of two mealybug species. The first, *Ferissia gilli* (or Gill's mealybug), and the second, tentatively identified as *Pseudococcus maritimus* (or the grape mealybug). In addition, identification based on morphological characteristics by Dr. Ian Stocks, as well as PCR results, revealed a third

species, the striped mealybug (*Ferissia virgata*). The Gill's mealybug was not expected to be in Virginia and is commonly associated with damage to pistachios (Gullan et al., 2003; Haviland et al., 2006). However, it has also been reported to occasionally damage almonds, grapes, and stone fruits (Haviland et al., 2006). Although we were able to detect GLRaV-3 from Gill's mealybug, the extent of the Gill's mealybug's ability to transmit or harbor GLRaV-2, GLRaV-3, or GFkV is currently unknown. The presence of the grape mealybug in Virginia was expected and it can transmit GLRaV-3 with ease (Tsai et al., 2010). This confirms that at least one, if not two, vectors of GLD are present in the state.

Future studies will focus on determining if other viruses (remaining GLRaVs, grapevine virus A, -B, tomato ringspot virus, and rupestris stem-pitting-associated virus-1) are present in the state and if so, to what extent. Along the same lines, data of viruses sampled will be compiled to analyze for mixed infection patterns that may be occurring. New methods of virus testing will be assessed such as membrane-based testing and qRT-PCR.

A full understanding of what viruses are present in the state and how they may be interacting is necessary in order to develop a virus-free certification program for the state and reduce economic losses that result from these viruses.

### 3.5 References

- BIOREBA. (2011). Product Information: AgriStrip-magnetic Grapevine leafroll-assoc. virus 3 (GLRaV-3) Retrieved November, 2012, from [http://www.cultek.com/inf/otros/perfil-provedores/Perfil\\_Bioreba/GLRaV3/Bioreba\\_GLRaV\\_AgriStrip\\_magnetic\\_e\\_V2.pdf](http://www.cultek.com/inf/otros/perfil-provedores/Perfil_Bioreba/GLRaV3/Bioreba_GLRaV_AgriStrip_magnetic_e_V2.pdf)
- Cabaleiro, C., Couceiro, C., Pereira, S., Cid, M., Barrasa, M., & Segura, A. (2008). Spatial analysis of epidemics of Grapevine leafroll associated virus-3. *European Journal of Plant Pathology*, 121(2), 121-130.
- Cabaleiro, C., Segura, A., & García-Berrios, J. J. (1999). Effects of grapevine leafroll-associated virus 3 on the physiology and must of *Vitis vinifera* L. cv. Albariño following contamination in the field. *American Journal of Enology and Viticulture*, 50(1), 40-44.
- Charles, J. G., Cohen, D., Walker, J. T. S., Forgie, S. A., Bell, V. A., & Breen, K. C. (2006). A review of the ecology of grapevine leafroll associated virus type 3 (GLRaV-3). *New Zealand Plant Protection*, 59, 330-337.
- Daane, K. M., Middleton, M. C., Sforza, R., Cooper, M. L., Walton, V. M., Walsh, D. B., Zaviezo, T., Almeida, R. P. P. (2011). Development of a multiplex PCR for identification of vineyard mealybugs. *Environmental Entomology*, 40(6), 1595-1603.
- Fuchs, M. (2007). Grape leafroll disease factsheet Retrieved June, 2012, from [http://www.nysipm.cornell.edu/factsheets/grapes/diseases/grape\\_leafroll.pdf](http://www.nysipm.cornell.edu/factsheets/grapes/diseases/grape_leafroll.pdf)
- Fuchs, M., Martinson, T. E., Loeb, G. M., & Hoch, H. C. (2009). Survey for the three major leafroll disease-associated viruses in Finger Lakes vineyards in New York. *Plant Disease*, 93(4), 395-401.
- Golino, D. A., Weber, E., Sim, S., & Rowhani, A. (2008). Leafroll disease is spreading rapidly in a Napa Valley vineyard. *California Agriculture*, 62(4), 156-160.
- Gullan, P. J., Downie, D. A., & Steffan, S. A. (2003). A new pest species of the mealybug genus *Ferissia fullaway* (Hemiptera: Pseudococcidae) from the United States. *Annals of the Entomological Society of America*, 96(6), 723-737.

- Haviland, D. R., Beede, R., Godfrey, K. E., & Daane, K. M. (2006). *Ferrisia gilli*: A new mealybug pest of pistachios and other deciduous crops Retrieved November, 2012, from <http://anrcatalog.ucdavis.edu/pdf/8207.pdf>
- Ingwell, L. L., Eigenbrode, S. D., & Bosque-Pérez, N. A. (2012). Plant viruses alter insect behavior to enhance their spread. *Scientific Reports*, 2(578), srep00578.
- Klaassen, V. A., Sim, S. T., Dangl, G. S., Osman, F., Al-Rwahnih, M., Rowhani, A., & Golino, D. A. (2011). *Vitis californica* and *Vitis californica* × *Vitis vinifera* hybrids are hosts for Grapevine leafroll-associated virus-2 and -3 and Grapevine virus A and B. *Plant Disease*, 95(6), 657-665.
- Kovacs, L. G., Hanami, H., Fortenberry, M., & Kaps, M. L. (2001). Latent infection by leafroll agent GLRaV-3 is linked to lower fruit quality in French-American hybrid grapevines Vidal blanc and St. Vincent. *American Journal of Enology and Viticulture*, 52(3), 254-259.
- Madden, L. V., Hughes, G., & Bosch, F. v. d. (2007). *The study of plant disease epidemics*. St. Paul: American Phytopathological Society (APS Press).
- Martelli, G. P., & Boudon-Padiou, E. (2006). *Directory of infectious diseases of grapevines*. (Vol. 55): Options Méditerranéennes, Series B. 55: 11-201.
- Naidu, R. A., Perry, E. M., Pierce, F. J., & Mekuria, T. (2009). The potential of spectral reflectance technique for the detection of Grapevine leafroll-associated virus-3 in two red-berried wine grape cultivars. *Computers and Electronics in Agriculture*, 66(1), 38-45.
- Nita, M., Ellis, M. A., & Madden, L. V. (2012). Spatial pattern of phomopsis cane and leaf spot symptoms in commercial vineyards in Ohio. *Journal of Phytopathology*, 160(1), 26-36.
- Perry, J. N. (1998). Measures of spatial pattern for counts. *Ecology*, 79(3), 1008-1017.
- Perry, J. N., Bell, E. D., Smith, R. H., & Woiwod, I. P. (1996). SADIE: software to measure and model spatial pattern. (46), 95-102.
- Perry, J. N., Winder, L., Holland, J. M., & Alston, R. D. (1999). Red-blue plots for detecting clusters in count data. *Ecology Letters*(2), 106-113.
- Rayapati, N., O'Neal, S., & Walsh, D. (2008). Grapevine leafroll disease. *Washington State University Extension Bulletin EB2027E* Retrieved 2012, April, from <http://pubs.wsu.edu>
- Stafford, C. A., Walker, G. P., & Ullman, D. E. (2011). Infection with a plant virus modifies vector feeding behavior. *Proceedings of the National Academy of Sciences of the United States of America*, 108(23), 9350-9355.
- Tsai, C., Rowhani, A., Golino, D. A., Daane, K. M., & Almeida, R. P. P. (2010). Mealybug transmission of grapevine leafroll viruses: an analysis of virus-vector specificity. *Phytopathology*, 100(8), 830-834.
- VWB. (2012). *The Economic Impact of Wine and Wine Grapes on the State of Virginia--2010*. St. Helena, CA: A Frank, Rimerman + Co. LLP.
- Wilcox, W. F., Jiang, Z. Y., & Gonsalves, D. (1998). Leafroll virus is common in cultivated American grapevines in western New York. *Plant Disease*, 82(9), 1062.
- Winder, L., Alexander, C. J., Holland, J. M., Woolley, C., & Perry, J. N. (2001). Modelling the dynamic spatio-temporal response of predators to transient prey patches in the field. *Ecology Letters*, 4(6), 568-576.



**Table 3.1.** Virus-specific oligonucleotide primers used in the RT-PCR and the size of the RT-PCR product

Virus	Forward Primer (5'—3')	Reverse Primer (5'—3')	PCR product size (bp)
Grapevine leafroll-associated virus-2	ATAATTCGGCGTACATCCCCACTT	GCCCTCCGCGCAACTAATGACAG	331
Grapevine leafroll-associated virus-3	CGCTAGGGCTGTGGAAGTATT	GTTGTCCCGGTACCAGATAT	546
Grapevine fleck virus	CTCAGCCTCCACCTTGCCCCGT	CAATTGGCTGGGCGAGAAGTACA	533

**Table 3.2:** Species-specific primers for vineyard mealybugs used in the multiplex PCR and the size of the products. (Daane et al., 2011)

Mealybug Species	Common Name	Primer Sequence (5'–3')	PCR product size (bp)
<i>Pseudococcus calceolariae</i>	Citrophilus mealybug	TGCAACAATAATTATTGCCATC	650
<i>Pseudococcus longispinus</i>	Long-tailed mealybug	CCATTTATCTTTGATCCACAG	600
<i>Planococcus ficus</i>	Vine mealybug	CTTTGTTGTAGCTCACTTTCAC	450
<i>Pseudococcus maritimus</i>	Grape mealybug	CTGATTTCCTTTATTAATTAATTCAAC	400
<i>Planococcus citri</i>	Citrus mealybug	TAATCTATTTTTATCTATCAATTTAACC	350
<i>Pseudococcus viburni</i>	Obscure mealybug	ATATTTCTTCTATTGGTTCATTC	250
<i>Ferissia gilli</i>	Gill's mealybug	GAATCATTAATTTCTAAACGTTTACTAA	150
Universal reverse primer for mealybug species		CAATGCATATTATTCTGCCATATTA	

**Table 3.3:** Mixed infections that occurred out of 415 total surveyed grapevines.

	Total	Percentage
Mixed infections	19	4.6
GLRaV-2 and GLRaV-3	16	3.9
GLRaV-2 and GFkV	1	0.2
GLRaV-3 and GFkV	2	0.5
GLRaV-2, GLRaV-3, and GFkV	0	0.0

**Table 3.4:** Percentages and numbers of vines testing positive for at least one virus by variety in Virginia.

Variety	Number GLRaV-2 (+) Vines	Number GLRaV-3 (+) Vines	Number GFkV (+) Vines	Mixed Infection Cases	Total number of vines sampled	Percent of vines positive for at least one virus
Cabernet Sauvignon <sup>X+</sup>	13	15	0	4	81	31
Cabernet franc <sup>X+</sup>	2	6	0	0	54	15
Chardonnay <sup>X*</sup>	4	16	1	5	36	47
Petit Verdot <sup>X+</sup>	0	7	0	0	36	19
Merlot <sup>X+</sup>	0	7	0	0	33	21
Viognier <sup>X*</sup>	0	8	0	0	27	30
Traminette <sup>Y+</sup>	1	1	0	1	15	7
Petit Manseng <sup>X*</sup>	0	1	0	0	14	7
Chambourcin <sup>Y+</sup>	1	2	0	1	12	17
Malbec <sup>X+</sup>	0	2	0	0	12	17
Norton <sup>Y+</sup>	0	6	0	0	11	55
Vidal blanc <sup>Y*</sup>	7	5	1	4	11	82
Syrah <sup>X+</sup>	0	4	0	0	8	50
Riesling <sup>X*</sup>	1	3	0	1	6	50
Chardonnay <sup>Y*</sup>	0	0	0	0	5	0
Pinot gris <sup>X*</sup>	0	2	1	1	5	40
Seyval blanc <sup>Y*</sup>	1	2	0	0	5	60
Gewurztraminer <sup>X*</sup>	0	2	0	0	4	50
Rkatsiteli <sup>X*</sup>	0	3	0	0	3	100
Roussanne <sup>X*</sup>	0	0	0	0	3	0
Sangiovese <sup>X+</sup>	1	1	0	1	3	33
Tannat <sup>X+</sup>	0	1	0	0	3	33
Albariño <sup>X*</sup>	0	0	1	0	2	50
Chancellor <sup>Y*</sup>	0	2	1	1	2	100
Concord <sup>Z+</sup>	0	1	0	0	2	50
Muscat <sup>X*</sup>	0	0	0	0	2	0
Nebbiolo <sup>X+</sup>	0	0	0	0	2	0
New York 95 <sup>Z+</sup>	0	0	0	0	2	0
Sauvignon blanc <sup>X*</sup>	0	1	0	0	2	50
Tempranillo <sup>X+</sup>	0	0	0	0	2	0
Touriga Nacional <sup>X+</sup>	0	1	0	0	2	50
American Table Grape <sup>Z+</sup>	0	0	0	0	1	0
Barbera <sup>X+</sup>	0	0	0	0	1	0
Catawba <sup>Z+</sup>	0	1	0	0	1	100
Cayuga White <sup>Y*</sup>	0	0	0	0	1	0
Fer Servadou <sup>X+</sup>	0	0	0	0	1	0
Mourvèdre <sup>X+</sup>	0	1	0	0	1	100
Muscadine <sup>Z*</sup>	0	0	0	0	1	0
Niagara <sup>Z*</sup>	0	1	0	0	1	100
Orange Muscat <sup>X*</sup>	0	0	0	0	1	0
Pinot noir <sup>X+</sup>	0	1	0	0	1	100

