Grapevine Viruses and Associated Vectors in Virginia: Survey, Vector Management, and Development of Efficient Grapevine Virus Testing Methods

Taylor Jones

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

Doctor of Philosophy
In
Plant Pathology, Physiology, and Weed Science

Mizuho Nita, Chair
Anton B. Baudoin
Douglas G. Pfeiffer
Xiaofeng Wang
Naidu A. Rayapati

May 3, 2016
Blacksburg, Virginia

Keywords: Grapevine leafroll disease; Grapevine red blotch associated virus; Nitrocellulose Membranes; real-time PCR; Mealybugs; Grapevines; Virginia

Grapevine Viruses and Associated Vectors in Virginia: Survey, Vector Management, and Development of Efficient Grapevine Virus Testing Methods

Taylor Jones

ABSTRACT

In order to aid the booming wine industry in the state of Virginia, U.S.A., we developed a series of studies to provide a deeper understanding of the viruses and vectors for management of virus diseases and development of better tools for grapevine virus diagnostics. A statewide survey for 14 different grapevine viruses between 2009 and 2014 was conducted: 721 samples were collected from 116 vineyards in the period. Among the 12 viruses identified, *Grapevine leafroll associated virus-3* (GLRaV-3), *Grapevine rupestris stem-pitting associated virus* (GRSPaV), and *Grapevine red blotch-associated virus* (GRBaV) were most commonly present. A new real-time PCR method for the detection of the V2 gene of GRBaV was developed. The resulting method takes less time for more accurate diagnostics than conventional PCR. Evaluation of insecticide effectiveness on GLRaV-3 vectors (mealybugs) and the spread of GLRaV-3 were examined: Four trials conducted from 2012 to 2014 revealed that despite successful control of mealybugs, GLRaV-3 is spread at a very rapid rate. A new sampling technique for efficient nucleic acid storage and testing was developed: the nitrocellulose membrane-based method allows simpler extraction of nucleic acid and provides a storage medium that can hold viable RNA/DNA at room temperature for up to 18 months. An investigation of multiple virus-infected vines and the impact of these co-infections on grapevine fruit chemistry was conducted. GLRaV-3, GRBaV, GRSPaV, and co-infections of the 3 all negatively impacted Brix, pH, titratable acidity, and anthocyanin levels.

Grapevine Viruses and Associated Vectors in Virginia: Survey, Vector Management, and Development of Efficient Grapevine Virus Testing Methods

Taylor Jones

GENERAL AUDIENCE ABSTRACT

In order to aid the booming wine industry in the state of Virginia, U.S.A., a series of studies were conducted. A statewide survey between 2009 and 2014 uncovered 12 viruses (with nine being reported for the first time in the state) among Virginia vineyards. Three viruses, *Grapevine leafroll-associated virus*, *Grapevine red blotch associated virus*, and *Grapevine rupestris stem pitting associated virus*, were prevalent ones. More than 90% of our surveyed vineyards (out of 116 visited) contained at least one virus-infected grapevine. This widespread nature of grapevine viruses in Virginia led to the development of new virus testing methods: a gene-based diagnostic tool that allows rapid and specific detection of grapevine red blotch-associated virus and a special membrane-based sampling method, which allows growers to sample at their own convenience and allows storage of virus genetic materials for multiple years. Because of its robustness, we envision that this technology can be applicable beyond grapevine viruses. We also found insects that can transmit these viruses from vine to vine: grape mealybugs, Gill's mealybugs, and obscure mealybugs. This is the first study to demonstrate the ability of Gill's mealybug to transmit grapevine leafroll-associated virus. For the management of insect vectors, four insecticide trials were implemented over multiple seasons. Some insecticides significantly reduced the existing mealybug populations; however, even the best treatments were not able to completely stop the spread of the virus to a new vineyard. Lastly, a study on the effects of co-infection of the three most common viruses on grape berry chemistry showed sugar levels, color pigments, and acidity of grape berries were all negatively impacted.

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, Amanda Bly, Sabrina Hartley, and Logan Howard. 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 continued help and support. I would also like to thank my source of funding: The Virginia Wine Board.

Table of Contents

List of Figures

Chapter 2.

Chapter 3:

Chapter 4:

Chapter 5:

List of Tables

Chapter 2.

Chapter 3:

Chapter 6:

List of Abbreviations

GLRaV-1	*Grapevine leafroll associated virus-1*
GLRaV-2	*Grapevine leafroll associated virus-2*
GLRaV-3	*Grapevine leafroll associated virus-3*
GLRaV-4	*Grapevine leafroll associated virus-4*
GLRaV-5	*Grapevine leafroll associated virus-5*
GLRaV-9	*Grapevine leafroll associated virus-9*
GRSPaV-1	*Grapevine rupestris stem pitting associated virus-1*
GVA	*Grapevine virus A*
GVB	*Grapevine virus B*
GVD	*Grapevine virus D*
GVE	*Grapevine virus E*
ToRSV	*Tomato ringspot virus*
GVCV	*Grapevine vein clearing virus*
GPgV	*Grapevine Pinot gris virus*
GRBaV	*Grapevine red blotch associated virus*
PCR	Polymerase chain reaction
RT-PCR	Reverse-transcription polymerase chain reaction
qPCR	Real-time polymerase chain reaction
NPN	Nitro-pure nitrocellulose

Chapter 1

1.1 Introduction to the topic

The wine industry: The grape is one of the most valuable horticultural crops in the world as approximately 80,000 km^2 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 (Myles et al. 2011). Cultivation of the domesticated grape (*Vitis vinifera* subsp. *vinifera*), began about 6-8,000 years ago after the discovery of wine in or near modern-day Iraq, Turkey, and Syria (McGovern 2003; Myles et al. 2011). The *Vitis* genus (family Vitaceae), consists of around 60 inter-fertile species that are located mostly in the Northern Hemisphere (This et al. 2006). Indigenous to Eurasia, *Vitis vinifera* is the species most extensively used in the global wine industry with thousands of cultivars currently in existence (Alleweldt and Dettweiler 1994; deSaporta 1879; Galet 2000; Levadoux 1956; This et al. 2006; Viala and Vermorel 1901-09). However, current wine marketing has led to global wine market domination by only a small number of these cultivars (This et al. 2006).

For years, European wines dominated the global wine market as Europe was one of the first successful wine production regions with little competition. In the 20th century however, new competition emerged from the "New World" (North America, South America, Australia, etc.) (Bisson et al. 2002). This new competition, compounded by the fact that wine consumption has been declining in traditional European wine producing countries, has posed new issues to European producers (Bisson et al. 2002). The United States per capita wine consumption has

nearly doubled (Bisson et al. 2002) in recent years, with "New World" producers increasing exports from 2% to 15% in the last 20 years (Aigrain 2001; Bisson et al. 2002). In 2013, the United States had a total of 987,100 acres devoted to vineyards, a 3.7% increase since 2010, placing the US in sixth place in terms of world vineyard acres by country (Institute 2015). In 2013 the United States was fourth (behind Italy, Spain, and France) in total world wine production, producing 3,234,300 liters that year--a 21.9% increase from 2010 (Institute 2015).

In recent years, the Virginia wine industry has been expanding and contributes an estimated $740 million to the state's economy annually (VWB 2012). This growth represents a 106% increase from the previous economic study conducted five years earlier (VWB 2012). In 2011, Virginia wine sales greatly increased with more than 5.5 million bottles sold, accounting for more than an 11% increase over the previous fiscal year (Caldwell 2012). Currently, Virginia ranks fifth in the United States with 1,100 growing hectares of wine grape cultivars and over 190 wineries (Caldwell 2012; VWB 2012). A variety of grapes can be successfully grown in Virginia's terroir; however, the most common cultivars grown in Virginia include: Cabernet franc, Cabernet Sauvignon, Merlot, Petit Verdot, Pinot noir, Syrah, Tannat, Chambourcin, Concord, Norton, Albarino, Chardonnay, Gewurztraminer, Petit Manseng, Pinot gris, Riesling, Sauvignon blanc, Viognier, Traminette, Vidal blanc, and Niagara (VWB 2012).

Grapevines and grapevine leafroll disease: Grapevines are susceptible to a significant number of diseases caused by bacteria, fungi, nematodes, insects, phytoplasmas, viruses, and virus-like agents (viroids), which all play important roles in infecting grapevines and affecting fruit production, berry chemistry, and vineyard management practices. Among perennial crops worldwide, the greatest number of viruses (around 70) have been detected from grapevines

(Martelli 2014; Naidu et al. 2015a). A majority of these viruses are considered minor threats to production, in that they either cause little economic injury or have limited geographical distribution; however, there are several viruses that are considered a major threat of high economic importance (Martelli and Boudon-Padieu 2006; Rayapati 2012). Examples are the grapevine leafroll complex, rugose wood complex, degeneration/decline disease complex, and fleck disease complex (Martelli and Boudon-Padieu 2006).

Grapevine leafroll disease (GLD), widely regarded as the most important grapevine virus disease, is present in all grapevine-growing regions of the world (Naidu et al. 2014; Naidu et al. 2015b). It was first seen and recorded in 1906 and again in 1924 as a physiological disorder in Italian vineyards and French vineyards, respectively, called "rossore" where grapevine leaves were experiencing early season reddening (Martelli and Boudon-Padieu 2006; Ravaz and Verge 1924). The first survey for GLD was conducted in 1936 by G. Scheu. In this survey the disease was found to be widespread throughout German vineyards and was shown to be graft transmissible (Scheu 1935, 1936). A disease known as "white emperor" disease in Europe (named due to the fruit color effects on the Emperor grape) was found to be graft-transmissible and was deemed a viral disease of grapevines, later to be confirmed as GLD in 1958 (Goheen et al. 1958; Harmon and Snyder 1946). Closterovirus-like particles were first found in infected grapevines in 1971 and were associated with GLD a few years later (Mendgen 1971; Namba et al. 1979). Since then, GLD has been found in 33 additional countries around the globe, bringing the total to 36 (Martelli, 2006).

The symptoms of GLD become more apparent as the season progresses. Symptoms on red-fruited varieties differ somewhat from those on white-fruited varieties. Red-fruited varieties typically show downward curling leaf margins and interveinal reddening with veins usually

remaining green. White-fruited varieties experience the same downward curling; however, there is only a slight chlorosis of the leaf blade and no reddening. Due to subtle symptoms in many grapevines, diagnosis by visual symptoms is very difficult as other diseases (i.e. trunk fungi) and disorders (i.e. nutrient deficiencies) also cause symptoms easily confusable with GLD symptoms and thus, molecular techniques are often employed.

Based on studies conducted in several countries, GLD significantly affected vine vigor and both fruit yield and berry quality (Kovacs et al. 2001; Alabi et al. 2016). Berry color intensity, titratable acidity (1g/L decrease on average), pH (slightly lower), and Brix (1° decrease on average) can be reduced and, thus, negatively affect the wine quality (Cabaleiro et al. 1999; Fuchs et al. 2009b; Kovacs et al. 2001; Rayapati et al. 2008). In severely infected vineyards planted with a susceptible variety, direct crop losses can range from 10% to 40% (Wolpert and Vilas 1992). In addition, uneven ripening of berries and reduced sugar level are commonly associated with the infection. Poor berry color due to reduced anthocyanin pigments are associated with infected red-fruited wine grape cultivars (Cabaleiro et al. 1999; Fuchs et al. 2009b; Rayapati et al. 2008). These traits can result in indirect crop loss in the form of poor wine quality (Cabaleiro et al. 1999; Fuchs et al. 2009b; Rayapati et al. 2008). GLD infection can also cause a loss in vine vigor making vines more susceptible to environmental conditions such as harsh winter temperatures. Fruit quality parameters of mix-infected vines suffer an increased reduction in berry chemistry quality. For example, grapevines 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. GLRaV-3 infected vines had a 5% reduction in berry weight and 5-9% increase in titratable acidity, whereas mix-infected vines had a 7% reduction in berry weight and a 14% increase in titratable acidity (Kovacs et al. 2001).

Multiple viruses are associated with GLD, and are referred to as the grapevine leafroll-associated viruses (GLRaVs) and named GLRaV-1, -2, -3, -4, and -7 (Martelli et al. 2012; Martelli et al. 2002a). These viruses belong to the family *Closteroviridae* whose members code for a homolog of the heat shock protein 70 (HSP70) protein (Dolja et al. 1994). GLRaV-2 is assigned to the genus *Closterovirus*, GLRaV-7 to the newly created genus *Velarivirus* and GLRaV-1, -3, and -4 and their strains grouped in the genus *Ampelovirus*. GLRaV-4, -5, -6, -9, -De, -Pr, and -Car were originally classified as separate species due to a lack of serological relationship (Martelli et al. 2012); however, GLRaV-4 is now considered as a consolidation of these divergent strains based on genomic sequences. Thus, these viruses are now referred to as GLRaV-4 strains 5, 6, 9, De, Pr, and Car (Ghanem-Sabanadzovic et al. 2012; Martelli et al. 2012; Thompson et al. 2012).

Closteroviruses contain a positive-sense single-stranded RNA [(+)RNA] genome and are morphologically non-enveloped and filamentous (King et al. 2011). The *Capillovirus*, *Trichovirus*, and *Vitivirus* genera have particle morphology (e.g. long flexuous filamentous particles) identical to the *Closteroviridae* (King et al. 2011). Viruses belonging to the family *Closteroviridae* are part of the superfamily of alpha-like viruses and subsequently 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 RNA-dependent RNA polymerase (RdRp) and helicase amino acid sequences (Gibbs et al. 2000). Similar viruses that are classified into the genus *Closterovirus* include *Beet yellow stunt virus* and *Citrus tristeza virus* (King et al. 2011).

Closteroviridae contains three genera distinguished by their genome composition and structure, each genera further distinguished by insect vectors: 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*, whitefly transmitted (Martelli et al. 2002a). GLRaV-2 is the only virus associated with GLD to be placed in the genus *Closterovirus* (Karasev 2000; Martelli et al. 2002a) along with *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* (Karasev 2000; Martelli et al. 2002a).

Some GLRaVs can be transmitted by insect vectors belonging to the families Pseudococcidae (mealybugs) and Coccidae (scales) while others are only graft-transmissable (Belli et al. 1994; Cabaleiro and Segura 1997; Douglas and Krüger 2008; Petersen and Charles 1997; Sforza et al. 2003; Tsai et al. 2008). GLRaV-1, GLRaV-3, GLRaV-4 strain 5, and GLRaV-4 strain 9 have been shown to be transmitted by insects (both mealybugs and scales) while GLRaV-2 and GLRaV-7 do not have a known vector (Fuchs et al. 2009a; Martelli et al. 2002a). Mealybugs are the primary vector of interest in Virginia (Jones and Nita 2016; Jones et al. 2015) and play an important role in GLD epidemiology. Only female mealybugs feed and are capable of transmitting the virus, making females the target for controlling virus spread (Fuchs 2007; Grimes and Cone 1985). These female mealybugs do not have wings, thus their movement is limited to walking between vines or being blown by air currents to neighboring vines (Fuchs 2007; Grimes and Cone 1985).

In Spain, slow vector transmission of GLRaV-3 has been observed while in Napa Valley, California, rate of GLD spread has been seen as high as 10% per year based on number of vines within an infected planting (Cabaleiro et al. 2008; D. A. Golino et al. 2008). Spatial distribution and dynamics of GLD in Western Cape, South Africa studied between 2001 and 2005 showed four main 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, 2006, 2010). These four types of GLD distribution dynamics are commonly recognized as typical GLD patterns.

Clean plant programs should be the first, and most important step to managing grapevine virus diseases such as GLD. Grapevine viruses infect vines systemically, and no remedies for plant virus infections exist (Agrios 2005). Many grape producing countries have certification programs for clean plant production to provide virus-free plants to nurseries or to growers. Meristem tip culture, first documented as successful in 1981 in Japan, has been used as a primary method for producing clean plants, and it is a successful method of eliminating GLRaVs from vines (Sasahara et al. 1981). Management options are limited to roguing of infected vines, planting of virus-tested certified vines, and insect vector 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.

Grapevine leafroll associated virus-1: GLRaV-1 has a (+)RNA genome of 18,659 nt in size with 9 open reading frames (ORFs) (Martelli et al. 2012). This virus is unique in that it contains a duplicate minor coat protein CPm1 and CPm2 creating 10 genes within the genome (Little et al.

2001; Martelli et al. 2012). Amino acid divergence within GLRaV-1 isolates ranges from 4-20% and divergence of the HSP70h gene is approximately 10% (Alabi et al. 2011; Martelli et al. 2012). GLRaV-1 is transmitted semi-persistently by mealybugs and soft scale insects belonging to the genera *Heliococcus, Phenacoccus, Planococcus, Pulvinaria, Neopulinaria,* and *Parthenolecanium* (Martelli and Boudon-Padieu 2006; Tsai et al. 2010). Phylogenetically, GLRaV-1 is most similar to GLRaV-3 when HSP70h, polymerase, or coat protein genes are analyzed (Martelli et al. 2012). However, these two viruses are serologically unrelated through monoclonal antibodies (Seddas et al. 2000).

Grapevine leafroll associated virus-2: GLRaV-2 was found by Gugerli et al. (1984) as the second virus with a closterovirus-like virus particle associated with GLD (Gugerli et al. 1984). GLRaV-2 has a (+)-sense RNA genome about 16.5kb in size that codes for nine ORFs (BaoZhong Meng et al. 2005; Zhu et al. 1998). GLRaV-2 is one of the viruses associated with GLD whose vector has not been currently identified although other members of the *Closterovirus* genus are transmitted by aphids (Karasev 2000). GLRaV-2 can be transmitted through vegetative propagation or grafting with infected materials (Martelli 2014). Several isolates of GLRaV-2 are mechanically transmissible (unlike all other GLRaVs) to herbaceous hosts like *Nicotiana benthamiana*, but transmission is very inefficient (Goszczynski et al. 1996). GLRaV-2 has been associated with other grapevine diseases such as graft incompatibility syndrome (Bonfiglioli et al. 2003; Greif et al. 1995), young vine decline (Golino et al. 2000), and rootstock stem lesion disease (Uyemoto et al. 2001).

Grapevine leafroll associated virus-3: GLRaV-3, the third closterovirus-like virus found to be associated with GLD (Rosciglione and Gugerli 1986) is the type member of the Ampelovirus genus in the Closteroviridae family (Martelli et al. 2012; Maree et al. 2013). Several other viruses, such as GLRaV-1, -4, *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* (PBNSPaV) (Maree et al. 2013; Martelli et al. 2002a), also belong to the Ampelovirus genus and are closely related to GLRaV-3. Taxonomically, GLRaV-3, GLRaV-1, LChV-W, and PMWaV-2 group together to form Subgroup I within the *Ampelovirus* genus (Maliogka et al. 2008; Martelli et al. 2012). GLRaV-3 is a (+)-sense RNA genome of 18,498 nucleotides and 12 ORFs (Martelli et al. 2012; Maree et al. 2013). Divergence of coat protein amino acid sequences does not reach above 6% while HSP70h divergence typically ranges between 3-4% (Martelli et al. 2012). GLRaV-3 is transmitted semi-persistently by mealybugs and soft scale insects (Martelli et al. 2012; Maree et al. 2013). *Planococcus ficus* (vine mealybug) was the first documented mealybug able to transmit GLRaV-3 (Rosciglione and Gugerli 1989), followed by the long-tailed mealybug (*Pseudococcus longispinus*) (Tanne et al. 1989). GLRaV-3 is 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 and Segura 1997; Golino et al. 2002; Mahfoudhi et al. 2009; Martelli et al. 2012; Petersen and Charles 1997; Sforza et al. 2003; Tsai et al. 2010; Maree et al.

2013). Transmission efficiency studies have shown that one mealybug first instar of *P. ficus* or *Ps. longispinus* is capable of infecting a healthy grapevine with GLRaV-3 (Douglas and Krüger 2008). Generally first instars are the most efficient transmitters, followed by second instars, and adults with little transmission efficiency (Mahfoudhi et al. 2009). Finally, GLRaV-3 can be transmitted through vegetative propagation and grafting.

Grapevine leafroll associated virus-4: GLRaV-4 has recently become a single species with multiple strains that includes the previous virus species of GLRaV-5, GLRaV-6, GLRaV–De, GLRaV-9, GLRaV-Pr, and GLRaV-Car (Martelli et al. 2012). This revision was due to full genome sequencing of GLRaV-4, -5, and -6 and the serological similarity found among GLRaV-5, -6, and -9 and GLRaV-4 (Ghanem-Sabanadzovic et al. 2012; Gugerli 2009; Martelli et al. 2012; Thompson et al. 2012). GLRaV-4 forms a phylogenetic clade (Subgroup II) within the ampeloviruses, along with Plum bark necrosis stem pitting associated virus (PBNSPaV), Pineapple mealybug wilt associated virus 1 (PMWaV-1) and Pineapple mealybug wilt associated virus 3 (PMWaV-3) (Maliogka et al. 2008; Maliogka et al. 2009; Martelli et al. 2012). In general, GLRaV-4 (and related strains) is around 13,700 nucleotides long and has 6 ORFs encoding 7 genes. GLRaV-4 has been thought to be the common ancestor to the family due to its simple genome (Dolja et al. 2006; Martelli et al. 2012). GLRaV-4 related viruses all cause mild leafroll symptoms when compared to other mealybug transmissible viruses such as GLRaV-1 or GLRaV-3 (Elbeaino et al. 2009; Martelli et al. 2012; Sim et al. 2003; Tsai et al. 2010).

Grapevine leafroll associated virus-7: GLRaV-7, first discovered in a symptomless grapevine, has a genome size of 16,496 nucleotides containing 10 ORFs (Al Rwahnih et al. 2012a;

Jelkmann et al. 2012; Martelli et al. 2012; Mikona and Jelkmann 2010). Most closely related to Little Cherry Virus 1 (LChV-1) but very different from the Ampelo-, Clostero-, and Crini-viruses, a new genus, *Velarivirus* that include GLRaV-7, LChV-1, and CoV-1 (Cordyline virus 1 (Melzer et al. 2011)), has been suggested (Al Rwahnih et al. 2012a; Jelkmann et al. 2012; Martelli et al. 2012). GLRaV-7 has no known vector, but replicates in certain species of dodder and transmits via dodder to herbaceous hosts like *Nicotiana occidentalis* (Mikona and Jelkmann 2010). Many times, GLRaV-7 is either symptomless or causes very mild leafroll symptoms, even on indicator vines (Al Rwahnih et al. 2012a; Avgelis and Boscia 2001).

Grapevine fleck virus: GFkV is a phloem-limited virus disease of grapevines worldwide, and most common in the United States in Washington and Missouri (Martelli 1993; Milkus and Goodman 1999; Naidu and Mekuria 2010). GFkV, or "grapevine marbrure virus" at the time, was first documented in 1966 in *Vitis vinifera* from France (Vuittenez et al. 1966) and later characterized under the name "grapevine phloem-limited isometric virus" (Boulila et al. 1990). GFkV causes latent, mostly 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* (the biological indicator for this virus) (Brunt et al. 1996; Hewitt et al. 1972). GFkV elicits characteristic multivesicular bodies in this indicator vine (Castellano and Martelli 1984). The agent is graft transmissible, non-mechanically transmissible and cannot be transmitted through seed (Boscia et al. 1991; Glasa et al. 2011; Martelli et al. 2002b; Martelli et al. 2002c). The spread of this disease has been documented in South Africa (Engelbrecht and Kasdorf 1990) and Italy (Fortusini et al. 1996) but the vector remains unknown (Glasa et al. 2011; Walter and Martelli 1997). Roguing of infected vines, clean plant programs, and not

sharing potentially contaminated materials through vegetative propagation are the only means of management of GFkV. GFkV can cause severe damage when it is present in a vine with other grapevine viruses (e.g. GLRaV-3) (Kovacs et al. 2001; Walter and Martelli 1997).

GFkV 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. 2002b; Martelli et al. 2002c). Members of the *Tymoviridae* family are non-enveloped, isometric particles about 30nm in diameter; made up of two capsids with and without RNA that can be separated by centrifugation (Martelli et al. 2002b; Martelli et al. 2002c). Members possess a (+)-stranded RNA genome with a high cytidine content that causes multivesicular body structures in the infected plant cells (Martelli et al. 2002b; Martelli et al. 2002c). GFkV is phylogenetically similar to Grapevine asteroid mosaic-associated virus (GAMaV) and Grapevine redglobe virus (GRGV) (Sabanadzovic et al. 2001; Walter and Martelli 1997) but remains the largest of the family with a genome size at 7.5kb encoding 4 open reading frames (Martelli et al. 2002b; Martelli et al. 2002c).

The rugose wood disease complex of viruses: The rugose wood disease complex is a group of viruses (mostly belonging to the family *Flexiviridae* genera *Vitivirus or Foveavirus*) that primarily affects grapevines on the woody cylinder (i.e. vascular system) (Martelli et al. 1993; Pearson and Goheen 1988; Rosa et al. 2011). This complex includes *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine virus D*(GVD), *Grapevine virus E* (GVE), and *Grapevine Rupestris Stem-pitting-associated virus* (GRSPaV). These viruses are believed to be the most important of the group since they are widespread and cause a slow decline of vines as they age.

Infection with age causes Kober stem grooving, corky bark, stem pitting, and LN33 stem grooving along with graft incompatibility (Garau et al. 1994; Rosa et al. 2011). Canes of severely infected vines will ripen irregularly or not at all (Martelli et al. 1993). Symptoms caused by these viruses develop slowly; however, when multiple viruses are found within a vine, symptoms may become more severe (Guidoni et al. 1997; Mannini et al. 1996). In the Finger Lakes region of the US, vineyards tend to have an average of 25 years (Atallah et al. 2012) before growers remove the planting and replant. Although grapevines commonly can live for 50-100 years, the typical lifespan of a wine grape vineyard is 25 to 35 years due to lower production by older vines (Wolf 2008). Due to the high initial investment on vineyards and relatively slow return on investment, it is critical for growers to have long, productive years before the needs for replanting arise.

Grapevine Virus A, the first phloem-limited grapevine virus found to transmit to herbaceous hosts through sap inoculation (Conti 1980), is a member of the genus *Vitivirus* (family *Betaflexiviridae*) associated with the rugose wood complex (du Preez et al. 2011; King et al. 2011). It is one of the most commonly detected viruses worldwide (Goszczynski and Jooste 2003). GVA produces flexuous, non-enveloped, filamentous virions containing a (+)-sense RNA genome approximately 7.3kb in size with 5 ORFs (du Preez et al. 2011). GVA can be transmitted in a semi-persistent manner, similar to GLRaVs, by mealybugs and scale insects in the genera *Cavariella, Heliococcus, Neopulvinaria, Parthenolecanium, Planococcus, Pseudococcus,* and *Ovatus* (du Preez et al. 2011; Engelbrecht and Kasdorf 1987; Garau et al. 1995; La Notte et al. 1997a; Rosciglione et al. 1983). GVA and GVB are most commonly associated with Kober stem grooving and corky bark, respectively (Rosa et al. 2011). Grapevine virus B (Martelli et al. 2007) is very similar to GVA in many aspects, including belonging to the *Vitivirus* genus, its similar

genome size (7.6kb), and number of ORFs (5) (Saldarelli et al. 1996). GVB is commonly found in a co-infection with other viruses within single grapevines (Shi et al. 2004) and can be transmitted by several species of mealybugs within the genera *Pseudococcus* and *Planococcus* (Engelbrecht and Kasdorf 1990; Golino et al. 1995). GVB is most commonly associated with corky bark (Bonavia et al. 1996; Boscia et al. 1993).

Grapevine rupestris stem pitting associated virus (GRSPaV), aptly named after its primary cause of rupestris stem-pitting disease (Azzam et al. 1991; Meng et al. 1999; Zhang et al. 1998), is also a member of the family *Flexiviridae*, but in the genus *Foveavirus* (type member *Apple stem pitting virus*) (Martelli and Jelkmann 1998). GRSPaV is not vectored by any insect, but is highly transmissible through grafting (Golino 1993). GRSPaV causes symptoms on *Vitis rupestris* cv. St. George rootstocks that is classified as pittings that extend down a line to the point where inoculated along with small pits on the woody cylinder. Infection reduces vine vigor over time causing a slow decline, and after a few years post-infection, vines are on average smaller than healthy vines (Zhang et al. 1998). This virus is asymptomatic on LN33 and Kober 5BB rootstocks (Martelli 1993). GRSPaV virions encapsidate a (+)-sense RNA genome that is approximately 8.7kb in genome size and encodes six ORFs (Zhang et al. 1998). Management for viruses associated with the rugose wood complex is limited to roguing of the infected vines and/or management of insect vectors in the cases of GVA and GVB.

Tomato ringspot virus: ToRSV (family *Comoviridae*, genus *Nepovirus*, members of which cause degeneration and grapevine decline) (Martelli and Boudon-Padieu 2006), has been present in Virginia, U.S. (Tuskan and Tolin 1984) but research on this virus has not been actively carried out in VA in recent years. It can cause uneven fruit development, a significant loss in yield, and

an overall vine decline (Powell et al. 1990). ToRSV is transmitted by the dagger nematode (*Xiphinema americanum*), is often a problem in fruit orchards (Braun et al. 1966; Rosenberger et al. 1983; Rosenberger and Meyer 1988). Infection causes severe apple union necrosis and decline on apples with MM106 rootstocks (Rosenberger et al. 1985; Stouffer and Uyemoto 1976). This virus has a very wide host range including apples, plums, peach, grape, raspberry, cherry, prune, blueberry, strawberry and numerous weed species found in vineyards and orchards, with the common dandelion serving as the most efficient reservoir (C. Powell et al. 1982; C. Powell et al. 1984). ToRSV is a spherical virus with a bipartite (+)-sense RNA genome. RNA2 (7.2kb) codes for the coat protein (Rott et al. 1991) and a cell-to-cell movement protein (Wieczorek and Sanfacon 1993). RNA1 (8.2kb) encodes a large polyprotein containing the RdRp, viral protein-genome linked (VPg) and nucleoside triphosphate (NTP)-binding domain (Rott et al. 1995). Management for ToRSV is limited to nematode management (if present and spreading the virus), roguing of infected vines, and weed management to discourage nematode spread.