<sup>X</sup> denotes *V. vinifera*; <sup>Y</sup> denotes inter-specific hybrid; and <sup>Z</sup> denotes *V. labrusca*; \* denotes white-fruited variety; + denotes red-fruited variety

**Table 3.5:** Viruses detected from sampled vines in Maryland, North Carolina, Pennsylvania, and Texas by variety.

Region	Variety	GLRaV-2	GLRaV-3	GFkV	Total number of vines sampled
Maryland	Cabernet franc	0	0	0	2
	Chardonnay	0	1	0	1
	Merlot	0	0	0	2
	Petit Verdot	0	1	0	1
North Carolina	Cabernet franc	0	0	0	6
Pennsylvania	Barbera	0	0	0	1
	Cabernet franc	0	0	0	1
	Chardonnay	0	0	0	2
	Malbec	0	0	0	2
	Merlot	0	0	0	2
	Syrah	0	0	0	1
	Tannat	0	0	0	1
Texas	Blanc Du Bois	0	0	0	6
	Lenoir	5	1	0	13

**Table 3.6:** Locations and years of intensive sampling including sampling grid size and variety tested.

Location	Year(s)	Matrix/Grid	Variety	Total # Samples
AHS Jr. AREC	2010, 2011, and 2012	10x10	Chardonnay + Merlot	100
Commercial Vineyard A	2009	20x5	Chardonnay	100
Commercial Vineyard B	2010	10x10	Cabernet Sauvignon + Viognier	100
Commercial Vineyard C	2009	10x10	Cabernet Sauvignon	100
Commercial Vineyard D	2009	5x20	Pinot gris + Gewurztraminer	100
Commercial Vineyard E	2009	20x5	Chardonnay	100

**Table 3.7:** Spatial analysis of disease incidence in intensive sampling plots in Virginia.

Location	Year	Total DI <sup>a</sup>	I <sub>a</sub> <sup>b</sup>
AHS Jr., AREC	2010	0.08	1.517*
AHS Jr., AREC	2011	0.3	1.896*
AHS Jr., AREC	2012	0.37	2.111*
Commercial Vineyard A	2009	1	1.369
Commercial Vineyard B	2009	0.3	2.419*
Commercial Vineyard C	2009	0.99	1.328
Commercial Vineyard D	2009	0.52	1.058
Commercial Vineyard E	2009	0.64	0.943

<sup>a</sup> Total DI (disease incidence) = Number of vines positive for a virus/total number of vines tested.

<sup>b</sup> Index of aggregation values (I<sub>a</sub>) from SADIEshell nonparametric analysis. Significant levels of aggregation if I<sub>a</sub>>1.5 (denoted by asterisk).

**Table 3.8:** Wild grapevines sampled in Virginia by region and species.

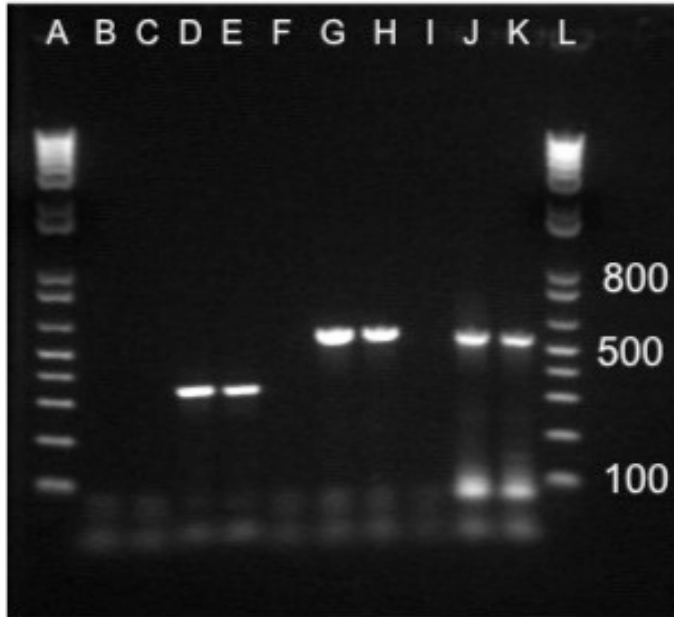
Location	Species	Number sampled
Central	<i>Vitis aestivalis</i>	3
	<i>Vitis cinerea</i>	1
	<i>Vitis labrusca</i>	1
	<i>Vitis riparia</i>	4
	<i>Vitis rotundifolia</i>	8
	<i>Vitis rupestris</i>	4
	<i>Vitis vulpina</i>	11
Eastern	<i>Vitis cinerea</i>	5
	<i>Vitis riparia</i>	1
	<i>Vitis rotundifolia</i>	2
	<i>Vitis rupestris</i>	6
	<i>Vitis vulpina</i>	2
Northern	<i>Vitis aestivalis</i>	4
	<i>Vitis cinerea</i>	1
	<i>Vitis riparia</i>	5
	<i>Vitis rotundifolia</i>	11
	<i>Vitis rupestris</i>	7
	<i>Vitis vulpina</i>	5
Southern	<i>Vitis aestivalis</i>	2
	<i>Vitis cinerea</i>	2
	<i>Vitis labrusca</i>	1
	<i>Vitis riparia</i>	1
	<i>Vitis rotundifolia</i>	3
	<i>Vitis vulpina</i>	2
Western	<i>Vitis aestivalis</i>	1
	<i>Vitis riparia</i>	2
	<i>Vitis rotundifolia</i>	3
	<i>Vitis rupestris</i>	2



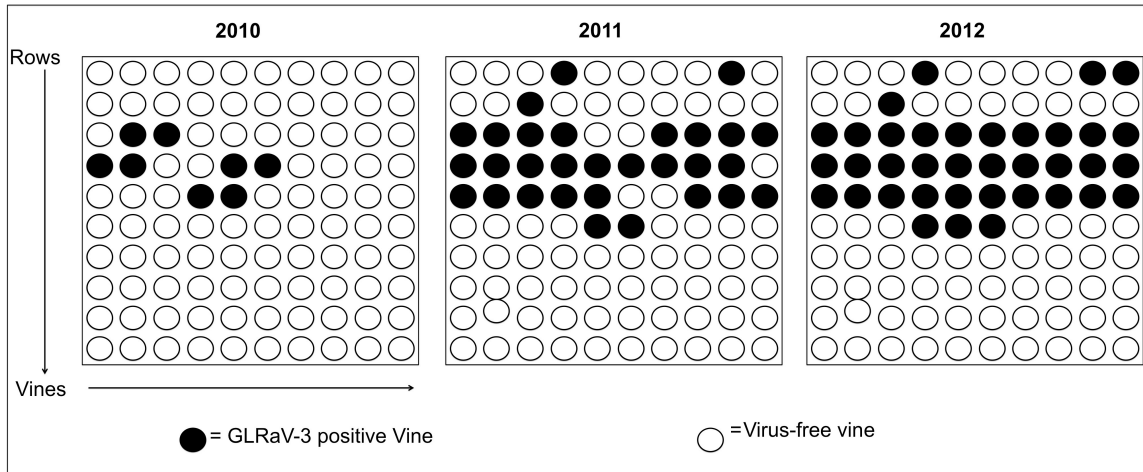
**Table 3.9:** Number of vines positive for cases of mixed infections (multiple viruses within the same vine).

Variety	GLRaV-2 + GLRaV-3	GLRaV-2 + GFkV	GLRaV-3 + GFkV	GLRaV-2 + GLRaV-3 + GFkV	Total Number of Mixed Infection Cases
Chardonnay <sup>X*</sup>	4	0	1	0	5
Cabernet Sauvignon <sup>X+</sup>	4	0	0	0	4
Vidal blanc <sup>Y*</sup>	3	1	0	0	4
Chambourcin <sup>Y+</sup>	1	0	0	0	1
Chancellor <sup>Y*</sup>	1	0	0	0	1
Pinot gris <sup>X*</sup>	0	0	1	0	1
Riesling <sup>X*</sup>	1	0	0	0	1
Sangiovese <sup>X+</sup>	1	0	0	0	1
Traminette <sup>Y*</sup>	1	0	0	0	1

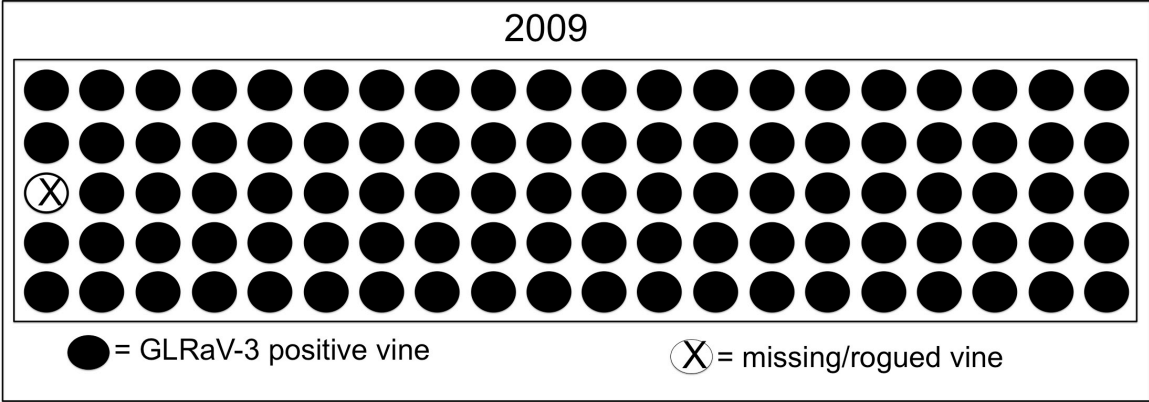
<sup>X</sup> denotes *V. vinifera*; <sup>Y</sup> denotes inter-specific hybrid; and <sup>Z</sup> denotes *V. labrusca*; \* denotes white-fruited variety; + denotes red-fruited variety



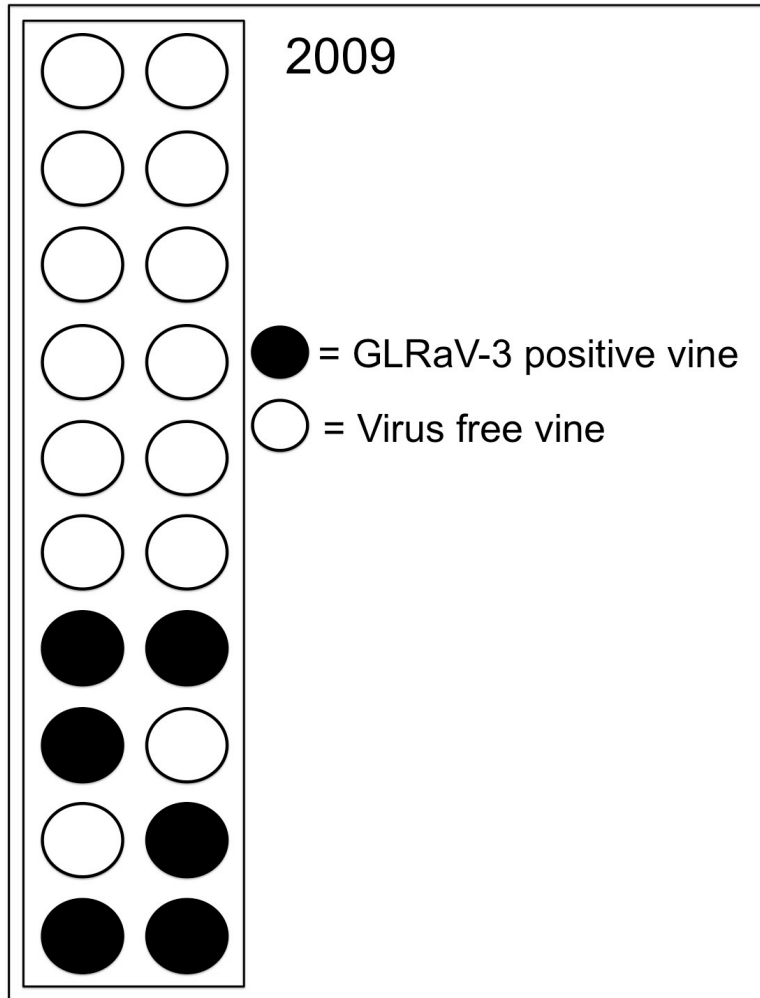
**Figure 3.1:** Agarose gel showing products from one-tube-one-step RT-PCR for three different viruses: (A + L) DNA 1kb ladder, (B) water blank control, (C) GLRaV-2 negative sample, (D) GLRaV-2 positive control sample, (E) GLRaV-2 positive sample, (F) GLRaV-3 negative sample, (G) GLRaV-3 positive control sample, (H) GLRaV-3 positive sample, (I) GFkV negative sample, (J) GFkV positive control sample, and (K) GFkV positive sample.



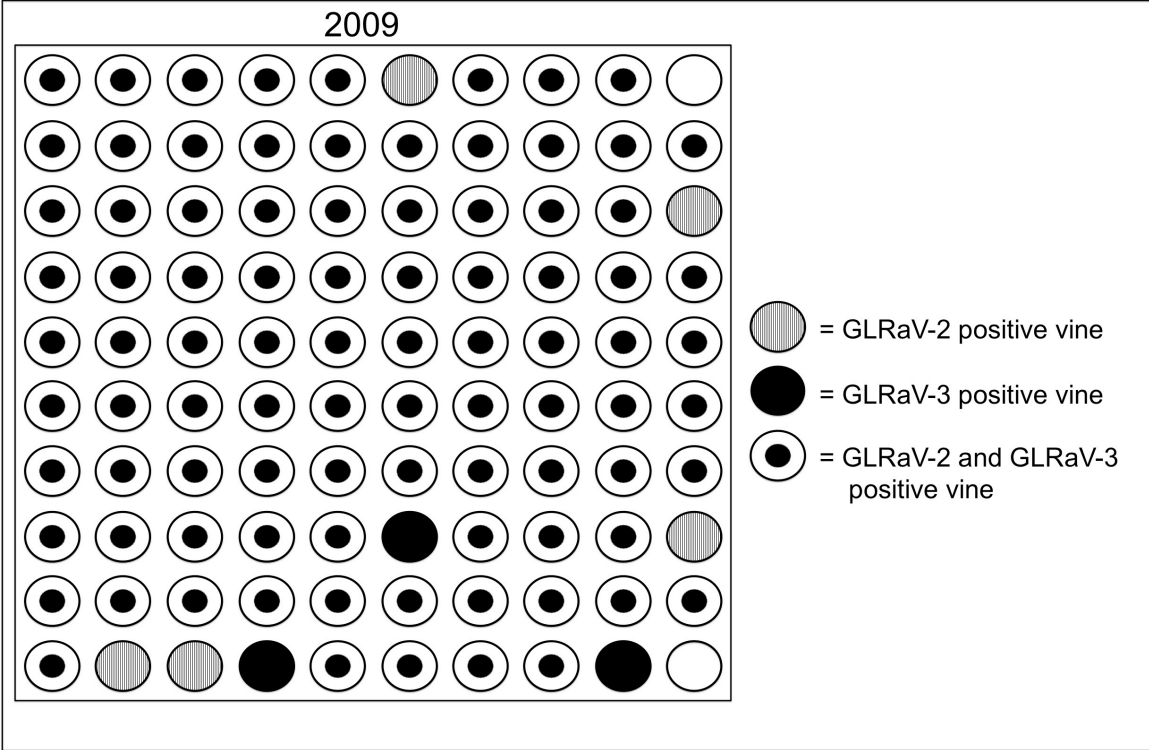
**Figure 3.2:** Within-field spread of GLRaV-3 at the AHS Jr., AREC in Winchester, VA over a three-year time span. The same 10-row by 10-vine matrix of Chardonnay and Merlot was used each year resulting in 100 total vines sampled and tested for GLRaV-3.



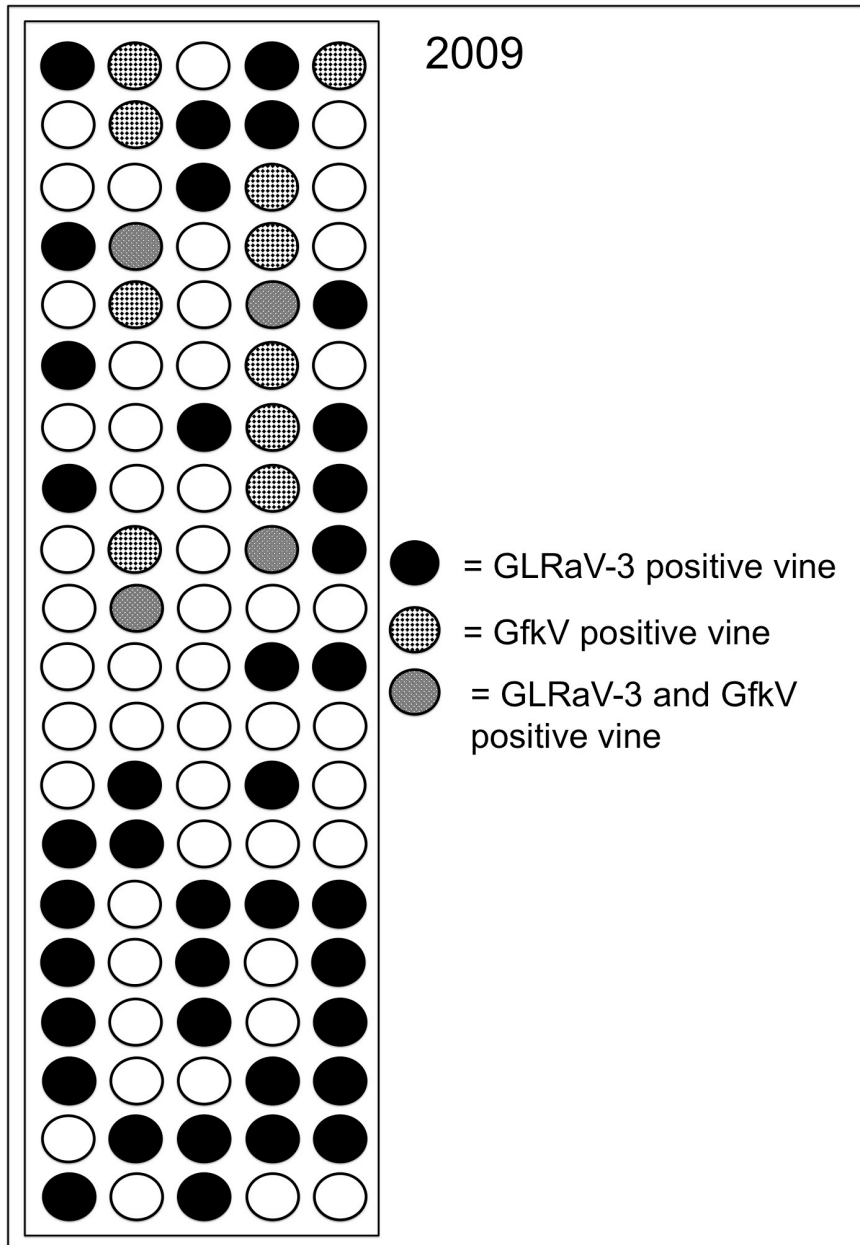
**Figure 3.3:** Intensive sampling-plot at commercial vineyard A. A 5-row by 20-vine matrix of Chardonnay was used (one vine out of the 100 had been rogued out by the vineyard owner prior to sampling). All samples tested positive for GLRaV-3.



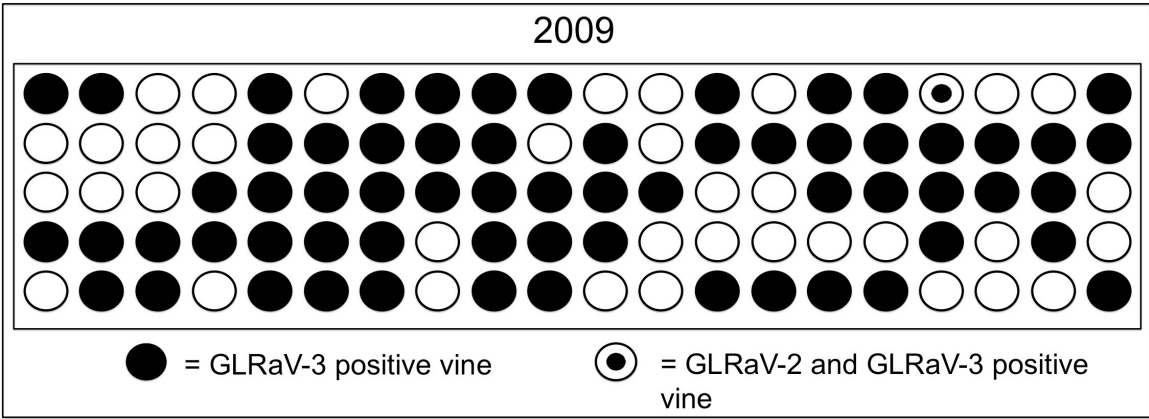
**Figure 3.4:** Intensive sampling-plot at commercial vineyard B. A 10-row by 10-vine matrix of Cabernet Sauvignon and Viognier was used; however, samples were combined during grinding (5 vines = one sample), resulting in a matrix of 10 rows by 2 vines). Six samples were positive for GLRaV-3.



**Figure 3.5:** Intensive sampling-plot at commercial vineyard C. A 10-row by 10-vine matrix of Cabernet Sauvignon was used. Majority of field was infected with a mixed infection of GLRaV-2 and GLRaV-3.

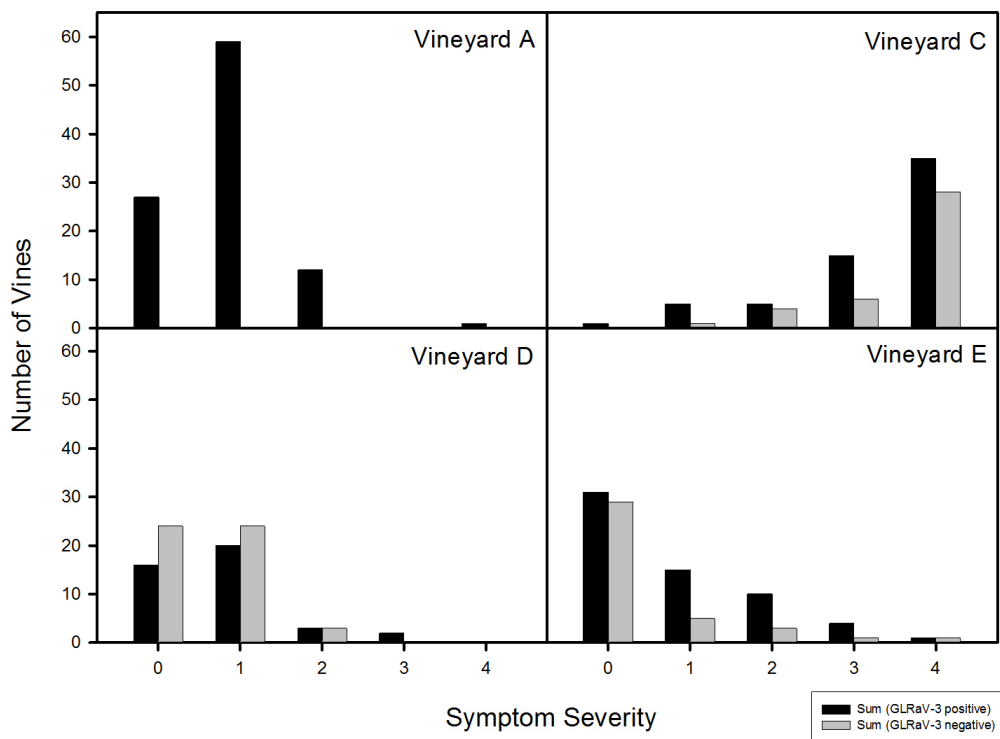


**Figure 3.6:** Intensive sampling-plot at commercial vineyard D. A 20-row by 5-vine matrix of Pinot gris and Gewurztraminer was used. The majority of the field was infected with either GLRaV-3 or GfKV. Mixed infection with both viruses within the same vine was also observed.