Grapevine red blotch-associated virus: The newly discovered DNA virus *Grapevine red blotch-associated virus* (GRBaV) (Al Rwahnih et al. 2013) (also known as *Grapevine Cabernet franc-associated virus* (Krenz et al. 2012) and *Grapevine redleaf-associated virus* (Poojari et al. 2013) is a growing concern among grapevine growers around the world. It has recently been discovered in historic grapevine specimens in California (Al Rwahnih et al. 2015), suggesting this virus has early routes in the wine industry. It was recently discovered when leafroll symptomatic grapevines continually tested negative for GLD (Al Rwahnih et al. 2013; Calvi 2011; Krenz et al. 2012). Deep sequencing was used to reveal the presence of a new virus of approximately 3.2

kb in size (Krenz et al. 2012). GRBaV is a single, circular ssDNA *Geminivirus* containing six ORFs with bi-directional orientation (Al Rwahnih et al. 2013; Al Rwahnih et al. 2015; Krenz et al. 2012; Sudarshana et al. 2015). Red blotch acts similarly to GLD in terms of leaf symptoms (red-fruited cultivars show red blotching of the leaves and leaf margins with secondary and tertiary veins also showing reddening and white fruited cultivars experience irregular chlorotic areas of the leaf blade (Sudarshana et al. 2015). Sugar reduction in infected vines is approximately 2.4° (Calvi 2011) up to 5° Brix (Habili 2013). *Geminiviridae* members can generally be transmitted by whiteflies and leafhoppers (genus *Erythroneura*) (they are also graft transmissible) and under experimental conditions, the Virginia creeper leafhopper (*Erythroneura ziczac*) has been shown to transmit GRBaV (Poojari et al. 2013). The overall epidemiology of this virus is unknown, but an in-field vector is thought to exist due to spatial-temporal increases of infected vines, consistent with short-distance spread compounded by patterns of aggregation (Sudarshana et al. 2015). Currently, no definitive insect vector has been identified for GRBaV; however, recent reports have surfaced that a tentative candidate vector has been found (Bahder et al. 2016) in the three-cornered alfalfa treehopper (*Spissistilus festinus*). In California, management options of infected vineyards have been limited to: 1) removal of vineyards that are 4-10 years old with greater than 50% GRBaV incidence and experiencing a loss of revenue and fruit quality; or 2) improving the nutritional status of infected grapevines through nutrient application, fruit load adjustments, and delayed harvesting (Calvi 2011; Sudarshana et al. 2015). Testing for this virus is also an issue, as only PCR is currently available as a routine test (Al Rwahnih et al. 2013; B. Krenz et al. 2014b). Methods such as ELISA and Real Time PCR have yet to be developed (Sudarshana et al. 2015) and could help aid in mass testing of potentially infected materials, such as those located in nursery stocks. Much is still unknown about cultivar,

rootstock, and yield effects. Red blotch is widespread in the U.S. and has been identified recently in California, New York, Virginia, Maryland, Pennsylvania, Texas, and Washington (B. Krenz et al. 2014b).

Grapevine vein clearing virus: GVCV is another newly discovered DNA virus (a provisional new species in the family *Caulimoviridae*, genus *Badnavirus*) found primarily in the midwestern United States, mainly Missouri, Illinois, and Indiana (Guo et al. 2014; Zhang et al. 2011). GVCV is a circular, dsDNA virus (similar to red blotch) with three open reading frames on the plus strand. This virus causes a severe translucent vein-clearing under sunlight, severe vine decline, and yield reduction in *V. vinifera* (Guo et al. 2014; Zhang et al. 2011). Within the field, horizontal spread has been noted in Midwest vineyards suggesting vine-to-vine spread via insect vectors (Zhang et al. 2011). Insect vectors currently being investigated are mealybugs, aphids, and whiteflies (Zhang et al. 2011). One concern is that GVCV is often found in co-infection cases with *Grapevine fanleaf virus*, *Tomato ringspot virus*, and *Grapevine Rupestris stem pitting-associated virus* (Lunden et al. 2010; Qiu et al. 2007). Recent genetic research with GVCV has shown its genetic populations might be diverse, and the interspecific hybrid cultivar Chambourcin might be resistant, while other hybrids (Vidal blanc, Cayuga White, and Traminette) are not (Guo et al. 2014). Current research is underway in the Midwest to look into wild grapevine hosts and possible vectors.

Grapevine Pinot gris virus: The newly characterized Grapevine Pinot gris virus (GPgV, genus *Trichovirus*, family *Betaflexiviridae*) has been found in Italy through deep sequencing, on varieties Pinot gris and Traminer, expressing chlorotic mottling and leaf deformation

(Giampetruzzi et al. 2012). GPgV also negatively affects cane numbers per vine as well as berry number and weight per vine (Malossini et al. 2012). Often the disease is overlooked because symptoms resemble that of frost damage and/or trunk diseases (such as Eutypa). A recent survey in Italy has shown this virus to be widespread in symptomatic and asymptomatic grapevines (Saldarelli et al. 2015). It has been recently found to have spread to other areas of Italy, France, Czech Republic, Slovenia, and more recently Canada (Beuve et al. 2015; Giampetruzzi et al. 2012; Glasa et al. 2014; Morelli et al. 2014; Plesko et al. 2014; Saldarelli et al. 2015; Xiao et al. 2015). GPgV has been predicted to have three main ORFs although its genome is not fully sequenced yet (Glasa et al. 2014).

Increases in global knowledge and advancements in grapevine virus detection: Recognizing the impacts of virus diseases on grapevine production, many grapevine-growing regions of the United States have implemented virus surveys to document the presence of various viruses in vineyards. These surveys conducted in California, Idaho, Missouri, New York, Oregon, and Washington State have shown the presence of GLRaV-1, -2, -3, -4, -7, and GLRaV-4 strains -5, -9, and -Car, *Grapevine rupestris stem pitting-associated virus* (RSPaV), *Grapevine fanleaf virus* (GFLV), *Grapevine fleck virus* (GFkV), *Grapevine virus A* (GVA), *Arabis mosaic virus* (ArMV), *Tomato ringspot virus* (ToRSV), *Tobacco ring spot virus* (ToRSV, Walker et al., 2015) and the newly reported *Grapevine red blotch-associated virus* and *Grapevine vein clearing virus* (GVCV) (Fuchs 2007; Martin et al. 2005; Mekuria et al. 2009a; Mekuria et al. 2009b; Milkus and Goodman 1999; Naidu and Mekuria 2010; Naidu et al. 2006; Al Rwahnih et al. 2013; Zhang et al. 2011; Poojari et al. 2013; Walker et al. 2015). However, until recently, very limited information has been available about grapevine viruses in Virginia vineyards.

A statewide survey of commercial vineyards in Virginia (VA) was conducted during 2009 through 2011 seasons for the presence of GLRaV-2, GLRaV-3, and GFkV (Jones et al. 2015). Out of 415 samples (with 41 wine grape varieties) from 77 locations (vineyards), GLRaV-2, GLRaV-3 and GFkV were detected in 8%, 25%, and 1% of total samples, respectively, and 64% of vineyards were positive for at least one of the three viruses (Jones et al. 2015). Samples from 100 wild grapevines of *Vitis sp*. tested negative for the three viruses, indicating that wild species of grapevines are unlikely to be alternative hosts or reservoirs for these viruses in Virginia (Jones et al. 2015).

Since the discovery of viruses, and the fact that symptoms of virus diseases are often not the best indicator of infection (Agrios 2005; Jones and Nita 2016), it is best to utilize molecular-based or serologically based detection methods. These methods can be used to track the movement of the virus within and among vines to further understand the mechanism of virus translocation and transmission to aid in development of management strategies. The conventional enzyme-linked immunosorbent assay (ELISA; a method based on antibody recognition of virus particles), reverse transcription-polymerase chain reaction (RT-PCR; amplification of viral genomic RNA), and quantitative assays, such as real-time PCR (qPCR; quantified polymerase chain reaction for amplified RNA/DNA) are all commonly used techniques to diagnose virus diseases.

The first polyclonal and monoclonal antibodies produced for detection of GLD using ELISA were for GLRaV-1 and GLRaV-3 (Gugerli et al. 1984; Gugerli and Ramel 1993). ELISA used to be the most commonly utilized method of detection for GLRaVs and viruses in general, and is still useful for screening large numbers of sample (Hu et al. 1991). PCR was first used for the detection of viruliferous mealybugs in 1994 and then was quickly adopted as the preferred

screening method for GLRaVs (Minafra and Hadidi 1994). Spot-PCR techniques (the use of charged nylon membranes to sample and test using PCR for viruses) (La Notte et al. 1997b) and degenerate primer techniques for PCR were developed (Routh et al. 1998; Saldarelli et al. 1998), shortly thereafter followed by sequencing of virus genomes.

A partial sequence of GLRaV-1 in 2000 by Fazeli & Rezaian was the first partial genomic data available for a GLRaV (Fazeli and Rezaian 2000). One-tube RT-PCR assays (Nassuth et al. 2000) and multiplex RT-PCR assays (Gambino and Gribaudo 2006) have also been developed and used frequently in recent years, along with a newer microarray for simultaneous detection of a wide range of different grapevine viruses (Engel et al. 2010). Conventional methods of PCR/RT-PCR for grapevine virus testing are less sensitive than their partner technique of real-time PCR (qPCR). The advantages of the qPCR procedures are higher sensitivity (Oberhansli et al. 2011), and capability to quantify the target DNA.

Osman et al. (2007) created one of the first sets of primers detecting different grapevine viruses. This RT-PCR primer set for Grapevine leafroll viruses is generally the most widely used. However, a global increase in genomic information has allowed for researchers to design primers better suited for detecting viruses with higher sequence diversity and making overall more sensitive detection methods. GLRaV-7 is a good example of a virus that has low to no reactivity to commercial monoclonal antibodies used in detection (Morales and Monis 2007) and a high sequence variability (Morales and Monis 2007). Al Rwahnih et al. (2012b) demonstrated that their conventional method of PCR for GLRaV-7 was inadequate, and developed a new qRT-PCR method. GLRaV-1, -2, -3, and -4 have isolates and strains with high sequence diversity (<30% divergency) and thus, better detection methods have been developed using universal primers (Ghanem-Sabanadzovic et al. 2012; Alabi et al. 2011). The recent change of GLRaV-4

to include former GLRaV-5, -9, and -Car as strains in the same species has posed new issues in testing. However, a new "universal" degenerate primer that can detect all strains of GLRaV-4 and qRT-PCR protocols was developed to detect the different strains of GLRaV-4 in a more effective manner (Ghanem-Sabanadzovic et al. 2012).

GLRaV-1 has been a major target of molecular data mining lately as this virus occurs in diverse populations and is the second most important grapevine leafroll virus after GLRaV-3 (Habili et al. 2007); therefore, many different primers have been developed for qRT-PCR and RT-PCR detection. A recent publication by Alabi et al. (2011) used four separate primers for four distinct genes within GLRaV-1 to investigate the effect of molecular diversity on detection Out of 34 isolates tested for GLRaV-1, the primers for HSP-70h, CP, CPd2, and p24 successfully detected 28, 16, 28, and 31 isolates, respectively. This shows that false negatives can be common and it is important to test using multiple primers or a more sensitive method (O. J. Alabi et al. 2011).

GLRaV-2 and -3 detection has been significantly improved in recent years. Increases in genomic data for these viruses have facilitated researchers in developing the tools to detect all variants within GLRaV-2 and -3. New universal primers have been developed for GLRaV-2 (Beuve et al. 2007) and GLRaV-3 (Chooi et al. 2013) that can be effectively used in conventional and real-time RT-PCR. For example, the older universal primers from Bertazzon and Angelini (2004) were tested against a new, universal detection qRT-PCR method by Beuve et al. (2007). Their results showed that four grapevines that were GLRaV-2-negative based on RT-PCR were actually positive when a newer, qRT-PCR method was used. Multiplex RT-PCRs have also been created for GLRaV-3 (that include the six known variants of GLRaV-3) that work

just as effectively as the universal primer in conventional methods that have been developed (Chooi et al. 2013).

Mealybugs and scale insects: Mealybugs and scale insects belong to the order Hemiptera, which is a large, diverse group including true bugs. All insects of this order have a unique piercing/sucking mouthpart used for sucking either plant sap or blood (Triplehorn et al. 2005). Mealybugs and scale insects are in the suborder Sternorrhyncha (superfamily Coccoidea, families Coccidea (scale) and Pseudococcidae (soft scale/mealybug), which are known to have very complex life cycles involving winged and wingless individuals (Triplehorn et al. 2005).

Scale insect females are wingless and usually legless and sessile; however, the males have a single pair of wings (they are rarely wingless) (Triplehorn et al. 2005). Males of this superfamily lack mouthparts and do not feed (Triplehorn et al. 2005). In terms of female development (the more pathologically important sex due to phloem feeding mouthparts), the first-instar nymphs (crawlers) are very active. Upon loss of legs and antennae following the first molt, the insect become sessile (Triplehorn et al. 2005). While sessile, waxy or scale-like covering is secreted, covering the body of the insect. When females mature into adults, eggs or live first instars are produced (Triplehorn et al. 2005).

Mealybugs, although similar to scale insects, differ slightly in development, where no hard scale-like covering is ever produced in the adults. Instead, mealybugs produce waxy secretions that cover and protect their bodies in what looks like small white fibers/hairs (Triplehorn et al. 2005). They are similar in that crawlers are the most active stage and that mealybugs can either lay eggs, which are placed in a cottony wax egg sack (like the grape mealybug), while others (like the Gill's mealybug) give birth to live 1st instars (Triplehorn et al.

2005). Mealybugs also excrete honeydew (Triplehorn et al. 2005), a major attractant to ants (Phillips and Sherk 1991), that will group, move, and protect mealybugs from predators in order to utilize this sugar source (Geiger and Daane 2001). Some mealybug species are known to cause extensive damages to vineyards by transmitting viral diseases, causing feeding damage, and excreting honeydew that promotes growth of other microorganisms that cause diseases such as sooty mold (Flaherty et al. 1992).

Mealybugs (Pseudococcidae) and scale insects (Coccidae) have been reported as vectors of grapevine-infecting ampeloviruses (Tsai et al. 2010) such as GLRaV-1 and -3. No insect vectors have yet been reported for GLRaV-2 and -7. GLRaV-3 is the most widely spread across grapevine-growing regions compared to other GLRaVs (Rayapati et al. 2008). Based on transmission studies with mealybugs, GLRaV-3 is transmitted in a semi-persistent manner (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). However, transmission efficiency among mealybugs is variable (at around 15-25%) (Charles et al. 2006). First instars of mealybugs are the most efficient vectors (Petersen and Charles 1997) and the ability of mealybugs to transmit the virus after manual transfer to a new plant is reduced significantly when compared to natural movement to new plants (Cabaleiro and Segura 1997).

Mealybugs themselves can cause feeding damage on grapevines and secondary damage such as growth of fungi on grape berry skin as a result of mealybug honeydew production. Moreover, the major concern with mealybugs and scale insects is their ability to transmit viruses, including the grapevine leafroll disease complex. *Planococcus ficus*, the vine mealybug, was the first mealybug found to successfully transmit GLD to a healthy grapevine in a laboratory setting (Engelbrecht and Kasdorf 1990; Rosciglione and Gugerli 1989; Tanne et al. 1989). Since then,

many more mealybug species have been exposed as vectors for GLD. Studies demonstrated that the grape (*Pseudococcus maritimus*), longtailed (*Pseudococcus longispinus*), citrophilus (*Pseudococcus calceolariae*), citrus (*Planococcus citri*), and obscure (*Pseudococcus viburni*) mealybugs are capable of transmitting GLRaV-3 (Tsai et al. 2010; Tsai et al. 2008).