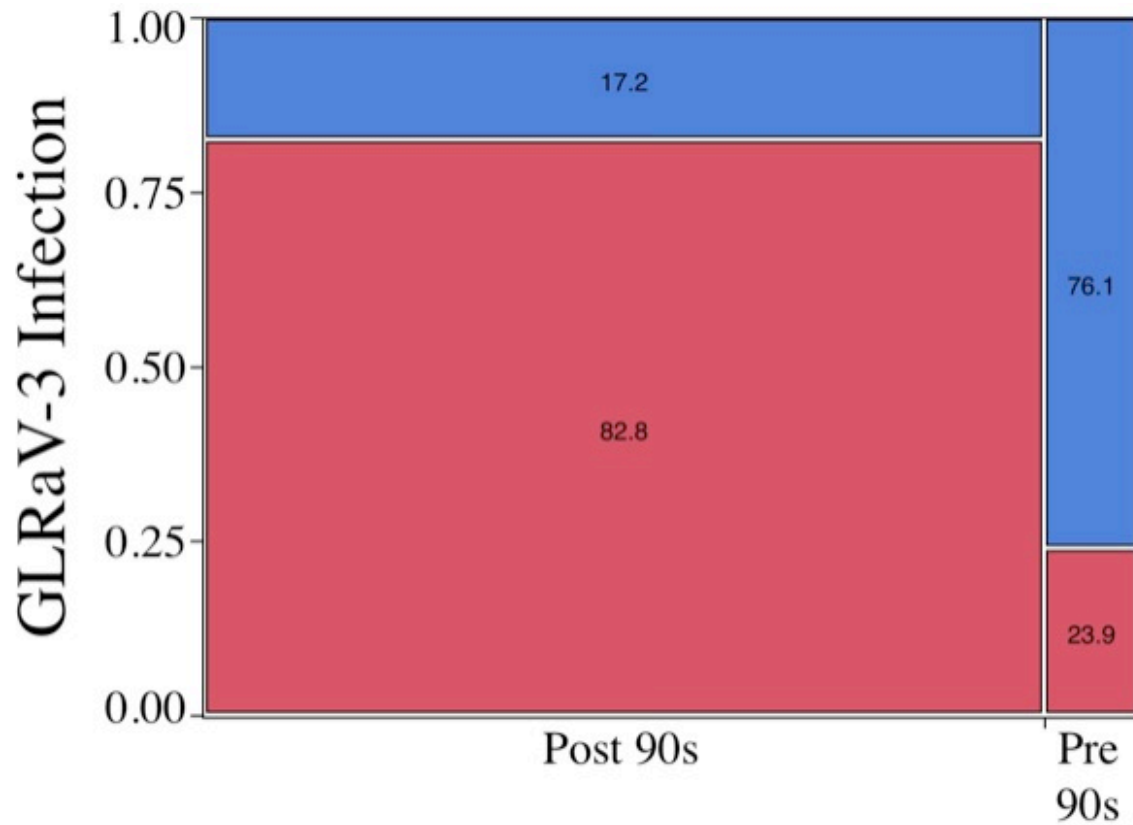


**Figure 3.7:** Intensive sampling-plot at commercial vineyard E. A 5-row by 20-vine matrix of Chardonnay was used. Majority of field was infected with GLRaV-3. Mixed infection with both GLRaV-2 and GLRaV-3 within the same vine was also observed.



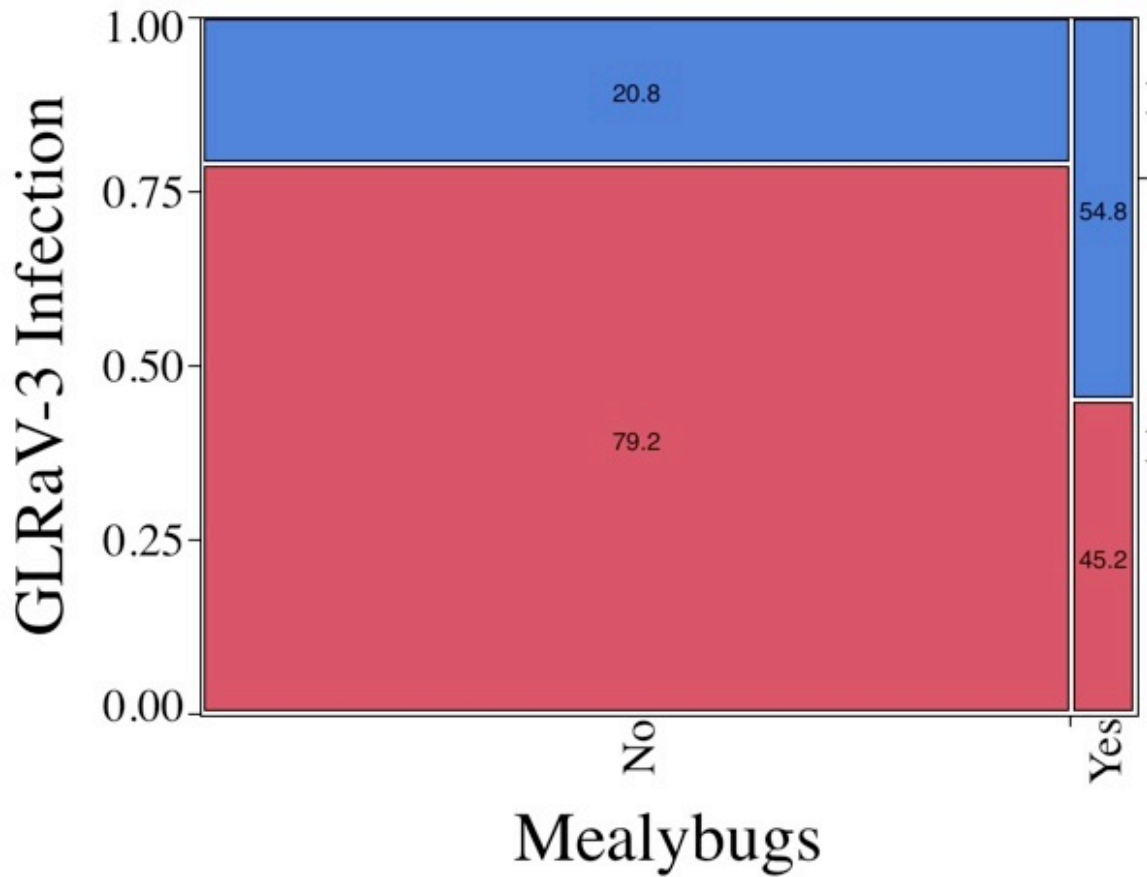


**Figure 3.8:** Symptom severity (0=no symptoms, to 4=100% foliar symptoms) by number of vines testing positive for GLRaV-3 at Commercial vineyard A, C, D, and E. Heights of black and grey bars represent number of GLRaV-3 positive and negative (respectively) vines per severity classification.

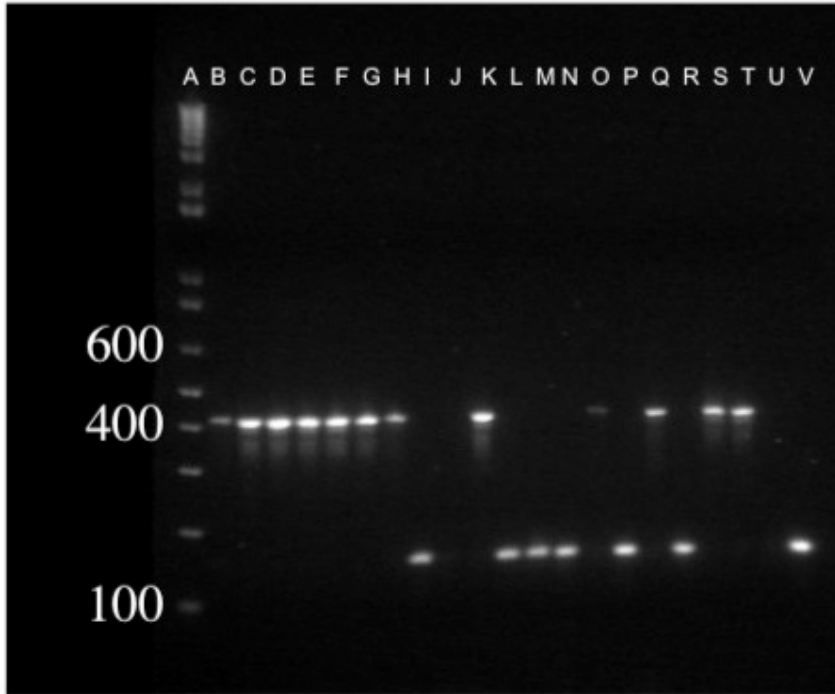


### Age of Grapevine

**Figure 3.9:** Contingency analysis of GLRaV-3 presence in vines planted both before and after 1990. Blue boxes represent GLRaV-3 positive vines and red boxes represent GLRaV-3 negative vines. Percentage of samples for each x-y combination is shown in the box.



**Figure 3.10:** Contingency analysis of GLRaV-3 presence in vines with and without mealybugs. Blue boxes represent GLRaV-3 positive vines and red boxes represent GLRaV-3 negative vines. Percentage of samples for each x-y combination shown is shown in the box.



**Figure 3.11:** Agarose gel showing products from multiplex PCR for several mealybug species. (A) DNA 1kb ladder, (B, C, D,E,F,G,H,K,O,Q,S, and T) ~425bp product (unknown), that falls in between the expected product sizes for Vine (450bp) and Grape (400bp) mealybug PCR products, (I, L,M,N,P,R, and V) ~150bp product (expected size for Gill's mealybug), (I and U) DNA concentrations too low for ID.

# Chapter 4

## Studies on mealybug biology, roles in GLD spread, and management

### 4.1 Introduction

Mealybugs and scale insects (order *Hemiptera*) are common vineyard pests throughout the world. Some mealybug species are known to cause extensive damage to vineyards by transmitting viral diseases, causing feeding damage, and excreting honeydew that promotes growth of other microorganisms such as sooty mold (Flaherty et al., 1992).

In general, mealybugs have multiple generations per year and overwinter as eggs or adults under protective layers of old bark on the grapevines trunk and cane (Varela, 2005). After hatching in the spring, mealybug 1<sup>st</sup> instars (the most mobile stage of development) move to emerging green tissues to find a feeding site (Cornwell, 1958; Furness, 1976; Grasswitz & James, 2008). As shoots grow and clusters develop, mealybugs move throughout the canopy to feed at the underside of leaves, along shoots, and on berries (Varela, 2005). In mid-summer, adult female mealybugs return to the old wood for a short period of time to lay eggs for the next generation (Varela & Smith, 2009). Environmental conditions, such as temperature and grapevine development, affect all stages of mealybug development and movement (Cornwell, 1958; Grasswitz and James, 2008). For example, higher temperatures seem to result in an increase in mealybug activity and thus, higher populations. (Cornwell, 1958).

In the commonwealth of Virginia (VA), little is known about the presence of these pests, or even what species exist. There have been a few reports of mealybug outbreaks in Virginia (Nita, *personal communication*). Although mealybugs themselves can cause feeding damage on grapevines and secondary damages such as growth of fungus on grape berry skin due to mealybug honey dew production, the primary concern with mealybugs and scale insects is their ability to transmit multiple viruses, including those that vector viruses associated with the grapevine leafroll disease complex.

Grapevine leafroll disease (GLD) is one of the most common viral disease affecting grapevines internationally. The disease itself has about ten viruses (the grapevine leafroll-associated viruses, or GLRaVs) that have been associated with it thus far (Hu et al., 1990; Fuchs, 2007). Of these, GLRaV-2 and GLRaV-3 are by far the most common. The vector for GLRaV-2 has not been identified; however, GLRaV-3 is known to have many mealybug and soft scale species as vectors (Tsai et al., 2010). Vines infected with GLRaV-3 can produce un-desirable grapes at harvest. Some of the traits are: decreased berry color and weight; increased pH; and decreased Brix (sugar level) and titratable acidity (Kovacs et al., 2001; Fuchs et al., 2009; Rayapati et al., 2008; Cabaleiro et al., 1999). No cases of mechanical transmission of GLRaVs have been reported, thus spread of this disease within a field is restricted to insect vectors. If a vineyard were to have a mealybug infestation, crop loss may be anticipated due to both mealybug activity and viral diseases transmitted by these insects.

Within the United States, VA has experienced 106% wine-related economic growth between 2005 and 2010 (VWB, 2012). Sales of VA wine have never been higher and wine-

related tourism continues to increase dramatically (VWB, 2012). Overall wine production has increased by 37% in recent years and the industry itself is predicted to expand even further (VWB, 2012). The VA wine industry contributed almost 750 million dollars annually in 2010 and there are over 200 wineries in VA in 2012 (VWB, 2012). With this expanding industry, an increased concern of GLD and its mealybug vectors has also surfaced.

GLRaV-3 is transmitted by mealybugs in a semi-persistent manner (Charles et al., 2006). Insecticides have been shown to be effective against vectors for all three types of transmission (persistent, semi-persistent, and non-persistent). One study by Gibson et al., 1982 showed that a pyrethroid (deltamethrin) reduced acquisition and infection rates in both semi-persistent (sugar beet yellows virus) and non-persistent (potato virus Y) cases and reduced the numbers of infected plants in the case of a persistently transmitted virus (beet mild yellowing virus).

By controlling the vector, it is possible to reduce the risk of the spread of the disease. Some insecticides that are labeled for control of mealybugs are acetamiprid (Assail), buprofezin (Applaud), cyfluthrin (Baythroid), dinotefuran (Venom), imidacloprid (Provado), and spirotetramat (Movento) (Schooley, 2010). Also, horticultural oil has been applied to dormant canes to reduce the insect populations and their activities in the spring (Flebut, 1922). Sprays of insecticide at a dormant and delayed dormant state can also help reduce the initial rise of the first instar population by 83-89% and 88-93% respectively (Flebut, 1922). In commercial vineyards in VA, insecticides are sprayed aerially using equipment such as an air blast sprayer. Some systemic insecticides (such as Movento, Scorpion, or Venom) can also be injected into the soil or applied through irrigation water; however, this requires special equipment or proper irrigation settings.

In this study, two field trials were conducted to determine effectiveness of control of mealybugs and to assess to the spread of grapevine leafroll disease under these conditions. The primary objectives of this study were to evaluate the efficacy of foliar insecticide treatments in two separate field experiments where we 1) determined the movement of GLRaVs and mealybugs from infected vines to a newly planted clean vine within the same row, and 2) determined the change in mealybug population in a commercial vineyard where an established mealybug population has been recorded.

## **4.2 Materials and Methods**

Two field experiments were conducted at geographically separated vineyards in Virginia. The first location was at an experimental field at the AHS Jr. AREC at Winchester, VA (AREC), located 39°N 6' 33.55", -78°W 16' 56.08". The cultivar was 'Cabernet Sauvignon' (*V. vinifera*) which was planted in 1990. The experiment was conducted from 2009 to 2011. The other experiment was conducted at a commercial vineyard in Orange County, Virginia (Orange), located at 38°N 13' 58.58", -78°W 6' 15.62". The cultivar was 'Chardonnay' (*V. vinifera*), which was planted in 1988. The experiment was conducted from 2011 to 2012. At both locations, presence of mealybugs was observed in multiple years prior to the start of the experiment.

### *4.2.1 Field Experiment 1, AREC vineyard*

Vines at the AREC location contained 3 rows with 13 panels of vines per row (each panel consists of three vines). Vines were trained in a divided canopy Lyre system, which is one of the commonly practiced vine training systems in Virginia. A majority of these vines were confirmed in 2008 to be infected with GLRaV-3 (M. Fuchs, *personal communication*) and mealybugs had also been observed in this plot (T. Wolf, *personal communication*).

The experimental design was a split-plot design with a total of six blocks (Figure 4.1). Each block consisted of three panels (row section between posts) that were separated by one empty panel (6.3 m). In order to restrict the movement of mealybugs between vines and panels, only every other panel was planted, and within-row vine space was treated with herbicide to remove weeds that may harbor mealybugs. In each panel, two vines were removed and replaced with virus-tested certified vines of Cabernet franc, which is known to be susceptible to GLRaVs and tends to show clear, visible symptoms. New Cabernet franc vines were planted at 1.5 m and 3.0 m from the old Cabernet Sauvignon vine. The position of the new vine relative to the infected vine was reversed in three blocks, so that we could examine potential effects from the prevailing wind direction. Between the plots, new plants were strategically placed so that none of the new vines were next to the infected vines except the one in the same panel (Figure 4.1).

There were three treatments: 1) two insecticide applications that consisted of acetamiprid (Assail, 0.182 L/ha, United Phosphorus, Inc. [UPI], King of Prussia, PA) at delayed dormant and a pyrethroid (Baythroid XL, 0.219 L/ha, Bayer CropScience LP, Research Triangle, NC) sprayed at bloom; 2) one insecticide application of acetamiprid (Assail, 0.182 L/ha) at delayed dormant; and 3) no insecticide spray as a control. The rate was based on 935.4 L/ha of water. The treatment was applied on a panel, and the assignment of treatment within a block was random. The same treatment was applied on the same vines in each of the three years. Fungal diseases were controlled by a standard fungicide application program that does not affect mealybug activities.

Petioles on these vines were sampled (21 petioles/vine taken randomly throughout the canopy) and then subjected to the following RT-PCR detection method annually in mid-September. The petiole samples were sealed in a plastic bag and stored in a cooler containing ice or in a refrigerator at 4°C until extraction of RNA. RNA was extracted as follows: 0.25 grams of each sample was placed into grinding bags (BIOREBA, Switzerland) containing a filter-sterilized grapevine extraction buffer (1.59g/liter Na<sub>2</sub>CO<sub>3</sub>, 2.93g/liter NaHCO<sub>3</sub>, 2% PVP-40, 0.2% BSA, and 0.05% Tween 20); the tissue was ground using a mechanical grinder (BIOREBA, Switzerland, Homex 6 [115V]); then crude extracts were transferred into 1.5-ml microcentrifuge tubes for storage at -80°C.

The crude extract was then used in a one-tube, one-step RT-PCR protocol that was developed by Dr. Rayapati (Washington State University) for the detection of multiple GLRaVs (Rowhani et al., 2000; Naidu et al., 2006; Rayapati et al., 2008). For each sample, 2 µl of crude extract was transferred to a 0.5ml microcentrifuge tube containing 25 µl 1X GES (0.1 M glycine, pH 9.0; 50 mM NaCl; 1 mM EDTA; 0.5% Triton X-100); then 1% β-mercaptoethanol was added to the 25-µl volume prior to addition of crude extract (Sigma-Aldrich Co. LLC, St. Louis, MO). Prior to a RT-PCR process, tubes containing the crude extract mixture were subjected to a denaturing temperature (95°C) for 10 min then placed immediately on ice for 5 min. Two µl of each sample's denatured extract was transferred to a 200 µl PCR tube containing the following reaction mixture: 13.4 µl nuclease-free H<sub>2</sub>O, 2.5 µl 10X PCR buffer containing 15mM Mg (Roche, Indianapolis, IN), 2.5 µl sucrose-cresol red (20% w/v sucrose, 1 mM cresol red) (Sigma-Aldrich Co. LLC, St. Louis, MO), 1.25 µl forward primer (20 µM) 1.25 µl reverse primer (20

$\mu\text{M}$ ), 1.25  $\mu\text{l}$  100mM dithiothreitol (Roche), 0.5  $\mu\text{l}$  dNTPs (10 mM) (Invitrogen, Grand Island, NY), 0.1  $\mu\text{l}$  RnaseOUT (40 U/ $\mu\text{l}$ ) (Invitrogen), 0.035  $\mu\text{l}$  Superscript III RTase (200 U/ $\mu\text{l}$ ) (Invitrogen), and 0.25  $\mu\text{l}$  Taq DNA polymerase (5 U/ $\mu\text{l}$ ) (Roche). The reactions were placed in a thermal cycler and subjected to the following cycles: 52°C for 1 h, 35 cycles of (94°C for 30 s, 54°C for 45 s, 72°C for 1 min) with a final extension at 72°C for 2 min. Ten  $\mu\text{l}$  of each reaction was run on a 1% TBE agarose gel for 1 h at 120 V and products were visualized with Gel Red™ (Phenix Research, Candler, NC). Specific primers were used for RT-PCR detection of different GLRaVs and other major viruses of grape (Table 4.1). Positive and negative (virus-free samples) controls were used to verify the test results.

Mealybugs were counted three times during the year in 2010 and eleven times in 2011. At each vine, 5 min was taken to visually count mealybugs. Visual counting included searching by peeling back bark, inspecting cordons and shoots, the underside of leaves, as well as inspection of clusters.

#### 4.2.2 *Field Experiment 2, Orange vineyard*

A second insecticide trial was conducted at a commercial vineyard in Orange County, VA. A row of Chardonnay, which was consistently infested with large populations of mealybugs, was used. A randomized block design with four replications was implemented as the experimental design for the field. Each block consisted of six vines that were trained in a Lyre system. Within each block, treatments were assigned to a vine randomly, and there was a buffer vine between each treated vine. The treatments consisted of foliar application of two different neonicotinoid insecticides: 1) dinotefuran (Scorpion, 0.292 L/ha, Gowan Company, Yuma, AZ); or 2) spirotetramat, (Movento, 0.439 L/ha, Bayer Crop Science LP)]; or 3) water as a control. Two treatments were applied at 3- and 2-week intervals during 2011 (June 10<sup>th</sup> and July 1<sup>st</sup>) and 2012 (May 16<sup>th</sup> and May 30<sup>th</sup>) seasons, respectively. The same treatment was applied to the same vine in each year. As with the AREC vineyard, within-row vine spaces were treated with an herbicide for weed control. Mealybugs were then assessed as above, through visual counting of live insects over a 5 min time span. The initial assessment was done prior to the first application of insecticide.

#### 4.2.3 *Statistical Analysis*

The AREC vineyard's data were analyzed using a linear mixed model in JMP (ver. 10, SAS institute, Cary, NC) for ANOVA, where treatment effects were considered as a fixed factor, and a blocking effect was considered as a random factor. Mealybug count data were transformed using natural log prior to the analysis. A linear mixed model was used for ANOVA for the Orange vineyard's data. Date and treatment were considered fixed factors, and repetition (block) was considered as a random factor. Mealybug count data were transformed using natural logs prior to the analysis. For both locations, the mean separation of factor effect was done using the Tukey-Kramer method.