Many mealybug species have multiple generations per year (Varela 2005). Mealybugs overwinter as eggs, or as adults under protective layers of old bark on the grapevines trunk and cane (Varela 2005), or underground (Varela 2005). After hatching in the spring, mealybug 1st instars, which are the most mobile stage of development, move to emerging green tissues to find a feeding site (Cornwell 1958; Furness 1976; Grasswitz and 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 and 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, with *Pseudococcus njalensis* Laing, a common mealybug on cacao, as ambient temperature reach 23.5 C, its mobility increases and continues to increase with temperature (Cornwell 1958). Mealybugs have been shown to be capable of walking on single vines in the field up to 90 cm from their point of release. The maximum distance recorded for aerial dispersal (wind driven) was 103 m, with the majority of studies documenting an average 10 m area for aerial dispersal (Barrass et al. 1994; Cornwell 1960; Grasswitz and James 2008; Strickland 1950).

Both grape mealybugs (*Pseudococcus maritimus*) and Gill's mealybugs (*Ferrisia gilli*) were commonly found in Virginia vineyards with isolated cases of the striped mealybug

(*Ferrisia virgata*) and the obscure mealybug (*Pseudococcus viburni*) (Jones et al. 2015). The vine mealybug is a major concern in CA due to its wide host range (grapes, figs, apples, citrus, and many tropical crops such as dates, bananas, mangos, and avocados), ability to have many generations per year, effectiveness of transmission of GLD, and ability to cause direct damage on the grapevine (Millar et al. 2002). The grape mealybug has the ability to transmit GLRaV-3 and has a wide host range that includes grapevines, figs, apples, and citrus crops (Millar et al. 2002). The striped and Gill's mealybugs are not known to transmit the viruses associated with GLD. Both species have been found in vineyards as well as in both pistachio and almond orchards in California, and are thought to be native to the southeastern United States (Gullan et al. 2003; Haviland et al. 2006). These two mealybugs also have a wide host range including a variety of woody tissue plants and have three generations per year in California (Gullan et al. 2003; Haviland et al. 2006). One of the main problems with these species is their excessive production of honeydew, which can lead to fungal disease buildup (Gullan et al. 2003; Haviland et al. 2006).

Chemical control, biological control, and cultivation methods can be combined or used individually to help aid in mealybug and scale insect management. Multiple management strategies may be necessary because mealybugs can be found in varying locations on vine throughout the season depending on the insect's stages of development (Geiger and Daane 2001). Not planting in mealybug-infested locations will help avoid the vectors and avoiding cultivars that tend to be more sensitive to mealybug infestations (like Chardonnay, Cabernet Sauvignon, and Merlot) (Walton 2001) can help aid in reducing risk. Cane-pruning vines may also aid in mealybug reduction in the following season due to large amounts of bark removal compared to traditional spur-pruning (Geiger and Daane 2001). Mealybugs can survive and breed in under-trellis cover crops, on grapevine roots, and on all above-ground grapevine tissues.

Weed management is a potential option for reducing the mealybug populations if present under the trellis. Ant population management with chemical sprays can also be considered if large numbers of ants are present and seem to be herding mealybugs on the vine (Daane et al. 2004). Protecting and promoting beneficial insects (parasitic wasps, spiders, green lacewings, transverse ladybirds, etc.) that target mealybugs will help reduce populations as well (Grimes and Cone 1985).

With chemical control, a dormant or delayed dormant oil spray can provide control of scale insects and mealybugs by lowering population emergence rates by suffocating the insects on the vine (Smith and Cowles 2009; Varela et al. 2012). In season applications of an insect growth inhibitor like buprofezin (Applaud), and neonicotinoids like acetamiprid (Assail), dinotefuran (Venom), and imidacloprid (Provado, Admire) all are stated to provide control of these insects (Schooley et al. 2010). Organophosphates like phosmet (Imidan) are also labeled to control mealybugs, but are not commonly used (Schooley et al. 2010). Spirotetramat (Movento), a tetramic acid derivative, can be used as an in-season foliar or drip applied treatment to knock back population levels (Schooley et al., 2010). Chemical management, although effective, can also cause aerial dispersal of mealybugs due to wind force when high power sprayer systems are used.

Parasitoids of mealybugs, such as parasitic wasps, green and brown lacewings, and coccinellid beetles are potential biological controls for mealybugs (Daane et al. 2006). The parasitic wasp *Anagyrus pseudococci* is the most effective (90% parasitism rate) (Daane et al. 2004). These wasps however, prefer large mealybugs, which would hinder early season parasitization when crawlers emerge, a critical time for effective management (Daane et al. 2006). Theoretically, a combination of wasp release and early season insecticides might provide

sufficient control. The downside of chemical treatments with fipronil, a-cypermethrin, and chlorpyrifos-methyl is that these active ingredients will reduce predator population levels (or beneficials) (Mansour et al. 2011; Mgocheki and Addison 2009). Ant population control via organophosphate chemicals can be applied in the early season at the base of the trunk. This method has been found to significantly reduce the obscure mealybug infestation levels, whose activity was directly related to the activity of the Argentine ant (Phillips and Sherk 1991). Pheromone-based mating disrupters are another option for vineyard managers attempting to control mealybugs as they have been found to significantly reduce mealybug population levels as well as inhibit egg production (Daane et al. 2006; Walton et al. 2006).

1.2 Conclusion and objectives

In this work, we aim to assess and further examine the prevalence of the aforementioned grapevine viruses: *Grapevine leafroll-associated virus-1* (GLRaV-1), *Grapevine leafroll associated virus-2* (GLRaV-2), *Grapevine leafroll associated virus-3* (GLRaV-3), *Grapevine leafroll associated virus-4* (GLRaV-4), *Grapevine leafroll associated virus-5* (GLRaV-5), *Grapevine leafroll associated virus-9* (GLRaV-9), *Grapevine rupestris stem pitting associated virus-1* (GRSPaV-1), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine fleck Virus* (GFkV), *Tomato ringspot virus* (ToRSV), *Grapevine vein clearing virus* (GVCV), *Grapevine Pinot gris virus* (GPgV), and *Grapevine red blotch associated virus* (GRBaV). We also aim to study the vectors (mealybugs) of these viruses and determine spatial and temporal aspects of GLD and their associated viruses. We also report on tests of management strategies by monitoring movements of these vectors to determine the efficacy of various insecticides. Better methods of detection are also being studied in this work, in terms of updated primer sets for PCR

detection, development of a membrane sampling method for grapevine viruses, as well as the development of a new qPCR method for the detection of the newly discovered GRBaV. Finally, examining the effects of main grapevine viruses on berry chemistry will be examined.

Objectives:

Chapter 2: To survey economically significant and newly discovered grapevine viruses among commercial vineyards in VA.

Chapter 3: To develop a suitable qPCR methodology for GRBaV.

Chapter 4: To evaluate foliarly applied insecticides for mealybug management and determine transmission of GLRaV-3 by the Gill's mealybug.

Chapter 5: To develop an efficient membrane-based sampling method for effective grapevine nucleic acid storage.

Chapter 6: To determine the effects of single and multiple virus infections of GLRaV-3, GRSPaV-1, and GRBaV on fruit chemistry.

This study is necessary due to a 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 futher work is needed to validate previous control attempts on their insect vectors within the state. Obtaining an understanding of viruses in Virginia is pertinent to keeping our wine industry in its current, expanding state. Knowledge of these virus disease complexes 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 as well as improve the current testing services available to growers. Results of this work will also aid growers who face the difficult decision-making process of removing virus affected vines.

1.3 References:

Agrios, G. N. (2005). *Plant Pathology*: Elsevier Academic Press.

Aigrain, P. (2001). Conjuncture vitiviniculture mondiale. *Office Int Vigne Vin Bull, 74*, 209-225.

Al Rwahnih, M., Dave, A., Anderson, M. M., Rowhani, A., Uyemoto, J. K., & Sudarshana, M. R. (2013). Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology, 103*(10), 1069-1076, doi:10.1094/PHYTO-10-12-0253-R.

Al Rwahnih, M., Dolja, V. V., Daubert, S., Koonin, E. V., & Rowhani, A. (2012a). Genomic and biological analysis of *Grapevine leafroll-associated virus 7* reveals a possible new genus within the family Closteroviridae. *Virus Research, 163*(1), 302-309, doi:10.1016/j.virusres.2011.10.018.

Al Rwahnih, M., Osman, F., Sudarshana, M., Uyemoto, J., Minafra, A., Saldarelli, P., et al. (2012b). Detection of *Grapevine leafroll-associated virus 7* using real time qRT-PCR and conventional RT-PCR. *Journal of Virological Methods, 179*(2), 383-389, doi:10.1016/j.jviromet.2011.11.026.

Al Rwahnih, M., Rowhani, A., & Golino, D. A. (2015). First report of *Grapevine red blotch associated virus* in archival grapevine material from Sonoma County, California. *Plant Disease*, 150127132800003, doi:10.1094/PDIS-12-14-1252-PDN.

Al Rwahnih, M., Rowhani, A., Golino, D. A., Islas, C. M., Preece, J. E., & Sudarshana, M. R. (2015). Detection and genetic diversity of *Grapevine red blotch-associated virus* isolates in table grape accessions in the National Clonal Germplasm Repository in California. *Canadian Journal of Plant Pathology, 37*(1), 130-135, doi:10.1080/07060661.2014.999705.

Alabi, O. J., Al Rwahnih, M., Karthikeyan, G., Poojari, S., Fuchs, M., Rowhani, A., Naidu, R.A. (2011). *Grapevine leafroll-associated virus 1* Occurs as Genetically Diverse Populations. *Phytopathology, 101*(12), 1446-1456, doi:10.1094/phyto-04-11-0114.

Alleweldt, G., & Dettweiler, E. (1994). *The Genetic Resources of Vitis: World List of Grapevine Collections. (2nd edition)*: Geilweilerhof.

Atallah, S. S., Gomez, M. I., Fuchs, M. F., & Martinson, T. E. (2012). Economic Impact of Grapevine Leafroll Disease on *Vitis vinifera* cv. Cabernet franc in Finger Lakes Vineyards of New York. [Article]. *American Journal of Enology and Viticulture, 63*(1), 73-79, doi:10.5344/ajev.2011.11055.

Avgelis, A., & Boscia, D. (2001). *Grapevine leafroll-associated closterovirus 7* in Greece. *Phytopathologia Mediterranea, 40*(3), 289-292.

Azzam, A. I., Gonsalves, D., & Golino, D. A. (1991). Detection of dsRNA in grapevines showing symptoms of rupestris stem pitting disease and the variabilities encountered. *Plant Disease, 75*(9), 960-964, doi:10.1094/PD-75-0960.

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, doi:10.1071/ea9941205.

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. Natural spread of grapevine leafroll disease in a vineyard of Northern Italy. In *Extended Abstracts 11th meeting of ICVG, 1993, Montreaux, 1993* (pp. 110)

Bertazzon, N., & Angelini, E. (2004). Advances in the detection of *Grapevine leafroll-associated virus 2* variants. *Journal of Plant Pathology, 86*(4, Special issue), 283-290.

Beuve, M., Candresse, T., Tannieres, M., & Lemaire, O. (2015). First Report of *Grapevine Pinot gris virus* (GPGV) in grapevine in France. *Plant Disease, 99*(2), 293-294, doi:10.1094/PDIS-10-14-1008-PDN.

Beuve, M., Sempe, L., & Lemaire, O. (2007). A sensitive one-step real-time RT-PCR method for detecting *Grapevine leafroll-associated virus 2* variants in grapevine. *Journal of Virological Methods, 141*(2), 117-124, doi:10.1016/j.jviromet.2006.11.042.

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. *Nature, 418*(6898), 696-699.

Bonavia, M., Digiaro, M., Boscia, D., Boari, A., Bottalico, G., Savino, V., et al. (1996). Studies on "corky rugose wood" of grapevine and on the diagnosis of *grapevine virus B*. *Vitis, 35*(1), 53-58.

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., Martelli, G. P., Savino, V., & Castellano, M. A. (1991). Identification of the agent of grapevine fleck disease. *Vitis, 30*(2), 97-105.

Boscia, D., Savino, V., Minafra, A., Namba, S., Elicio, V., Castellano, M. A., et al. (1993). Properties of a filamentous virus isolated from grapevines affected by corky bark. *Archives of Virology, 130*(1-2), 109-120, doi:10.1007/BF01319000.

Boulila, M., Boscia, D., Terlizzi, B. d., Castellano, M. A., Minafra, A., Savino, V., et al. (1990). Some properties of a phloem-limited non mechanically-transmissible grapevine virus. *Journal of Phytopathology, 129*(2), 151-158, doi:10.1111/j.1439-0434.1990.tb04298.x.

Braun, A., Palmiter, D., & Keplinger, J. (1966). Nematodes found in apple orchards in the Hudson Valley, 1956-1965. *Plant Dis. Reptr, 50*(10), 783-786.

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. http://biology.anu.edu.au/Groups/MES/vide/August 1996.

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, doi:10.1007/s10658-007-9254-1.

Cabaleiro, C., Segura, A., & Garcia-Berrios, J. J. (1999). Effects of *grapevine leafroll-associated virus 3* on the physiology and must of Vitis vinifera L. cv. Albarino 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, doi:10.1094/pdis.1997.81.3.283.

Caldwell, J. (2012). Virginia wine industry jobs grow by 50%; Economic impact doubles, new study finds. http://www.governor.virginia.gov/News/viewRelease.cfm?id=1114.

Calvi, B. L. (2011). *Effects Of Red-leaf Disease On Cabernet Sauvignon At The Oakville Experimental Vineyard And Mitigation By Harvest Delay And Crop Adjustment*. ProQuest Dissertations Publishing,

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). *59*, 330-337.

Chooi, K. M., Cohen, D., & Pearson, M. N. (2013). Generic and sequence-variant specific molecular assays for the detection of the highly variable *Grapevine leafroll-associated virus 3*. *Journal of Virological Methods, 189*(1), 20-29, doi:10.1016/j.jviromet.2012.12.018.

Conti, M. (1980). A Closterovirus from a Stem-Pitting-Diseased Grapevine. *Phytopathology, 70*(5), 394, doi:10.1094/Phyto-70-394.

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, doi:10.1017/s0007485300053888.

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, doi:10.1017/s0007485300055048.

Daane, K. M., Bentley, W. J., Walton, V. M., Malakar-Kuenen, R., Millar, J. G., Ingels, C. A., et al. (2006). New controls investigated for vine mealybug. *California Agriculture, 60*(1), 31-38, doi:10.3733/ca.v060n01p31.

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, doi:10.1016/j.biocontrol.2004.04.010.

de Saporta, G. (1879). *Le monde des plantes avant l'apparition de l'homme*.: G. Masson.

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, doi:10.1146/annurev.py.32.090194.001401.

Dolja, V. V., Kreuze, J. F., & Valkonen, J. P. T. (2006). Comparative and functional genomics of closteroviruses. *Virus Research, 117*(1), 38-51, doi:10.1016/j.virusres.2006.02.002.

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, doi:10.1007/s10658-008-9269-2.

du Preez, J., Stephan, D., Mawassi, M., & Burger, J. T. (2011). The grapevine-infecting vitiviruses, with particular reference to grapevine virus A. *Archives of Virology, 156*(9), 1495-1503, doi:10.1007/s00705-011-1071-3.