### 4.3 Results

#### 4.3.1 *AREC vineyard*



#### *GLRaV-3 transmission:*

Within the first year of the new planting (2009), one newly planted vine tested positive for GLRaV-3 on non-treated vines (Figure 4.2). By the end of the 2010 season, GLRaV-3 was confirmed in four additional new, young vines. Finally, by the end of the experiment (Fall 2011), eleven total vines from the new plantings of Cabernet franc tested positive for GLRaV-3, four new infections from the previous year. Although the prevailing wind comes from the west (from the direction of panel 1 in Figure 4.1), there was no clear trend in the direction of the movement of GLRaV-3 within the field (Figure 4.2).

#### *Mealybug movement:*

Mealybug counting initiated in 2010 (Table 4.2) confirmed the movement of female mealybugs to newly planted vines. Mealybug counts per vine ranged from 0 to 35, with the average number per vine across treatment, vine location, and sampling date being 2.3. The effect of the orientation of new vines relative to the old vine was not statistically significant at any of the sampling dates ( $P < 0.05$ ), indicating that the effect of the prevailing wind (coming toward the direction of panel 1 in Fig. 4.1) had no significant effect on mealybug spread. Additionally, movement occurred regardless of the treatments that were being applied (Table 4.3). In other words, the treatment effect on the number of mealybugs was not significant ( $P < 0.05$ ) in 2010. The significant effect of vine age was that older vines had higher number of mealybugs than the younger ones. The difference in mealybug counts between two younger vines at different distances from the old vine in the same panel was not significant. Mealybug counts were very high early in the season (before and at bloom); however, it became increasingly harder to find the insect as the season progressed.

In 2011, the mean mealybug count per vine ranged from 0 to 24, and an average count per vine across treatment, vine location, and sampling date was 0.8 (Fig. 4.3). As in 2010, the effect of the location of new vines relative to the old vines was not significant ( $P < 0.05$ ); however, the effect of vine location was significant ( $P < 0.05$ ) on most sampling dates (Table 4.3), except at the early sampling dates where the number of mealybugs was very low on every vine. Also, there was a consistent significant difference in mealybug count numbers between the three vines within a panel (e.g. Old vine, Young Vine 1 (1.5 m away), Young Vine 2 (3.0 m away)), as was observed in 2010 (Table 4.3, Figure 4.3). The mealybug count was significantly higher on old vines than young vines, but there were no significant difference between young vines.

Treatments affected the number of mealybugs over the season in 2011. There was no difference among treatments until 22 June (Table 4.3), but on 30 June (about a month after application of the at-bloom treatment), the twice-sprayed treatment (at bud break and at bloom) showed a significantly higher number of mealybugs per vine ( $P < 0.05$ ) than the other treatments (Fig. 4.3).

In addition to in-season counts of mealybugs, the root systems of 38 vines, which were planted at the beginning of this study, were excavated at the end of the study during late winter. The roots were examined under a dissecting microscope at 100x for the presence of mealybugs, but none were found.

#### *4.3.2 Orange vineyard*

Due to different insecticide treatments applied, the Orange vineyard trial yielded different results from the AREC vineyard. In 2011, mealybug counts ranged from 0 to 26 and an average count per vine across date and treatments was 7.1 (Figure 4.4). When effects of date, treatment, and their interaction were examined using ANOVA, there was no statistically significant ( $P < 0.05$ ) interaction between date and treatment (Table 4.4). Both date and treatment significantly ( $P < 0.05$ ) affected the number of mealybug on vines. The differences in dates were basically the declining trend in number of mealybugs following application of insecticide treatments (Figure 4.4). There was a significant difference of mealybug counts ( $P < 0.05$ ) between treated and non-treated vines; however, spirotetramat and dinotefuran were not significantly different (Table 4.5).

In 2012, the overall population of mealybugs was lower than 2011 (Figure 4.4). The range of mealybugs counted per vine varied from 0 to 17, and an average count per vine across date and treatment was 1.9. (Figure 4.4) As in 2011, there was no interaction between date and treatment for mealybug counts, but both date and treatment were significant ( $P < 0.05$ ) (Table 4.4). The difference in dates was due to small peaks at the beginning of the trial and a peak that happened after 5 July (Figure 4.4). Although the difference between spirotetramat and dinotefuran was small (~1.2 mealybugs per vine), it was significant ( $P < 0.05$ ), and vines treated with dinotefuran harbored lower number of mealybugs per vine (Table 4.5).

#### 4.4 Discussion

The results from the AREC vineyard demonstrated how quickly mealybugs and GLRaV-3 could be transmitted to nearby vines regardless of insecticide treatments. The discovery of GLRaV-3 in a newly planted vine six months after planting showed that mealybugs were efficiently transmitting GLRaV-3 to new vines, even though their mobility is somewhat limited. Female mealybugs (the only sex that can vector these viruses) do not have wings and, thus, they can only crawl short distances to a neighboring vine. Alternatively, they can be moved across rows by strong wind events, spray induced air flows, or even human- or other animal-assisted movement (Grimes & Cone, 1985; Fuchs, 2007). However, maintenance sprays for these vines were made with a backpack sprayer, with only 145 kPa (21 psi) at the nozzle. In addition, the usual seasonal maintenance of young vines during the experiment was very limited. Thus, the movement of mealybugs was probably due to either crawling or naturally occurring wind-related events.

The difference between counts among vines within the same plot showed that mealybugs originated from old vines and moved to the newly planted vines. There was no difference in terms of the count of mealybugs between vines planted 1.5 m and 3.0 m distant from an old vine; therefore, the movement was more likely by wind rather than crawling. With crawling, the vines closer to the old vine should have a higher number of mealybugs than those further away. A study by Grasswitz and James (2008) showed that the female grape mealybug (*P. maritimus*) crawl only about 90 cm over three years. Our plot layout was designed to minimize wind effects (e.g., the minimum distance between plots was 21 m), but with other research concluding that mealybugs can be aerielly dispersed from 10-103 meters (Strickland, 1950; Cornwell, 1960; Barrass et al., 1994), our design might not have been able to restrict wind dispersal. Since the movement of mealybugs onto new vines was not significantly influenced by the direction of the prevailing wind, occasional storms, which likely would provide a much stronger force, might be responsible for the movement.

Mealybugs and scale insects are semi-persistent transmitters (Charles et al., 2006), making these insects a problematic target for insecticide control. Insecticides can effectively control insects that are persistent feeders since these insects must feed for longer durations of time in order to acquire and transmit the virus (Ogle and Brown, 1997). Semi-persistent transmitters, such as mealybugs, feed and transmit much faster; thereby, their actual feeding time in one area on the plant tissue is less (Ogle and Brown, 1997). Although the insecticide will kill the insect, the quick acquisition of the virus can allow transmission of the virus to a new plant before the insecticide affects the insect (Ogle and Brown, 1997). Therefore, a combination of contact and systemic insecticide is thought to be best to control mealybugs.

However, our study demonstrated that the use of a contact insecticide could actually increase mealybug populations. In the AREC plot, the delayed-dormant application of acetamiprid, that has translaminar movement, did not provide a significant reduction in mealybug numbers. At delayed-dormant stage, buds were either about to break or barely breaking and there were no large foliar tissue areas to uptake the material. In addition, the movement of the material from bark tissues to green tissues are less likely since the bark tissues are essentially dead cells. Moreover, there was a spike of mealybug population after the second application of insecticide in the two-spray treatment. This second spray (a pyrethroid) most likely reduced populations of beneficial insects, allowing for the mealybug populations to rise much higher compared to the other two treatments. The treatment in question (Baythroid XL) has a suggested use for control of grape berry moth, grape cane girdler, leafhoppers, mealybug crawlers, and the grape flea beetle in Virginia (Schooley et al., 2010). Since the application timing (at bloom) was also suitable for grape berry moth control, it is likely that growers would choose this insecticide to control both insects. It is, however, a broad-spectrum, contact insecticide that targets crawling insects; thus spiders and wasps, which are predators of mealybugs, will be affected.

While the treatment program in the AREC vineyard experiment used a whole-season approach, the approach taken at the Orange vineyard was a rescue approach, where action was taken after observing development of mealybug population within a vineyard. Although the timing of application might not be ideal, the effects of the systemic insecticides produced promising results. Both spirotetramat and dinotefuran treatments worked well in controlling the mealybug populations. With significant population declines in both treatments compared to the untreated check, these two treatments seem to effectively control the population. Spirotetramat may have residual effects on the following years population levels as well. The same treatments were applied on the same vines two years in a row. The number of mealybugs treated with dinotefuran was numerically lower (difference not statistically significant) than spirotetramat in 2011, and the overall counts of mealybugs in 2012 were statistically lower ( $P < 0.05$ ) in spirotetramat-treated vines than dinotefuran-treated vines.

Spirotetramat was developed mainly for sucking insect pests on annual and perennial crops in the United States (Bell et al., 2008). A group in Santa Rosa, CA found that a single, early season application of a low rate of spirotetramat significantly reduced and controlled mealybug populations throughout the year (Varela, 2008). Researchers at Cornell University also found that spirotetramat, when applied prior to bloom and 30 days post-bloom, provided a 70% decrease in mealybug population numbers over the 2011 season (Loeb, 2012). In our study, the reduction in mealybug numbers was 55% for dinotefuran and 37% for spirotetramat in 2011, and 37% and 52% for dinotefuran and spirotetramat, respectively in 2012.

Foliar sprays of fungicides and insecticides are common practice in Virginia vineyards. Alternative methods such as drenching require special equipment; however, aerial application can be done with existing equipment. In addition, the choice of material seems to be very important because foliar sprays of contact materials were shown to be non-effective, and counter-effective at the AREC vineyard in 2010 and 2011, respectively. With the mealybug populations residing deep under the bark tissues, foliar sprays are less likely to efficiently penetrate bark tissues and contact mealybugs. Systemic insecticides, on the other hand, were demonstrated to be a better choice and this is likely because direct contact is not required.

Studies in California on the vine mealybug have shown that buprofezin (Applaud, Nichino America) used in irrigation systems provides a much better control of the vectors when compared to imidacloprid (Admire, Bayer) and chloropyifos (Lorsban, Dow Chemical) (Daane et al., 2006). Buprofezin works best if applied early in the season, as it is a growth regulator and affects mostly young instars (Daane et al., 2006). The imidacloprid tested in this study also performed well when applied at around 70% bloom; however, it allowed mealybug populations to recover. This is probably due to the fact that the chemical is not able to reach all parts of the plant including areas of mealybugs colonization (Daane etl a., 2006). In another study (Morandi et al., 2009), age of vines affected the efficacy of insecticides against the citrus mealybug (*P. citri*). Soil applications of three neonicotinoids (acetamiprid, imidacloprid, and thiamethoxam) to newly planted vines (1 year old) all significantly controlled mealybug populations; however, only the imidacloprid significantly controlled mealybugs in the older vines (15-years old). Foliar sprays of the same neonicotinoids and additional growth regulators (buprofezin and pyriproxyfen) were not effective at controlling the population levels (Morandi et al., 2009).

Timing of foliar sprays in Virginia needs to be considered as well. There is little known about the timing of migration of mealybugs from bark to green tissues in the spring under VA conditions. In California, the vine mealybug starts its spring movements in February (Daane et al., 2003). In our study, there was no mealybug activity during the early part of the season at the AREC vineyard. The number of mealybugs did not increase until more than a month after bloom. At the Orange vineyard we observed a higher number of mealybugs in mid-May (about a month after bud-break) in 2012; however, the number declined and did not increase until mid-June. The low count of mealybugs may indicate the slow development of crawlers due to relatively colder winters in Virginia when compared with California.

In this study, detailed observations of mealybug populations were taken throughout the year in two vineyard locations, and more general observations were taken where mealybug samples were taken. Typically, mealybugs were found around the cordon wood, shoots, and leaves. The inability to find mealybugs on the roots of rogued vines suggests that, unlike the vine mealybug (Daane et al., 2008), grape and Gill's mealybugs found in the two locations do not likely overwinter on roots. We also noted that mealybug populations differed significantly each season, making it hard to predict in which years a mealybug outbreak might occur.

Thus, based on movement of crawlers, initial mealybug control sprays should occur when first few leaves are unfolding to target the migration of the initial population. At this early stage one recommend either a systemic or a growth regulator-type insecticide, as it is targeting crawlers that are not very visible or exposed. Then as the population increases toward mid-season (mid- to late- May in VA), another application of a systemic insecticide might be granted to suppress the population. However, as we observed in the dataset, there were large fluctuations of population within and between years. In order to select the best material and determine the best timing for application, more studies on the biology of mealybugs, especially monitoring

techniques that are tailored to the regional difference in mealybug species and climate, are necessary.