Elbeaino, T., Numic, F., Digiaro, M., Sabanadzovic, S., & Martelli, G. P. (2009). Partial characterization of a grapevine leafroll-associated virus isolated from an infected cypriot vine of cv. Mavro. *Journal of Plant Pathology, 91*(2), 479-484.

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, doi:10.1016/j.jviromet.2009.11.009.

Engelbrecht, D., & Kasdorf, G. (1987). Occurrence and transmission of *Grapevine virus A* in South African grapevines. *South African Journal for Enology and Viticulture, 8*(1), 23-29.

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.

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.

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. http://www.nysipm.cornell.edu/factsheets/grapes/diseases/grape_leafroll.pdf. Accessed June 2012.

Fuchs, M., Marsella-Herrick, P., Loeb, G. M., Martinson, T. E., & Hoch, H. C. (2009a). Diversity of ampeloviruses in mealybug and soft scale vectors and in grapevine hosts from leafroll-affected vineyards. *Phytopathology, 99*(10), 1177-1184, doi:10.1094/phyto-99-10-1177.

Fuchs, M., Martinson, T. E., Loeb, G. M., & Hoch, H. C. (2009b). Survey for the three major leafroll disease-associated viruses in Finger Lakes vineyards in New York. *Plant Disease, 93*(4), 395-401, doi:10.1094/pdis-93-4-0395.

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, doi:10.1071/zo9760237.

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, doi:10.1094/phyto-96-1223.

Garau, R., Prota, V. A., Boscia, D., Fiori, M., & Prota, U. (1995). *Pseudococcus-affinis* (Mask.), new vector of grapevine trichovirus-A and trichovirus-B. *Vitis, 34*(1), 67-68.

Garau, R., Prota, V. A., Piredda, R., Boscia, D., & Prota, U. (1994). On the possible relationship between Kober stem grooving and *grapevine virus A*. *Vitis, 33*(3), 161-163.

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, doi:10.1603/0022-0493-94.1.291.

Ghanem-Sabanadzovic, N., Sabanadzovic, S., Gugerli, P., & Rowhani, A. (2012). Genome organization, serology and phylogeny of *Grapevine leafroll-associated viruses 4* and *6*: Taxonomic implications. *Virus Research, 163*(1), 120-128, doi:10.1016/j.virusres.2011.09.001.

Giampetruzzi, A., Roumi, V., Roberto, R., Malossini, U., Yoshikawa, N., La Notte, P., et al. (2012). A new grapevine virus discovered by deep sequencing of virus- and viroid-derived small RNAs in Cv Pinot gris. *Virus Research, 163*(1), 262-268, doi:10.1016/j.virusres.2011.10.010.

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., Predajňa, L., & Komínek, P. (2011). *Grapevine fleck virus* isolates split into two distinct molecular groups. *Journal of Phytopathology, 159*(11/12), 805-807, doi:10.1111/j.1439-0434.2011.01845.x.

Glasa, M., Predajňa, L., Komínek, P., Nagyová, A., Candresse, T., & Olmos, A. (2014). Molecular characterization of divergent *grapevine Pinot gris virus* isolates and their detection in Slovak and Czech grapevines. *Archives of Virology, 159*(8), 2103-2107, doi:10.1007/s00705-014-2031-5.

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. (1995). Transmission studies of grapevine leafroll associated virus and grapevine corky bark associated virus by the obscure mealybug. *American Journal of Enology and Viticulture, 46*, 408.

Golino, D., Sim, S., & Rowhani, A. Identification of the latent viruses associated with young vine decline in California. In *In: Extended Abstracts of the 13th meeting of ICVG, Adelaide, 2000, 85-86, 2000* (pp. 85-86)

Golino, D. A. (1993). Potential Interactions Between Rootstocks and Grapevine Latent Viruses. *American Journal of Enology and Viticulture, 44*(2), 148-152.

Golino, D. A., Sim, S. T., Gill, R., & Rowhani, A. (2002). California mealybugs can spread grapevine leafroll disease. *California Agriculture, 56*(6), 196-201, doi:10.3733/ca.v056n06p196.

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, doi:10.3733/ca.v062n04p156.

Goszczynski, D. E., & Jooste, A. E. C. (2003). Identification of divergent variants of *Grapevine virus A*. *European Journal of Plant Pathology, 109*(4), 397-403, doi:10.1023/A:1023555018700.

Goszczynski, D. E., Kasdorf, G. G. F., Pietersen, G., Tonder, H. v., Goszczynski, D. E., Kasdorf, G. G. F., et al. (1996). Detection of two strains of *grapevine leafroll-associated virus 2*. *Vitis, 35*(3), 133-135.

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, doi:10.1111/j.1570-7458.2008.00786.x.

Greif, C., Garau, R., Boscia, D., Prota, V. A., Fiori, M., Bass, P., et al. (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.

Gugerli, P. 25 years of serological identification of grapevine leafroll-associated viruses: antiserum and monoclonal antibodies to GLRaV-1 to GLRaV-9. In *16th Meetig of ICVG, Dijon, France, 2009* (16 ed., pp. 24-28, Extended Abstracts 16th Meetig of ICVG, Dijon, France)

Gugerli, P., Brugger, J. J., & Bovey, R. (1984). L'enroulement de la vigne: mise en evidence de particules virales et drsveloppement dune methode immuno-enzymatique pour le diagnostic rapide. *Revue Suisse de Viticulture Arboriculture et Horticulture, 16*, 299-304.

Gugerli, P., & Ramel, M. E. Grapevine leafroll associated virus II analyzed by monoclonal antibodies. In *Extended Abstracts 11th Meeting ICVG, Montreux, 1993* (pp. 23-24, Vol. 1993)

Guidoni, S., Mannini, F., Ferrandino, A., Argamante, N., & Di Stefano, R. (1997). The effect of grapevine leafroll and rugose wood sanitation on agronomic performance and berry and leaf phenolic content of a Nebbiolo clone (Vitis vinifera L.). *American Journal of Enology and Viticulture, 48*(4), 438-442.

Gullan, P. J., Downie, D. A., & Steffan, S. A. (2003). A new pest species of the mealybug genus *Ferrisia fullaway* (Hemiptera : Pseudococcidae) from the United States. *Annals of the Entomological Society of America, 96*(6), 723-737, doi:10.1603/0013-8746(2003)096[0723:anpsot]2.0.co;2.

Guo, Q., Honesty, S., Xu, M. L., Zhang, Y., Schoelz, J., & Qiu, W. P. (2014). Genetic diversity and tissue and host specificity of *Grapevine vein clearing virus*. *Phytopathology, 104*(5), 539-547, doi:10.1094/PHYTO-03-13-0075-R.

Habili, N. (2013). Highlights of a newly-emerging grapevine virus: *Grapevine red blotch-associated virus*.

Habili, N., Kominek, P., & Little, A. (2007). *Grapevine leafroll-associated virus 1* as a common grapevine pathogen. *PLant Viruses, 1*, 63-68.

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.

Haviland, D. R., Beede, R., Godfrey, K. E., & Daane, K. M. (2006). *Ferrisia gilli*: A new mealybug pest of pistachios and other deciduous crops. http://anrcatalog.ucdavis.edu/pdf/8207.pdf. Accessed November 2012.

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.

Jelkmann, W., Mikona, C., Turturo, C., Navarro, B., Rott, M. E., Menzel, W., et al. (2012). Molecular characterization and taxonomy of *grapevine leafroll-associated virus 7*. *Archives of Virology, 157*(2), 359-362.

Jones, T. J., & Nita, M. (2016). Spatio-temporal association of GLRaV-3-infected grapevines, and effect of insecticidal treatments on mealybug populations in Virginia vineyards. [journal article]. *European Journal of Plant Pathology*, 1-16, doi:10.1007/s10658-016-0878-x.

Jones, T. J., Rayapati, N., & Nita, M. (2015). Occurrence of *Grapevine leafroll associated virus-2, −3* and *Grapevine fleck virus* in Virginia, U.S.A., and factors affecting virus infected vines. *European Journal of Plant Pathology*, 1-14, doi:10.1007/s10658-015-0605-z.

Karasev, A. V. (2000). Genetic diversity and evolution of closteroviruses. *Annual Review of Phytopathology, 38*, 293-324, doi:10.1146/annurev.phyto.38.1.293.

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.

Krenz, B., Thompson, J. R., Fuchs, M., & Perry, K. L. (2012). Complete genome sequence of a new circular DNA virus from grapevine. *Journal of Virology, 86*(14), 7715-7715.

Krenz, B., Thompson, J. R., McLane, H. L., Fuchs, M., & Perry, K. L. (2014). *Grapevine red blotch-associated virus* Is Widespread in the United States. *Phytopathology, 104*(11), 1232-1240, doi:10.1094/phyto-02-14-0053-r.

La Notte, P., Buzkan, N., Choueiri, E., Minafra, A., & Martelli, G. P. (1997a). Acquisition and transmission of *grapevine virus A* by the mealybug Pseudococcus longispinus. *Journal of Plant Pathology, 78*(1), 79-85.

La Notte, P., Minafra, A., & Saldarelli, P. (1997b). A spot-PCR technique for the detection of phloem-limited grapevine viruses. *Journal of Virological Methods, 66*(1), 103-108, doi:10.1016/s0166-0934(97)00038-4.

Levadoux, L. (1956). Les populations sauvages et cultivees de *Vitis vinifera* L.. *Ann. Amelior. Plantes, 6*, 59-117.

Little, A., Fazeli, C. F., & Rezaian, M. A. (2001). Hypervariable genes in *Grapevine leafroll associated virus 1*. *Virus Research, 80*(1), 109-116, doi:10.1016/S0168-1702(01)00343-4.

Lunden, S., Meng, B., Avery, J., & Qiu, W. (2010). Association of *Grapevine fanleaf virus*, *Tomato ringspot virus* and *Grapevine rupestris stem pitting-associated virus* with a grapevine vein-clearing complex on var. Chardonnay. *European Journal of Plant Pathology, 126*(2), 135-144.

Mahfoudhi, N., Digiaro, 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, doi:10.1094/pdis-93-10-0999.

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, doi:10.1016/j.virusres.2008.02.015.

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, doi:10.1007/s00705-008-0290-8.

Malossini, U., Moscon, R., Ferrazza, M., Bianchedi, P., Varner, M., & Credi, R. Caratteristiche vegeto-produttive di viti Pinot grigio e Traminer aromatico affette da una nuova virosi segnalata in Trentino. In *IV Convegno Nazionale di Viticoltura (Conavi), 2012* (pp. 37 (032))

Mannini, F., Argamante, N., & Credi, R. (1996). Improvements in the quality of grapevine 'Nebbiolo' clones obtained by sanitation. In S. Poni, E. Peterlunger, F. Iacono, & C. Intrieri (Eds.), *First Ishs Workshop on Strategies to Optimize Wine Grape Quality* (pp. 319-324, Acta Horticulturae, Vol. 427). Leuven 1: International Society Horticultural Science.

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. near pseudococci , with implications for integrated pest management in vineyards. *Phytoparasitica, 39*(4), 369-376, doi:10.1007/s12600-011-0170-8.

Martelli, G. P. (1993). *Graft-transmissible diseases of grapevines: handbook for detection and diagnosis*. Rome: Food and Agriculture Organization (FAO).

Martelli, G. P. (2014). Directory of virus and virus-like diseases of the grapevine and their agents. *Journal of Plant Pathology, 96*(1), 1-136.

Martelli, G. P., Abou Ghanem-Sabanadzovic, N., Agranovsky, A. A., Al Rwahnih, M., Dolja, V. V., Dovas, C. I., et al. (2012). Taxonomic revision of the family Closteroviridae with special reference to the grapevine leafroll-associated members of the genus Ampelovirus and the putative species unassigned to the family. *Journal of Plant Pathology, 94*(1), 7-19.

Martelli, G. P., Adams, M. J., Kreuze, J. F., & Dolja, V. V. (2007). Family Flexiviridae: a case study in virion and genome plasticity. *Annual Review of Phytopathology, 45*, 73-100, doi:10.1146/annurev.phyto.45.062806.094401.

Martelli, G. P., Agranovsky, A. A., Bar-Joseph, M., Boscia, D., Candresse, T., Coutts, R. H. A., et al. (2002a). The family Closteroviridae revised. *Archives of Virology, 147*(10), 2039-2044, doi:10.1007/s007050200048.

Martelli, G. P., & Boudon-Padieu, E. (2006). *Directory of infectious diseases of grapevines*. (Vol. 55): International Centre for Advanced Mediterranean Agronomic Studies. Options Méditerranéennes, Ser. B, Studies and Research. 55: 59-75.

Martelli, G. P., & Jelkmann, W. (1998). Foveavirus, a new plant virus genus. *Archives of Virology, 143*(6), 1245-1249, doi:10.1007/s007050050372.

Martelli, G. P., Sabanadzovic, S., Abou Ghanem-Sabanadzovic, N., & Saldarelli, P. (2002b). Maculavirus, a new genus of plant viruses. *Archives of Virology, 147*(9), 1847-1853, doi:10.1007/s007050200046.

Martelli, G. P., Sabanadzovic, S., Abou-Ghanem Sabanadzovic, N., Edwards, M. C., & Dreher, T. (2002c). The family Tymoviridae. *Archives of Virology, 147*(9), 1837-1846, doi:10.1007/s007050200045.

Martelli, G. P., Viruses, I. C. f. t. S. o., Grapevine, V. D. o. t., Food, & Nations, A. O. o. t. U. (1993). *Graft-transmissible Diseases of Grapevines: Handbook for Detection and Diagnosis*: Food and Agriculture Organization of the United Nations.

Martin, R. R., Eastwell, K. C., Wagner, A., Lamprecht, S., & Tzanetakis, I. E. (2005). Survey for viruses of grapevine in Oregon and Washington. *Plant Disease, 89*(7), 763-766, doi:10.1094/pd-89-0763.

McGovern, P. E. (2003). *Ancient wine The search for the origins of viniculture*. Princeton: Princeton University Press.

Mekuria, Karasev, A. V., Martin, R. R., & Naidu, R. A. (2009a). First report of *Grapevine leafroll-associated virus-3* in six wine grape cultivars in Idaho. *Plant Disease, 93*(11), 1218, doi:10.1094/pdis-93-11-1218c.

Mekuria, Soule, M. J., Jarugula, S., & Naidu, R. A. (2009b). Current status of grapevine viruses in Washington State vineyards. *Phytopathology, 99*, S83.

Melzer, M. J., Sether, D. M., Borth, W. B., Mersino, E. F., & Hu, J. S. (2011). An assemblage of closteroviruses infects Hawaiian ti (Cordyline fruticosa L.). *Virus Genes, 42*(2), 254-260, doi:10.1007/s11262-010-0537-9.

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., 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, doi:10.1007/s11262-004-2197-0.

Meng, B., Zhu, H. Y., & Gonsalves, D. (1999). Rupestris stem pitting associated virus-1 consists of a family of sequence variants. *Archives of Virology, 144*(11), 2071-2085, doi:10.1007/s007050050623.

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.

Mikona, C., & Jelkmann, W. (2010). Replication of Grapevine leafroll-associated virus-7 (GLRaV-7) by *Cuscuta* species and its transmission to herbaceous plants. *Plant Disease, 94*(4), 471-476.

Milkus, B. N., & Goodman, R. N. (1999). A survey of Missouri vineyards for the presence of five grape viruses. *American Journal of Enology and Viticulture, 50*(1), 133-134.

Millar, J. G., Daane, K. M., McElfresh, J. S., Moreira, J. A., Malakar-Kuenen, R., Guillen, M., et al. (2002). Development and optimization of methods for using sex pheromone for monitoring the mealybug *Planococcus ficus* (Homoptera : Pseudococcidae) in California vineyards. *Journal of Economic Entomology, 95*(4), 706-714, doi:10.1603/0022-0493-95.4.706.

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, doi:10.1016/0166-0934(94)90076-0.

Morales, R. Z., & Monis, J. (2007). First detection of *Grapevine leafroll associated virus-7* in California vineyards. *Plant Disease, 91*(4), 465-465, doi:10.1094/pdis-91-4-0465b.

Morelli, M., Catarino, A. D. M., Susca, L., Saldarelli, P., Gualandri, V., & Martelli, G. P. (2014). First report of *Grapevine Pinot gris virus* from table grapes in Southern Italy. *Journal of Plant Pathology, 96*(2), 439-439.

Myles, S., Boyko, A. R., Owens, C. L., Brown, P. J., Grassi, F., Aradhya, M. K., et al. (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, doi:10.1073/pnas.1009363108.
Naidu, R. A., Maree, H. J., & Burger, J. T. (2015). Grapevine Leafroll Disease and Associated Viruses: A Unique Pathosystem. *Annual Review of Phytopathology*, *53*, 613-634).
Naidu, R. A., & Mekuria, T. A. (2010). First report of *Grapevine fleck virus* from Washington vineyards. *Plant Disease, 94*(6), 784, doi:10.1094/pdis-94-6-0784a.
Naidu, R. A., Soule, M. J., & Jarugula, S. (2006). Single and mixed infections of *Grapevine leafroll-associated viruses* in Washington State vineyards. *Phytopathology, 96*, S83.
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, doi:10.3186/jjphytopath.45.497.
Nassuth, A., Pollari, E., Helmeczy, 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, doi:10.1016/s0166-0934(00)00211-1.
Oberhansli, T., Altenbach, D., & Bitterlin, W. (2011). Development of a duplex TaqMan real-time PCR for the general detection of phytoplasmas and 18S rRNA host genes in fruit trees and other plants. [Article]. *Bulletin of Insectology, 64*, S37-S38.
Osman, F., Leutenegger, C., Golino, D., & Rowhani, A. (2007). Real-time RT-PCR (TaqMan®) assays for the detection of *Grapevine Leafroll associated viruses 1-5* and *9*. *Journal of Virological Methods, 141*(1), 22-29, doi:10.1016/j.jviromet.2006.11.035.
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, doi:10.1046/j.1365-3059.1997.d01-44.x.
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. 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. Accessed May 2012.
Pietersen, G. Spatio temporal distribution dynamics of grapevine leafroll disease in western cape vineyards. In *Extended Abstracts 15th Meeting of ICVG, Stellenbosch, South Africa, April 2006, pp 126-127, 2006* (pp. 126-127)
Pietersen, G. (2010). Grapevine leafroll disease control in South Africa. https://41.203.19.179/index.php?option=com_zine&view=article&id=548%3Agrapevine-leafroll-disease-control-in-south-africa&Itemid=10. Accessed May 2012.
Plesko, I. M., Marn, M. V., & Zezlina, I. (2014). First Report of *Grapevine Pinot gris virus* Infecting Grapevine in Slovenia. *Plant Disease, 98*(7), 1014-1015, doi:10.1094/PDIS-11-13-1137-PDN.
Poojari, S., Alabi, O. J., Fofanov, V. Y., & Naidu, R. A. (2013). A leafhopper-transmissible DNA virus with novel evolutionary lineage in the family Geminiviridae implicated in grapevine redleaf disease by next-generation sequencing. *PLoS ONE, 8*(6), e64194.
Powell, C., Forer, L., & Stouffer, R. (1982). Reservoirs of *tomato ringspot virus* in fruit orchards. *Plant Disease, 66*(7), 583-584.

Powell, C., Forer, L., Stouffer, R., Cummins, J., Gonsalves, D., Rosenberger, D., et al. (1984). Orchard weeds as hosts of *tomato ringspot* and *tobacco ringspot viruses*. *Plant Disease, 68*(3), 242-244.

Powell, C. A., Longenecker, J. L., & Forer, L. B. (1990). Incidence of *Tomato Ringspot Virus* and *Tobacco Ringspot Virus* in grapevines in Pennsylvania. *Plant Disease, 74*(9), 702-704, doi:10.1094/pd-74-0702.

Qiu, W., Avery, J., & Lunden, S. (2007). Characterization of a severe virus-like disease in Chardonnay grapevines in Missouri. *Plant Health Progress*.

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. http://wine.wsu.edu/research-extension/plant-health/virology/virus-diseases/. Accessed November 2012.

Rayapati, N., O'Neal, S., & Walsh, D. (2008). Grapevine leafroll disease. http://pubs.wsu.edu. Accessed 2012 April.

Rosa, C., Jimenez, J. F., Margaria, P., & Rowhani, A. (2011). Symptomatology and Effects of Viruses Associated with Rugose Wood Complex on the Growth of Four Different Rootstocks. *American Journal of Enology and Viticulture, 62*(2), 207-213, doi:10.5344/ajev.2011.10104.

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. Transmission of grapevine leafroll disease and an associated closterovirus to healthy grapevine by the mealybug *Planococcus ficus* Signoret. In *Proceedings 9th Meeting of ICVG, Kiryat Anavim, 1989* (pp. 1987, 1967-1969)

Rosenberger, D., Cummins, J., & Gonsalves, D. Development of apple union necrosis and decline in apple-trees inoculated with *Tomato Ringspot Virus*. In *Phytopathology, 1985* (Vol. 75, pp. 1312-1312, Vol. 11): Amer Phytopathological Soc 3340 Pilot Knob Road, St Paul, Mn 55121

Rosenberger, D., Harrison, M., & Gonsalves, D. (1983). Incidence of apple union necrosis and decline, *tomato ringspot virus*, and *Xiphinema* vector species in Hudson Valley orchards. *Plant Disease, 67*(4), 356-360.

Rosenberger, D., & Meyer, F. (1988). Control of dagger and lesion nematodes in apple and plum orchards with fenamiphos, carbofuran, and carbosulfan. *Plant Disease, 72*(6), 519-522.

Rott, M. E., Gilchrist, A., Lee, L., & Rochon, D. A. (1995). Nucleotide sequence of tomato ringspot virus RNA1. *Journal of General Virology, 76*(2), 465-473.

Rott, M. E., Tremaine, J. H., & Rochon, D. M. (1991). Nucleotide sequence of *tomato ringspot virus* RNA-2. *Journal of General Virology, 72*(7), 1505-1514, doi:10.1099/0022-1317-72-7-1505.

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, doi:10.1094/phyto.1998.88.11.1238.

Sabanadzovic, S., Abou Ghanem-Sabanadzovic, N., 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., Giampetruzzi, A., Morelli, M., Malossini, U., Pirolo, C., Bianchedi, P., et al. (2015). Genetic Variability of *Grapevine Pinot gris virus* and Its Association with Grapevine Leaf Mottling and Deformation. *Phytopathology, 105*(4), 555-563, doi:10.1094/PHYTO-09-14-0241-R.

Saldarelli, P., Minafra, A., & Martelli, G. P. (1996). The nucleotide sequence and genomic organization of grapevine virus B. *Journal of General Virology, 77*(10), 2645-2652, doi:10.1099/0022-1317-77-10-2645.

Saldarelli, P., Rowhani, A., Routh, G., Minafra, A., & Digiaro, 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, doi:10.1023/a:1008608506699.

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, doi:10.2503/jjshs.50.169.

Scheu, G. (1935). Die Rollkrankheit des Rebstockes. *Der Deutsche Weinbau., 14*, 222-223, 345-346, 356-358.

Scheu, G. (1936). *Mein Winzerburch*. Berlin: Reichnährstand Verlag.

Schooley, N., Baudoin, A. B., Derr, J. F., Pfeiffer, D. G., Weaver, M. J., & Wolf, T. K. (2010). Crop profile for grapes in Virginia. http://www.ipmcenters.org/cropprofiles/docs/vagrapes.pdf.

Seddas, Haidar, Greif, Jacquet, Cloquemin, & Walter (2000). Establishment of a relationship between grapevine leafroll closteroviruses 1 and 3 by use of monoclonal antibodies. *Plant Pathology, 49*(1), 80-85, doi:10.1046/j.1365-3059.2000.00415.x.

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, doi:10.1023/b:ejpp.0000003750.34458.71.

Shi, B.-j., Habili, N., Gafny, R., & Symons, R. H. (2004). Extensive Variation of Sequence within Isolates of *Grapevine Virus B*. *Virus Genes, 29*(2), 279-285, doi:10.1023/B:VIRU.0000036388.41242.c1.

Sim, S. T., Rowhani, A., Alkowni, R., & Golino, D. A. Experimental transmission of *Grapevine leafroll associated virus 5* and *9* by longtailed mealybugs. In *Extended Abstract 14th Meeting of ICVG, Locorotondo, Italy. 211-212, 2003* (pp. 211-212)

Smith, H., & Cowles, R. (2009). Scale insect pests of Connecticut trees and ornamentals. http://www.hort.uconn.edu/ipm/nursery/caes_fs/caes_fs_scalepests.htm. Accessed November 2012.

Stouffer, R., & Uyemoto, J. Association of *tomato ringspot virus* with apple union necrosis and decline. In *X International Symposium on Fruit Tree Virus Diseases 67, 1976* (pp. 203-208)

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.

Sudarshana, M., Perry, K. L., & Fuchs, M. (2015). Grapevine red blotch-associated virus, an emerging threat to the grapevine industry. *Phytopathology*, doi:10.1094/phyto-12-14-0369-fi.

Tanne, E., Ben-Dov, Y., & Raccah, 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, doi:10.1016/j.tig.2006.07.008.
Thompson, J. R., Fuchs, M., & Perry, K. L. (2012). Genomic analysis of *Grapevine leafroll associated virus-5* and related viruses. *Virus Research, 163*(1), 19-27, doi:10.1016/j.virusres.2011.08.006.
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, doi:10.1094/phyto-100-8-0830.
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, doi:10.1094/phyto-98-10-1093.
Tuskan, R. G., & Tolin, S. A. (1984). Properties of grape and soybean isolates of *Tomato Ringspot Virus*. *Phytopathology, 74*(7), 808-808.
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, doi:10.3733/ca.v055n04p28.
Varela, L. G. (2005). Grape and Vine Mealybug Life Cycles in the North Coast. http://cesonoma.ucdavis.edu/files/27229.pdf.
Varela, L. G., Elkins, R. B., Van Steenwyk, R. A., & Ingels, C. A. (2012). Pear Mealybugs. http://www.ipm.ucdavis.edu/PMG/r603301211.html. Accessed 2012 November.
Varela, L. G., & Smith, R. J. (2009). Mealybugs. http://ucanr.org/sites/SoCo/files/27649.pdf. Accessed November 2012.
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.
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. 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, doi:10.1603/0022-0493-99.4.1280.
Wieczorek, A., & Sanfacon, H. (1993). Characterization and subcellular localization of tomato ringspot nepovirus putative movement protein. *Virology, 194*(2), 734-742, doi:10.1006/viro.1993.1314.
Wine Institute. (2015). Wine Institute Statistics for California and the World. www.wineinstitute.org

Wolf, T. K. (2008). *Wine grape production guide for eastern North America*. (NRAES-145). Ithaca, NY.: PALS Publishing (formerly NRAES).

Wolpert, J. A., & Vilas, E. P. (1992). Effect of mild leafroll disease on growth, yield, and fruit maturity indices of Riesling and Zinfandel. *American Journal of Enology and Viticulture, 43*(4), 367-369.

Zhang, Y., Singh, K., Kaur, R., & Qiu, W. P. (2011). Association of a novel DNA virus with the grapevine vein-clearing and vine decline syndrome. *Phytopathology, 101*(9), 1081-1090, doi:10.1094/PHYTO-02-11-0034.

Zhang, Y.-P., Uyemoto, J. K., Golino, D. A., & Rowhani, A. (1998). Nucleotide sequence and RT-PCR detection of a virus associated with grapevine rupestris stem-pitting disease. *Phytopathology, 88*(11), 1231-1237.

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.

Chapter 2

Occurrence of grapevine viruses in Virginia, U.S.A. vineyards and factors affecting virus infected vines

2.1 Introduction

Among perennial crops worldwide, the greatest number of viruses and virus-like agents (around 70) have been detected in grapevines (Martelli 2014; Naidu et al. 2015a). However, only a few of these viruses are considered to have high economic importance to wine grape production (Martelli and Boudon-Padieu 2006; Rayapati 2012). Few examples include the grapevine leafroll complex, rugose wood complex, degeneration/decline disease complex, and fleck disease complex (Martelli and Boudon-Padieu 2006).

Grapevine leafroll disease, commonly found throughout all grapevine growing regions worldwide (Fuchs et al. 2009b), causes crop loss ranging from 10% to 40% (Wolpert and Vilas 1992). Grapevine leafroll associated viruses (GLRaVs, family *Closteroviridae*) taxonomically include GLRaV-2 (genus *Closterovirus*), GLRaV-7 (genus *Velarivirus*), and GLRaV-1, -3, and -4 (genus *Ampelovirus*). Originally, GLRaV-4, -5, -6, -9, -De, -Pr, and -Car were all considered separate species; however, these strains are now classified as divergent strains of GLRaV-4 (Ghanem-Sabanadzovic et al. 2012; Martelli et al. 2012; Thompson et al. 2012) and currently referred as GLRaV-4 strains 5, 6, 9, De, Pr, and Car based on genomic sequences.

The rugose wood disease complex is a group of viruses (mostly belonging to the family *Flexiviridae* genera *Vitivirus* and *Foveavirus*) that primarily affect grapevines on the woody cylinder (i.e. vascular system) (Martelli et al. 1993; Pearson and Goheen 1988; Rosa et al. 2011). This complex includes *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), and *Grapevine rupestris stem-pitting associated virus-1* (GRSPaV). All of these viruses are widely spread and cause a slow decline of vines as they age combined with stem grooving (GVA), corky bark (GVB), and graft incompatibility (Rosa et al. 2011). Irregular to no ripening of berries (Martelli et al. 1993) can occur with the infections of these viruses and may become more severe when multiple viruses infect the same vine (such as GLRaV-3) (Guidoni et al. 1997; Mannini et al. 1996).

Tomato ringspot virus (ToRSV) is transmitted by the dagger nematode (*Xiphinema americanum*) (Braun et al. 1966; Rosenberger et al. 1983; Rosenberger and Meyer 1988). ToRSV infection causes uneven fruit development, a significant loss in yield, and overall vine decline (Powell et al. 1990). ToRSV has historically been present in Virginia (Tuskan and Tolin 1984), but has not been actively investigated since that time period. The risk this virus poses in Virginia vineyards is potentially high with susceptible own-rooted hybrid cultivars, such as Vidal blanc (60.7 bearing hectares producing 687 tons in 2015) and Seyval (12.95 bearing hectares producing 116 tons in 2015) (VWB 2016).