This study showed that spread of GLD can happen within the first year of planting if infected vines and mealybugs are in the field. This study also confirmed that the choice and timing of application of insecticide is critical for mealybug control. Systemic insecticides applied in a season as a foliar application showed promising results on mealybugs control. However, these insecticide treatments cannot totally stop the movement of GLD because mealybugs can feed for a while after exposure to the insecticide treatment, resulting in the potential for virus transmission. More epidemiological studies are needed to determine location- and species-specific lifecycles of mealybugs, as well as examination of the efficacy of a combination of control strategies, such as management of ants and release of parasitic agents in conjunction with insecticide applications.

#### 4.5 References

- Barrass, I. C., Jerie, P., & Ward, S. A. (1994). Aerial dispersal of first- and second-instar longtailed mealybug, *Pseudococcus longispinus* (Targioni Tozzetti) (Pseudococcidae: Hemiptera). *Australian Journal of Experimental Agriculture*, 34(8), 1205-1208.
- Cabaleiro, C., Segura, A., & García-Berrios, J. J. (1999). Effects of grapevine leafroll-associated virus 3 on the physiology and must of *Vitis vinifera* L. cv. Albariño following contamination in the field. *American Journal of Enology and Viticulture*, 50(1), 40-44.
- Charles, J. G., Cohen, D., Walker, J. T. S., Forgie, S. A., Bell, V. A., & Breen, K. C. (2006). A review of the ecology of grapevine leafroll associated virus type 3 (GLRaV-3). *New Zealand Plant Protection*, 59, 330-337.
- Cornwell, P. B. (1958). Movements of the vectors of virus diseases of cacao in Ghana. I. Canopy movement in and between trees. *Bulletin of Entomological Research*, 49, 613-630.
- Cornwell, P. B. (1960). Movements of the vectors of virus diseases of cacao in Ghana, II. Wind movements and aerial dispersal. *Bulletin of Entomological Research*, 51, 175-201.
- Daane, K. M., Bentley, W. J., Walton, V. M., Malakar-Kuenen, R., Millar, J. G., Ingels, C. A., . . . Gispert, C. (2006). New controls investigated for vine mealybug. *California Agriculture*, 60(1), 31-38.
- Daane, K. M., Cooper, M. L., Triapitsyn, S. V., Walton, V. M., Yokota, G. Y., Haviland, D. R., . . . Wunderlich, L. R. (2008). Vineyard managers and researchers seek sustainable solutions for mealybugs, a changing pest complex. *California Agriculture*, 62(4), 167-176.
- Daane, K. M., Malakar-Kuenen, R., Guillén, M., Bentley, W. J., Bianchi, M., & González, D. (2003). Abiotic and biotic pest refuges hamper biological control of mealybugs in California vineyards (pp. 389-398). Washington: United States Department of Agriculture, Forest Service.
- Daane, K. M., Malakar-Kuenen, R. D., & Walton, V. M. (2004). Temperature-dependent development of *Anagyrus pseudococci* (Hymenoptera: Encyrtidae) as a parasitoid of

- the vine mealybug, *Planococcus ficus* (Homoptera: Pseudococcidae). *Biological Control*, 31(2), 123-132.
- Daane, K. M., Sime, K. R., Cooper, M. L., & Battany, M. C. (2004). Ants in your vineyard? *UC Plant Protection Quarterly*, 11(2), 3.
- Flaherty, D. L., Christensen, L. P., Lanini, W. T., Marois, J. J., Phillips, P. A., & Wilson, L. T. (1992). *Grape pest management*. Oakland: University of California.
- Flebut, A. J. (1922). U.S. Bur. Ent. The Grape Mealybug. *Monthly Bulletin. California Department of Agriculture*, 11(7), 6-11 pp.
- Fuchs, M. (2007). Grape leafroll disease factsheet Retrieved June, 2012, from [http://www.nysipm.cornell.edu/factsheets/grapes/diseases/grape\\_leafroll.pdf](http://www.nysipm.cornell.edu/factsheets/grapes/diseases/grape_leafroll.pdf)
- Fuchs, M., Martinson, T. E., Loeb, G. M., & Hoch, H. C. (2009). Survey for the three major leafroll disease-associated viruses in Finger Lakes vineyards in New York. *Plant Disease*, 93(4), 395-401.
- Furness, G. O. (1976). The dispersal, age-structure and natural enemies of the long-tailed mealybug, *Pseudococcus longispinus* (Targioni-Tozzetti), in relation to sampling and control. *Australian Journal of Zoology*, 24(2), 237-247.
- Geiger, C. A., Daane, K. M., Bentley, W. J., Yokota, G. Y., & Martin, L. A. (2001). Sampling program for grape mealybugs improves pest management. *California Agriculture*, 55(3), 19-27.
- Gibson, R. W., Rice, A. D., & Sawicki, R. M. (1982). Effects of the pyrethroid deltamethrin on the acquisition and inoculation of viruses by *Myzus persicae*. *Annals of Applied Biology*, 100(1), 49-54.
- Grasswitz, T. R., & James, D. G. (2008). Movement of grape mealybug, *Pseudococcus maritimus*, on and between host plants. *Entomologia Experimentalis et Applicata*, 129(3), 268-275.
- Hu, J. S., Gonsalves, D., & Teliz, D. (1990). Characterization of closterovirus-like particles associated with grapevine leafroll disease. *Journal of Phytopathology*, 128(1), 1-14.
- Kovacs, L. G., Hanami, H., Fortenberry, M., & Kaps, M. L. (2001). Latent infection by leafroll agent GLRaV-3 is linked to lower fruit quality in French-American hybrid grapevines Vidal blanc and St. Vincent. *American Journal of Enology and Viticulture*, 52(3), 254-259.
- Loeb, G. M. (2012). Grape Insect and mite pests-2012 field season Retrieved November, 2012, from <http://www.fruit.cornell.edu/grape/pdfs/Loeb-Grape Insect Mite Pests 2012.pdf>
- Mansour, R., Suma, P., Mazzeo, G., Grissa Lebdi, K., & Russo, A. (2011). Evaluating side effects of newer insecticides on the vine mealybug parasitoid &i>Anagyrus sp. near &i>pseudococci , with implications for integrated pest management in vineyards. *Phytoparasitica*, 39(4), 369-376.
- Mgocheki, N., & Addison, P. (2009). Effect of contact pesticides on vine mealybug parasitoids, *Anagyrus sp. near pseudococci* (Girault) and *Coccidoxenoides perminutus* (Timberlake) (Hymenoptera: Encyrtidae). *South African Journal of Enology and Viticulture*, 30(2), 110-116.
- Morandi Filho, W. J., Grützmacher, A. D., Botton, M., & Bertin, A. (2009). Chemical control of mealybugs *Planococcus citri* (Risso, 1813) (Homoptera: Pseudococcidae) in vineyards of different ages. *Arquivos do Instituto Biológico (São Paulo)*, 76(3), 427-435.

- Ogle, H., & Brown, J. (1997). Vector-Borne Inoculum *Plant Pathogens and Plant Diseases* (pp. 231-241). Armidale, NSW: Rockvale Publications.
- Phillips, P. A., & Sherk, C. J. (1991). To control mealybugs, stop honeydew-seeking ants. *California Agriculture*, 45(2), 26-28.
- Rayapati, N., O'Neal, S., & Walsh, D. (2008). Grapevine leafroll disease. *Washington State University Extension Bulletin EB2027E* Retrieved 2012, April, from <http://pubs.wsu.edu>
- Schooley, N., Baudoin, A. B., Derr, J. F., Pfeiffer, D. G., Weaver, M. J., & Wolf, T. K. (April 2010). Crop profile for grapes in Virginia, from <http://www.ipmcenters.org/cropprofiles/docs/vagrapes.pdf>
- Strickland, A. H. (1950). The dispersal of Pseudococcidae (Hemiptera-Homoptera) by air currents in the Gold Coast. *Proceedings of the Royal Entomological Society, London*, 25, 1-9.
- Tsai, C., Rowhani, A., Golino, D. A., Daane, K. M., & Almeida, R. P. P. (2010). Mealybug transmission of grapevine leafroll viruses: an analysis of virus-vector specificity. *Phytopathology*, 100(8), 830-834.
- Varela, L. G. (2008). Grape Mealybug Control Trial 2008 Retrieved November, 2012, from <http://cesonoma.ucanr.edu/files/27728.pdf>
- Varela, L. G. (May 2005). Grape and Vine Mealybug Life Cycles in the North Coast, from <http://cesonoma.ucdavis.edu/files/27229.pdf>
- Varela, L. G., & Smith, R. J. (2009). Mealybugs Retrieved November, 2012, from <http://ucanr.org/sites/SoCo/files/27649.pdf>
- VWB. (2012). The Economic Impact of Wine and Wine Grapes on the State of Virginia--2010. St. Helena, CA: A Frank, Rimerman + Co. LLP.
- Walton, V. M., Daane, K. M., Bentley, W. J., Millar, J. G., Larsen, T. E., & Malakar-Kuenen, R. (2006). Pheromone-based mating disruption of *Planococcus ficus* (Hemiptera: Pseudococcidae) in California vineyards. *Journal of Economic Entomology*, 99(4), 1280-1290.

**Table 4.1.** Virus specific oligonucleotide primers used in the RT-PCR and the sizes of their RT-PCR fragments.

Virus	Forward Primer (5'—3')	Reverse Primer (5'—3')	Expected PCR product size (bp)
Grapevine leafroll-associated virus-2	ATAATTCGGCGTACATCCCCACTT	GCCCTCCGCGCAACTAATGACAG	331
Grapevine leafroll-associated virus-3	CGCTAGGGCTGTGGAAGTATT	GTTGTCCCGGGTACCAGATAT	546
Grapevine fleck virus	CTCAGCCTCCACCTTGCCCCGT	CAATTTGGCTGGGCGAGAAGTACA	533



**Table 4.2:** Counts of mealybugs on insecticide treated grapevines at AREC vineyard.

<b>Treatment<sup>x</sup></b>	<b>Vine<sup>y</sup></b>	<b>6/18/10<sup>z</sup></b>	<b>7/8/10<sup>z</sup></b>
No Spray	Old	84	24
	Young 1	0	1
	Young 2	1	3
Spray at Bud Break	Old	34	9
	Young 1	0	0
	Young 2	0	4
Spray at Bud Break and at Bloom	Old	60	23
	Young 1	0	1
	Young 2	0	2

<sup>x</sup> No spray = untreated check; Spray at Bud Break = Assail was applied 4/5/10 and 4/14/11; Spray at Bloom = Baythroid XL was applied 5/25/10 and 5/28/11.

<sup>y</sup> Old = Cabernet Sauvignon vines planted in 1990; Young 1 = Cabernet franc vines planted in 2009 1.5m from Old; Young 2 = Cabernet franc vines planted in 2009 3m from Old

<sup>z</sup> Assessment date. Assessment was done visually by counting the number of female mealybugs/vine

**Table 4.3.** Effect of treatment and vine location to the number of mealybugs per vine at AREC vineyard in 2010 and 2011 season.

Date	Source	F Ratio	Prob > F <sup>z</sup>	
6/18/10	Treatment	0.69	0.51	
	Vine	35.15	<0.0001	**
7/8/10	Treatment	0.13	0.88	
	Vine	21.67	<0.0001	**
5/25/11	Treatment	0.54	0.59	
	Vine	4.50	0.02	**
6/7/11	Treatment	1.35	0.27	
	Vine	0.72	0.49	
6/22/11	Treatment	2.03	0.14	
	Vine	7.24	0.00	**
6/30/11	Treatment	4.48	0.02	**
	Vine	4.37	0.02	**
7/6/11	Treatment	3.69	0.03	**
	Vine	12.94	<0.0001	**
7/15/11	Treatment	3.82	0.03	**
	Vine	10.66	0.00	**
7/20/11	Treatment	5.16	0.01	**
	Vine	9.90	0.00	**

<sup>z</sup> \*\* = significant at  $\alpha = 0.05$  level

**Table 4.4:** ANOVA results of the effect of sampling date and neonicotinoid insecticides at the Orange vineyard in 2011 and 2012.