Three recently discovered viruses, *Grapevine vein clearing virus* (GVCV, family *Caulimoviridae*, genus *Badnavirus*) (Zhang et al. 2011), *Grapevine red blotch-associated virus* (Al Rwahnih et al. 2013) [GRBaV, family *Geminiviridae* also known as *Grapevine Cabernet franc-associated virus* (Krenz et al. 2012) and *Grapevine redleaf disease* (Poojari et al. 2013)], and *Grapevine Pinot gris virus* (GPgV, genus *Trichovirus*, family *Betaflexiviridae*)

(Giampetruzzi et al. 2012) might also pose a concern for Virginia vineyards as the current state of these viruses is mostly unknown. GRBaV has been found to be widespread in the US, including some confirmed infections in Virginia (Krenz et al. 2014b). Vector(s) of GRBaV are being investigated, but no formal conclusions have been made. With similar symptoms as grapevine leafroll disease, GRBaV could be masked in GLRaV-positive samples. GVCV has been found primarily in the Mid-West United States, and caused severe translucent vein-clearing, vine decline, and yield reduction in *V. vinifera* (Guo et al. 2014; Zhang et al. 2011). Within field, horizontal spread of this virus has been noted, but a causal vector is yet to be found (Zhang et al. 2011). GVCV is often found in co-infection cases with *Grapevine fanleaf virus*, *Tomato ringspot virus*, and *Grapevine Rupestris stem pitting-associated virus* (Lunden et al. 2010; Qiu et al. 2007) causing an increase in symptom severity. GPgV has been found in Italy through deep sequencing in vines expressing chlorotic mottling and leaf deformation, but no pathogenicity has been proven (Giampetruzzi et al. 2012). GPgV negatively affects shoot production as well as berry number and weight per vine (Malossini et al. 2012). A recent survey in Italy has shown this virus to be widespread in symptomatic and asymptomatic grapevines (Saldarelli et al. 2015) and it has been recently found to spread to other areas of Italy, France, Czech Republic, and Slovenia (Beuve et al. 2015; Giampetruzzi et al. 2012; Glasa et al. 2014; Morelli et al. 2014; Plesko et al. 2014; Saldarelli et al. 2015).

Symptoms of virus diseases are often not the best indicator of infection (Agrios 2005; Jones et al. 2015), making it best to utilize molecular-based diagnostic methods. Recent genomic information has allowed researchers to design primers better suited for detecting viruses with higher sequence diversity to help develop less specific, but more sensitive detection methods. For example, GLRaV-1, -2, -3, and -4 have isolates and strains with high sequence diversity

(<30% divergency) and thus, detection methods have been created based on "universal" primers, which will detect all known strains of the target virus (Ghanem-Sabanadzovic et al. 2012; Alabi et al. 2011). Universal primers have now been developed for GLRaV-1 (Alabi et al. 2011), GLRaV-2 (Beuve et al. 2007), GLRaV-3 (Chooi et al. 2013), and GLRaV-4 (Ghanem-Sabanadzovic et al. 2012) and show great promise in their ability to detect these viruses.

The state of Virginia, USA, home to a rapidly expanding wine industry with a 106% increase in state economic impact from 2007 to 2012 (VWB 2012), has seen a noticeable impact from grapevine viruses within the last few years. A recent survey showed the wide distribution of three important grapevine viruses, GLRaV-2, GLRaV-3, and Grapevine fleck virus (GFkV) throughout the state (Jones et al. 2015). Moreover, vineyard age has a significant effect on the probability of detecting GLRaV-2 and -3 from the field, indicating these viruses were in the field for more than four decades based on samples taken from grapevines 30-40 years old, and potential positive effect of clean plant materials that became available in recent years (Jones et al. 2015). However, current understanding of other grapevine viruses in Virginia is very limited. High initial investment on vineyards and relatively slow return on the investment makes it critical for growers to have long, productive years with virus free grapevines.

Therefore, in this study, the previous statewide survey (Jones et al. 2015) was expanded in both the number of grapevine samples and viruses to be tested. Existing and new samples were tested for the following grapevine viruses: GLRaVs (1, 2, 3, 4, 5, and 9), GFkV, GVA, GVB, GRSPaV, GVCV, ToRSV, GRBaV, and GPgV. The survey also increased the number of mealybugs sampled and tested for determination of species present in VA vineyards.

2.2 Materials and Methods

Extended Virus and Mealybug Survey. Vineyards were selected arbitrarily from across the major grape growing regions of Virginia (VA). Samples were collected throughout the 2012-2014 seasons. At each vineyard, multiple cultivars were randomly selected and sampled. Each sample contained 10 total petioles taken arbitrarily across the canopy from a single, symptomatic vine. This sampling method was used due to uneven virus distribution of GLRaV-3 in grapevines (Charles et al. 2006). Sampled petioles were placed into a Ziploc bag, immediately stored in an iced cooler box, and taken back to the laboratory. Samples are then immediately processed into crude extract or stored at -20°C for a maximum of one week before being processed. At the time of sampling, vine locations were recorded using a GPS to help return to the sample site if needed as well as obtain geographic region and elevation data. Sample information such as cultivar, presence/absence of mealybugs and age of planting were also recorded along with the location data. These samples were subjected to detection of all viruses listed in Table 2.1. In addition to the samples collected in 2012-14, there were 456 samples collected in 2009-2011 (Jones et al. 2015), that were subjected to detection of GLRaVs (1, 2, 3, 4, 5, and 9), GFkV, GVA, GVB, and GRSPaV.

When mealybugs were present in the sampled vineyard, several female mealybugs were sampled and placed into a 1.5-ml microcentrifuge tube containing 95% ethanol. Samples were stored at -20°C until the end of the field season. All collected samples were tested via a vineyard mealybug multiplex PCR (Daane et al. 2011) to determine species collected. Morphological identification was also initially attempted; however, as *Ferissia* is a newly described species and has been historically difficult to identify through morphology (Gullan et al. 2003), thus, the decision was made to utilize PCR techniques for mealybug identification. Additionally, seven

vineyards with known infestations of mealybugs and GLRaV-3 infected vines were visited in 2013. A total of 100 mealybugs were sampled across all sites, stored, and subjected to the vineyard mealybug multiplex PCR for species identification.

Crude extraction, Reverse Transcription PCR, and conventional PCR for virus samples. Samples were processed in the same manner as described previously for one-step reverse-transcription PCR (Jones et al. 2015; Rowhani et al. 2000) (hereafter referred as RT-PCR). Petiole samples were removed from their Ziploc bags and 0.25 g of petiole sections, cut using sterile razor blades, were placed into grinding bags (BIOREBA, Switzerland) containing 5 ml of a filter-sterilized grapevine extraction buffer (1.59 g/liter Na_2CO_3, 2.93 g/liter $NaHCO_3$, 2% polyvinylpyrrolidone-40, 0.2% bovine serum albumin, and 0.05% Tween 20) (Sigma-Aldrich Co. LLC, St. Louis, MO) (Rowhani et al. 2000). The samples were then homogenized using a mechanical grinder (BIOREBA, Switzerland, Homex 6 [115V]) and crude extracts were transferred into 1.5-ml microcentrifuge tubes. Samples were stored at -80°C until used in future molecular diagnostic assays.

For routine virus detection using conventional and RT-PCR, 4 μl crude extract was added to 50 μl GES denaturing buffer (0.1 M glycine, pH 9.0; 50 mM NaCl; 1 mM EDTA; 0.5% Triton X-100) in a 0.5-ml microcentrifuge tube. Following incubation at 95°C for 10 minutes, samples were placed on ice for a minimum of 5 minutes before adding 2 μl of the GES denatured homogenate to PCR tubes containing: 13.4 µl nuclease-free H_2O, 2.5 µl 10X PCR buffer containing (New England Biolabs, Ipswitch, MA), 2.5 µl sucrose/cresol red (20% w/v sucrose, 1 mM cresol red) (Sigma-Aldrich Co. LLC, St. Louis, MO), 1.25 µl virus specific forward primer (20 µM), 1.25 µl virus specific reverse primer (20 µM), 1.25 µl 100mM dithiothreitol (Sigma-Aldrich Co. LLC, St. Louis, MO), 0.5 µl dNTPs (10 mM) (Invitrogen, Grand Island, NY), 0.1 µl

RnaseOUT (40 U/µl) (Invitrogen), 0.035 µl Superscript III RTase (200 U/µl)(Invitrogen), and 0.25 µl Taq DNA polymerase (5 U/µl) (New England Biolabs) (Naidu et al. 2006; Osman et al. 2007; Rowhani et al. 2000). For conventional PCR reactions, an additional 0.135 µl H20 was used to substitute for the RTase and RNaseOUT.

The PCR tubes were placed in a thermal cycler (Bio-Rad C1000 thermocycler, Hercules, CA) and subjected to the cycles outlined in Table 2.1 for each primer pair set. For GLRaV-1, -2, -3, and -4, two sets of primers were used to compare new universal primer sets with older and more specific primer sets (Table 2.1). Following amplification, gel electrophoresis using a 1.2% agarose gel resolved PCR products, and DNA bands were visualized using a Fotodyne digital imaging system (Fotodyne, Hartland, WI, U.S.). Positive and negative controls were used to validate test results. Amplified DNA from randomly selected PCR-positive samples for each virus was purified using the QIAquick PCR Purification kit (Qiagen Inc USA, CA) using the manufacturer's protocol, and then sequenced to confirm identity and technique at the Virginia Biocomplexity Institute of Virginia Tech (VBI) (Blacksburg, VA, U.S.) using an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA).

Clustal X (Conway Institute, UK) was used to perform multiple sequence alignments for the HSP70h of GLRaV-1, GLRaV-4, GLRaV-5, and GLRaV-9, the replicase gene of GRSPaV-1, the coat protein of GVA and GVB, and the V2 gene of GRBaV under default parameters. Corresponding sequences from GenBank were used in these alignments and comparisons of nucleotide identity were inferred.

Mealybugs: genomic DNA extraction, PCR, and sequencing for species determination. Collected and stored mealybugs were separated and individually ground in liquid nitrogen. The powdered tissue was transferred to a new 1.5-ml microcentrifuge tube, and then subjected to total

genomic DNA extraction through the QIAGEN DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) following the manufacturers protocol. The extracted genomic DNA was stored at -80°C until used in the vineyard mealybug multiplex PCR for vineyard mealybug identification which was used in our previous study (Daane et al. 2011; Jones et al. 2015). Multiplex-PCR products were purified following the manufacturers standard protocol for the QIAquick PCR purification kit (Qiagen), and select samples were sent to the VBI for sequencing. In addition to the vineyard mealybug multiplex PCR, a simplex PCR for the amplification of the cytochrome oxidase subunit 1 gene was used to confirm mealybug species from the multiplex results. Primers for amplification were C1-J-2183 (also known as Jerry, 5'-CAACATTTATTTTGATTTTTTGG-3') (Simon et al. 1994) and C1-N-2568 (also known as BEN3R, 5'-GCWACWACRTAATAKGTATCATG-3') (Brady et al. 2000). The parameters for the cytochrome oxidase subunit 1 PCR were 95 C for 7 min, followed by 40 cycles of 95 C for 1 min, 45 C for 1 min, and 72 C for 1.5 min, final extension at 72 C for 5 min (Gullan et al. 2003). These PCR products were purified following the manufacturers standard protocol for the QIAquick PCR purification kit (Qiagen), and select samples were sent to the VBI for sequencing.

Assessment of potential factors: Hierarchical analysis. A hierarchical linear mixed model was used to determine effects of region, vineyard, and cultivar on disease incidence (Nita et al. 2008; Jones et al. 2015). The state of Virginia was broken down into the five main wine growing regions of North, South, East, West, and Central (Fig. 2.1). The model used the data that were collected at the lowest spatial hierarchy (= sampling site within a cultivar block) to identify higher-level effects in the survey. The model can be written as:

$$y=\mu+R+V+C+e \qquad \text{(Equation 1)}$$

where *y* is disease incidence (= detection of a virus) for a vine, *R* is the effect of region, *V* is the effect of vineyard within a region, *C* is the effect of cultivar within a vineyard, and *e* is a residual. The model was fitted with PROC MIXED of SAS (v. 9.4; SAS Institute, Cary, NC), using the restricted maximum likelihood method. All variables were considered random-effect factors in the model, except region, which was considered as a fixed-effect factor. The effect of region, *R*, was determined with an *F* test. The other random effects were evaluated with χ^2 tests based on differences of –2 times the log-likelihoods, which describes a degree of fit, for sequential fits of the model (Littell et al. 2006). For example, to determine the effect of the *C* term, equation 1 (as written) and a version of equation 1 without *C* term were fitted to the data, and then models were compared using log-likelihoods statistics for the models. The lower number of –2 times the log-likelihoods indicates better fit of the model to the data. If the difference of two statistics was greater than 3.84 (i.e., -2 times the log-likelihoods value of a model with the *C* term is at least 3.84 less than the one without it), which corresponding to the upper 95th percentile of the χ^2 distribution with 1 degree of freedom, it was determined that the model with the *C* term had significantly better fit with 95% confidence.

Effect of vine age, elevation, and mealybugs. Samples were classified into different vine age groups based on approximate planting year, which was collected from growers by the time of sampling. The classification we used were "Pre-1990", "1990s", and "post-2000". The effect of the age group on the number of virus-positive vines was examined using a generalized linear mixed model (PROC GLIMMIX, SAS ver. 9.4), where the age group was considered a fixed factor. In addition, a generalized linear mixed model was utilized to examine potential effects of elevation of sampling site. The elevation varied from 4m to 797m above sea level. The dataset was divided into three groups (each group containing the same number of samples) <137m, 137-

252m, and >252m, and virus incidence were examined using elevation as a fixed factor. Presence of visual symptoms and mealybugs were recorded at the time of sampling, and their effects on the number of virus-positive vines were examined using PROC FREQ of SAS for χ^2analysis

Association between viruses: In order to examine pairwise association of the significance of detection abilities of the old and new primers for detection of GLRaV-1, 2, 3, and 4, a matched pairs analysis was estimated using JMP Pro 11 (SAS institute, Cary, NC). In order to examine pairwise association of viruses, Kendall's Tau-b and Pearson's correlation was estimated using PROC CORR of SAS 9.4. Kendall's Tau-b is a nonparametric measure of association where paired observations' concordance (= paired observations vary together) and discordance (= paired observations vary differently) is calculated.

2.3 Results

Primer comparisons. Prior to data analysis, primer comparisons for GLRaV-1, -2, -3, and -4 were conducted. Using two primer sets per virus species, their efficacy for proper diagnosis of viruses was examined. A total of 721 samples from 2009-2014 were used in this primer comparison study. For GLRaV-1, there was no significant difference between primer sets HSP70-417/HSP70-737R (Osman et al. 2007) and dCP1-1/dCP1-2 (Esteves et al. 2013) as both produced the same results in terms of detecting the presence of virus. There were nine GLRaV-1-positive samples in our dataset, and both primer sets were successful at detection of these samples.

For GLRaV-2, the universal primer set of P19qtF4 and p24qtR (Beuve et al. 2007) was significantly better ($P < 0.03$) at detecting GLRaV-2 isolates than L2 F/U2 R (Bertazzon and

Angelini 2004). Thirteen samples with an originally undetectable GLRaV-2 infection were found to be positive with the updated primer set, and these results were confirmed via sequencing. For GLRaV-3, the universal primer set of GEN-11112F/GEN-11233R (Chooi et al. 2013) was significantly better ($P < 0.001$) at detecting positive samples when compared to primer set LC1 F/LC1 R (Osman and Rowhani 2006). A total of 41 samples that previously tested negative using the LC1 F/LC1 R primer set were found to be positive, and results were confirmed via sequencing.