2011	Source	<i>F</i> ratio	<i>P</i> > <i>F</i>
	Date	6.36	<.0001
	Treatment	13.74	<.0001
	Date*Treatment	1.23	0.26
2012	Source	<i>F</i> ratio	<i>P</i> > <i>F</i>
	Date	18.73	<.0001
	Treatment	14.76	<.0001
	Date*Treatment	1.96	0.10

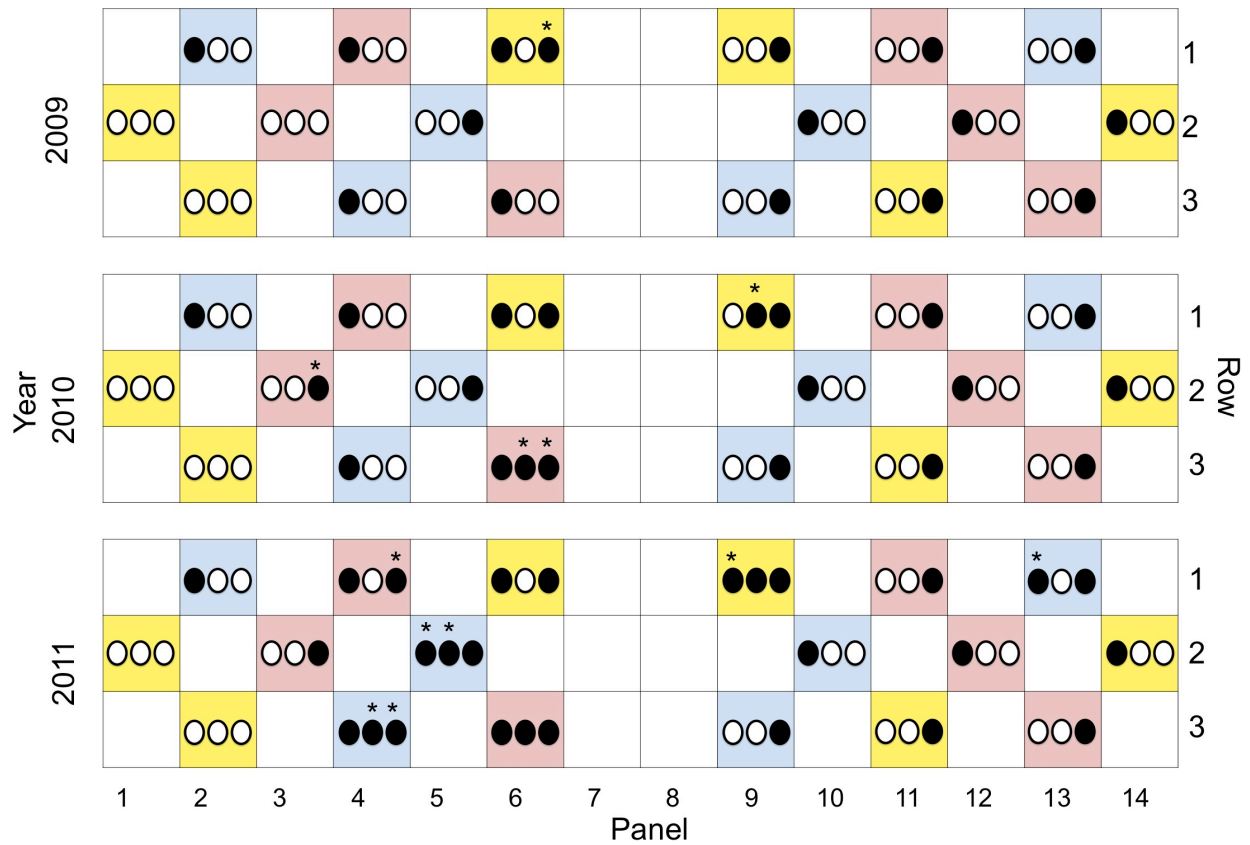
**Table 4.5:** Effects of two in-season foliar applications of a neonicotinoid insecticide on the number of mealybugs at the Orange vineyard in 2011 and 2012.

2011	Treatment	LS mean <sup>z</sup>	
	Control	10.25	A
	Dinotefuran	4.63	B
	Spirotetramat	6.44	B
2012	Treatment	LS mean <sup>z</sup>	
	Control	3.05	A
	Dinotefuran	1.88	B
	Spirotetramat	0.73	C

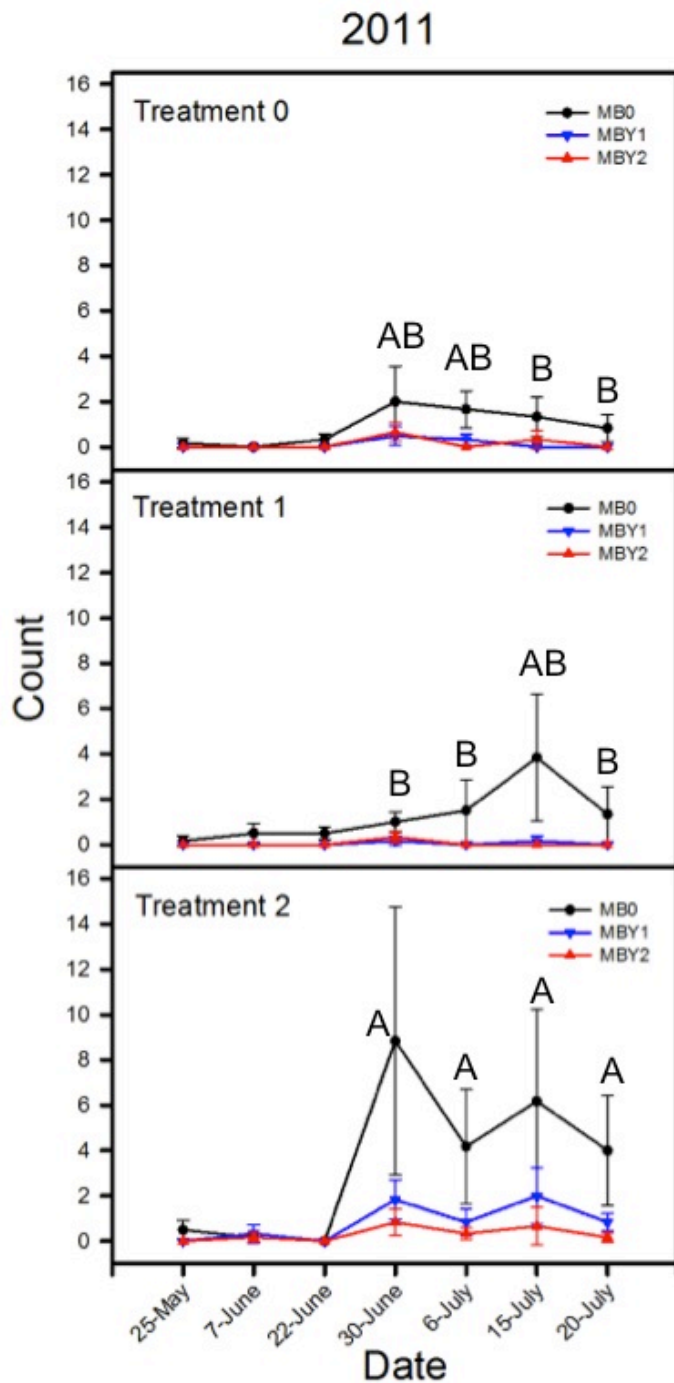
<sup>z</sup> Values with different letter indicates statistically significant differences at  $P < 0.05$ .

Panel	1	1	1	2	2	2	3	3	3	4	4	4	5	5	5	6	6	6	7	7	7	8	8	8	9	9	9	10	10	10	11	11	11	12	12	12	13	13	13	14	14	14								
Vine	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3								
up																																																		
Row 17	-	-	-	XC	O	O	-	-	-	X	O	O	-	-	-	X	O	O	-	-	-	-	-	-	O	O	XD	-	-	-	O	O	X	-	-	-	O	O	X	-	-	-	-	-	-	-				
down																																																		
up																																																		
Row 18	O	O	XB	-	-	-	O	O	X	-	-	-	O	O	X	-	-	-	-	-	-	-	-	-	-	-	-	XE	O	O	-	-	-	X	O	O	-	-	-	X	O	O	-	-	-	-				
down																																																		
up																																																		
Row 19	-	-	-	XA	O	O	-	-	-	X	O	O	-	-	-	X	O	O	-	-	-	-	-	-	-	O	O	XF	-	-	-	O	O	X	-	-	-	O	O	X	-	-	-	-	-	-	-			
down																																																		

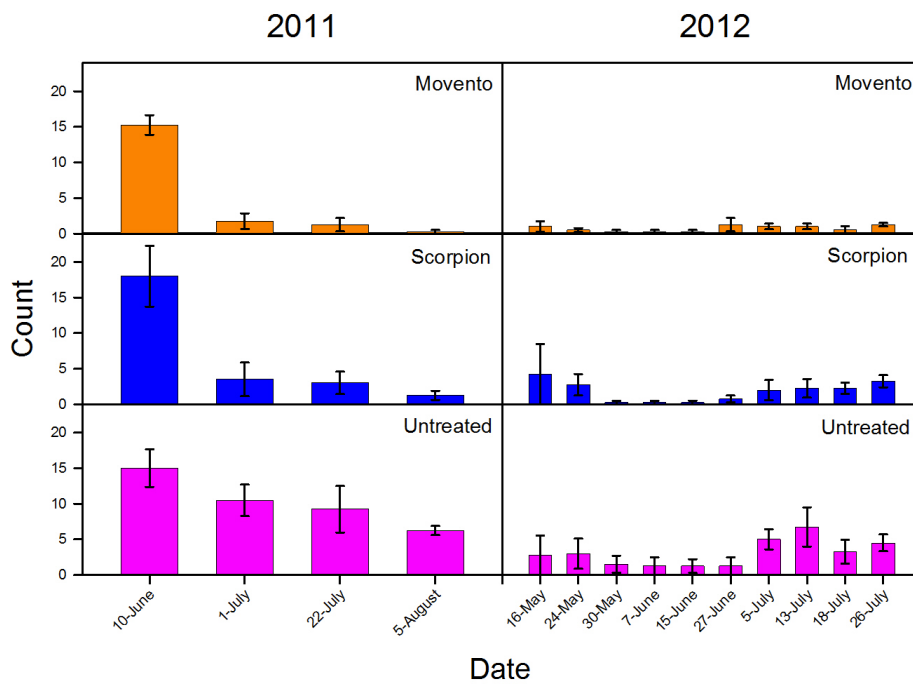
**Figure 4.1:** Field layout of the AREC plot. Panels, rows, and vines are shown. Letters A, B, C, D, E, F represent the different treatment blocks. Vines labeled as ‘X’ are old vines; vines labeled as ‘O’ are newly planted, young vines. Yellow shaded regions represent the control treatment, Blue regions the single spray treatment, and red regions the twice-sprayed treatments.



**Figure 4.2:** Spread of GLRaV-3 within field between 2009 and 2011. Colored treatments and vine placement same as Figure 4.1. White ovals represent clean (virus-negative vines). Black ovals represent GLRaV-3 positive vines. New infections that occurred during the experiment are marked with an asterisk.



**Figure 4.3:** Mealybug (MB) counts on three different vines by treatment in 2011 at the AREC vineyard. Black line = MB counts on old vines. Blue line = MB counts on young vines 1.5m away from old. Red line = MB counts 3m away from old. Different letters indicate significant differences between treatments per sampling date ( $P=0.05$ , bar = standard error) based on Tukey HSD mean separation (JMP 9, SAS Institute). Prior to ANOVA, data were transformed using square root.



**Figure 4.4:** Box plot of mealybug counts on the same treated and untreated Chardonnay vines at a commercial vineyard in 2011 and 2012. Count data on the y-axis and date of counts on the x-axis. Heights of boxes represent number of mealybugs counted; standard error bars are shown.