There was no significant difference between GLRaV-4 primer sets HSPV F/HSPC R (Osman et al. 2007) and LRAmp-F/LRAmp-R (Ghanem-Sabanadzovic et al. 2012) in detecting GLRaV-4 as all six positive samples were detected using both primer sets. However, the LRAmp-F/LRAmp-R primer set additionally detected three GLRaV-4 strain-5s and three GLRaV-4 strain-9s while HSPV F/HSPC R could not; therefore, this primer set is a better choice in practice since all strains of GLRaV-4 can be detected with one primer set.

Extended Virus and Mealybug Survey. Between 2012 and 2014, a total of 265 additional samples were tested via RT-PCR and conventional PCR for all of the viruses listed in Table 2.1. Using the combined samples obtained through the 2009-11 (Jones et al. 2015) and 2012-2014 surveys, 456 samples from our survey were tested for GLRaVs (1, 2, 3, 4, 5, and 9), GfkV, GVA, GVB, and GRSPaV using primers listed in Table 2.1. Throughout the entire period (2009-14), a total of 116 vineyards in VA were visited and a total of 721 grapevine samples were obtained.

Results of the virus survey are in Table 2.2. A total of 49 different cultivars were sampled and tested, the fifteen most common cultivars and their numbers in our sample were Cabernet franc (98 total samples), Cabernet Sauvignon (97), Merlot (75), Chardonnay (65), Petit Verdot

(49), Viognier (31), Vidal blanc (25), Chambourcin (20), Malbec (20), Traminette (19), Chardonel (18), Riesling (17), Petit Manseng (15), Syrah (13), and Norton (12). Numbers of vineyards visited by regions of Virginia (Fig. 2.1) were: Northern (40), Southern (8), Eastern (22), Western (12), Central (34).

More than 75% of our samples were positive for at least one virus (Table 2.2). GRSPaV-1 was the most common virus detected (Table 2.2). GLRaV-3 and GRBaV were also very common while GLRaV-2, ToRSV, GVA, GVB, GLRaV-1, GLRaV-4, and GFkV were detected in smaller numbers (Table 2.2). GPgV and GVCV were not detected in any samples. Single infection of GRSPaV-1 was the most common, followed closely by single infections of GRBaV and GLRaV-3.

Many types of co-infection cases were found (samples containing more than one virus) (Table 2.3). A total of 316 out of 721 samples were positive for one virus, 159 were positive for two viruses, 58 were positive for three viruses, nine were positive for four viruses, and one was positive for six viruses, and lastly, one was positive for seven viruses (Table 2.3). Samples containing both GLRaV-3 and GRSPaV-1 were the most commonly found co-infection type followed by GRBaV mixed with GRSPaV-1 and GLRaV-2 mixed with GRSPaV-1 (Table 2.3). GLRaV-1, -4, -5, and -9 were always found with another virus(es) within the same sample. The highest number of viruses was found from an 18 year old Syrah, which contained seven different viruses (GLRaV-2, GLRaV-3, GLRaV-4, GLRaV-4 strain 5, GRSPaV-1, GVA, and GRBaV). When the data are grouped by the 103 sampled vineyards, 72 vineyards were positive for two or more viruses, 21 were positive for just one virus, and only 10 had no virus detected in samples from those vineyards.

When pair-wise association was examined among six commonly found grapevine viruses from this survey, results from both Kendall's Tau-b and Pearson's correlation were very similar (Table 2.4). Significant association was found between GLRaV-2 and -3, GLRaV-3 and GVA and GVB, GVA and GVB and RSPaV-1. Significant disassociation was found between GLRaV-3 and GRBaV and RSPaV-1 and GRBaV.

Assessment of potential factors: Hierarchical analysis. Due to the sample size, only GLRaV-2, -3, GRSPaV-1, GVA, GVB, and GRBaV were subjected to the hierarchical analysis (Table 2.4). Results from a linear mixed model indicated that incidence of all tested viruses were significantly affected ($P < 0.05$) by cultivar (C) effect (Table 2.5). On the other hand, regions (R) were not significantly different with any test viruses (Table 2.5), indicating that incidence of these viruses did not systematically vary across the state.

Effect of vine age, elevation, and mealybugs. Due to the sample size, only GLRaV-2, -3, GRSPaV-1, GVA, GVB, and GRBaV were subjected to assessment of the effect of vine age, elevation, and mealybugs. The effect of age of the vine on the prevalence of viruses was examined using three cutoff points: planted before 1990, in the 1990's, and in the 2000's (Table 2.6). Age was a significant factor for all six viruses tested. In all cases, older vines had significantly higher percentages of viruses with an exception of GRSPaV-1 where the youngest vines that were planted in 2000's had significantly higher percentage than vines planted before 1990 (Table 2.5).

The effects of elevation were also tested using three elevation classifications: <137m, 137-252m, and >252m. Significant effect of elevation was found with GLRaV-2, GRSPaV, GVA, and GRBaV (Table 2.7). In many cases, lower elevation resulted in lower number of

viruses, but not with GRBaV where the highest elevation resulted in the lowest percentage (Table 2.7).

When the effect of mealybugs present in a vineyard was tested against the probability of vines in the vineyard being infected with viruses, the result was significant ($P<0.05$) with GLRaV-3, GVA, and to our surprise, GRBaV (Table 2.8).

Species identification of viruses and mealybugs. Nucleotide identity of our virus-positive samples was confirmed by sequencing. Table 2.8 shows the number of virus isolates sequenced, and nucleotide identities were compared among the VA samples, as well as compared to reference sequences deposited in GenBank. Most viruses had relatively high identity matches among themselves, and with sequences in GenBank, with the exception of the Rugose wood complex viruses (GRSPaV-1, GVA, GVB) (Table 2.9).

A total of 100 mealybugs sampled between 2013 and 2015 were tested for species identification via the vineyard mealybug multiplex PCR. Species identification resulted in 67 grape mealybugs (*P. maritimus*), 31 Gill's mealybugs (*F. gilli*), and two obscure mealybugs (*Pseudococcus viburni*). Five vineyards visited and sampled for mealybugs had both grape and Gill's mealybugs present together on the same vine. Mealybugs sampled in vineyards were found along trunks, cordons, shoots, petioles, within clusters, and the undersides of leaves.

2.4 Discussion:

Our results from the primer comparison study were in line with previously reported primer results (Beuve et al. 2007; Chooi et al. 2013) for GLRaV-2 and -3. In this case the newer primer sets that account for multiple virus variants were significantly better than those previously used. On the other hand, newer primer sets tested for GLRaV-1 and -4 did not significantly

improve detection levels, in contrast to what was expected based on previous research (Ghanem-Sabanadzovic et al. 2012; Esteves et al. 2013); however our sample size for these two viruses were relatively small and don't allow a firm conclusion.

This current study added more accurate and broader information on grapevine virus infection in the state of Virginia compared to our previous survey study (Jones et al. 2015) with increased numbers of samples and viruses tested. We have shown the high percentage of grapevine virus infections in all areas of the state, especially for GLRaV-3, GRBaV, and GRSPaV. With 90% of vineyards positive for at least one virus infection, and 70% of vineyards positive for two or more virus infections, it is clear virus infections are widespread in VA and management strategies are needed to control potential spread.

The high incidence (25%) of GLRaV-3 was similar to many other regions across the US (Fuchs et al. 2009b; Martin et al. 2005; Mekuria et al. 2009a; Mekuria et al. 2009b; Milkus and Goodman 1999; Naidu et al. 2006) and the rate remains almost unchanged from our previous study (Jones et al. 2015). The association between the age of the vine and occurrence of GLRaV-3 positive vines suggests the positive effect of clean plant materials in recent years; prior to the 1990's, such materials were not available through the Clean Plant Network (Alley and Golino 2000; Martin et al. 2000). In addition, our data also suggests that the mealybug vector(s) are efficiently transmitting GLRaV-3 in Virginia vineyards.

The most commonly found virus in Virginia was GRSPaV; in contrast, Oregon and Washington (Martin et al. 2005) reported very low incidences of GRSPaV. Regardless, this high rate of GRSPaV is not surprising as this virus is not commonly regulated in many grapevine virus-free certification programs (Martin et al. 2000). Symptoms caused by GRSPaV develop slowly and symptoms may become more severe only when multiple viruses are found infecting

the same vine (Guidoni et al. 1997; Mannini et al. 1996). In the Finger Lakes region of the US, an average lifespan of a vineyard is estimated to be about 25 years before growers remove the planting and replant (Atallah et al. 2012). The limiting factor for the life of a wine grape vineyard is the low production by older vines (Wolf 2008). Given the high percentage of infestation, the importance of GRSPaV to long-term vineyard production might need to be re-evaluated.

The newly discovered DNA virus GRBaV (Al Rwahnih et al. 2013), also known as *Grapevine Cabernet franc-associated virus* (Krenz et al. 2012) and *Grapevine redleaf disease* (Poojari et al. 2013), is a growing concern among grapevine growers around North America. Currently GRBaV is found in California, Maryland, New York, New Jersey, Oregon, Pennsylvania, Virginia, and Washington (Krenz et al. 2014b). It has been found in historic grapevine specimens in California (Al Rwahnih et al. 2015), Canada, Switzerland (Krenz et al. 2014b), and most recently Korea (Lim et al. 2016). This virus was recently discovered from symptomatic grapevines that tested negative for GLRaVs (Al Rwahnih et al. 2013; Calvi 2011; Krenz et al. 2012).

The high incidence of GRBaV in Virginia is concerning because this disease can impact fruit quality parameters and has been speculated to be spread by a currently unknown vector (Al Rwahnih et al. 2013; Al Rwahnih et al. 2015; Al Rwahnih et al. 2015; Krenz et al. 2014b). The lack of difference in the incidence of GRBaV based on the vine age group suggests that infested planting material were available for a long time. With almost a quarter of the grapevines sampled in this study positive for GRBaV, results alone indicate cause for concern for the industry as a whole.

Our survey supports previous studies that show GLRaV-3 is the most common of the GLRaVs and that other viruses, such as GVA, GVB, and GFkV, are generally found less often and in co-infection instances (Fuchs et al. 2009b; Milkus and Goodman 1999; Naidu et al. 2014; Naidu et al. 2015b; Naidu et al. 2006). The finding that GVCV and GPgV are not present in any of our survey samples is a strong indication that these viruses have yet to enter the Virginia vineyards system.

The results from pairwise association showed that GLRaV-2 and -3, GLRaV-3 and GVA and GVB, GVA and GVB and RSPaV-1 were significantly associated with each other. An association between GLRaV-2 and GLRaV-3 seems counterintuitive, as GLRaV-2 has no known insect vector; however, 10 out of 11 cases of GLRaV-2 found in our survey were co-infections between GLRaV-2 and -3. Moreover, significantly higher incidence of GLRaV-2 was found with pre-1990's planting. This could be an example of co-infection at the time of planting (viruses found in planting stock) or older GLRaV-2-infected vines became infected with GLRaV-3 in the field.

Significant association shown between GLRaV-3 and GVA and GVB was most likely due to their vector(s). GVA, GVB and GLRaV-3 are transmitted by mealybug vectors (Tsai et al. 2010; Boscia et al. 1997). When mealybug populations were found within a vineyard, the probability of also finding two mealybug-transmissible viruses (GLRaV-3 and GVA) was significantly higher. In our previous work (Jones et al. 2015), GLRaV-3 was found to be associated with mealybug presence within the same vineyard. These two viruses, both able to be transmitted together and by multiple mealybug species (Almeida et al. 2013; Boscia et al. 1997; Tsai et al. 2010), make this result somewhat expected. Previous research has shown GVA is dependent on GLRaV-3 for its transmission by *Planococcus ficus* (Engelbrecht and Kasdorf

1987). However, a more recent study submitted contradicting evidence that GVA could be transmitted independently from GLRaV-3 (Tsai et al. 2010). Nevertheless, GVA and GLRaV-3 share a common vector, thus, a strong association between two is not surprising. GVA, GVB, and GRSPaV should also be associated with each other. They are all genetically related, and members of the rugose wood complex of viruses are typically spread by mealybug insect vectors (Boscia et al. 1997). In our case, we found GVA and GRSPaV and GVA and GVB were significantly associated with each other.

The association of GRBaV and its relationship to mealybugs was unexpected. Currently, no definitive insect vector has been identified for GRBaV; however, recent reports have surfaced that a tentative candidate vector has been found (Bahder et al. 2016) in the three-cornered alfalfa treehopper (*Spissistilus festinus*), which is a minor alfalfa pest in Virginia. Thus, the association of GRBaV to mealybugs might be due to the high prevalence of the virus observed and the then increased probability of finding mealybugs in a vineyard with GRBaV, not representative of a true relationship to mealybugs. The results from the pair-wise association showed that GRBaV and GLRaV-3 were negatively associated, or the pattern of variability for these two viruses were different from each other, despite both viruses found in close to 25% of our samples.

The hierarchical analysis yielded only a cultivar effect for GLRaV-2, -3, GRSPaV-1, GVA, GVB, and GRBaV. The results did not directly link the influence of cultivar; rather, it was probably the effect of the same source material within a vineyard. A planted cultivar could already have a high rate of virus infection or it could be located in a vineyard with other cultivars that were infected with virus(es) and vectors were present. We did not find any noticeable differences among main cultivars grown in Virginia (thus, the most sampled) such as Cabernet franc, Cabernet Sauvignon, Chardonnay, Merlot, Petit Verdot, Petit Manseng, Viognier, Vidal

blanc). The lack of regional effect suggested a minimal effect of the differences in the environmental conditions. Grape growing conditions vary considerably among grape growing regions in Virginia due to mountain ranges in the west and the Atlantic Ocean in the east.

When the effect of age of infected vines was examined, it was a significant factor for GLRaV-2, -3, GRSPaV-1, GVA, GVB, and GRBaV. This confirms results from our previous study where older grapevines tend to have a higher risk of infection (Jones et al. 2015). The lower incidence of viruses in younger vines compared to older vines can be partially attributed to the clean nursery stock programs lead by the National Clean Plant Network in the past two decades. Advanced detection techniques (ELISA, PCR, qPCR) were not available to the few clean plant programs that existed prior to 1988 (Alley and Golino 2000). Old programs relied heavily on using indicator vines for virus detection to help in removing contaminated stock (Alley and Golino 2000). Additionally, it was common for growers to share materials in the past. As detection techniques advance, and more high-throughput methods are developed, we should see a continuing trend towards cleaner and cleaner planting materials.

The effect of elevation on virus incidence was found in only four out of six viruses in this study: GLRaV-2, GRSPaV-1, GVA, and GRBaV. For GLRaV-2, GRSPaV-1, and GVA lower elevation sampling sites had lower numbers of infections from these viruses while GRBaV had its lowest percentage at high elevation sites. These results are interesting as no other plausible explanations can help explain the link between elevation and virus presence.

Mealybug identification further confirms (Jones et al. 2015) that two main species exist in Virginia vineyards: *Ferissia gilli* (Gill's mealybug) and *Pseudococcus maritimus* (grape mealybug). In addition to one previous case of the striped mealybug (*Ferrisia virgata*) (Jones et al. 2015), two mealybugs from the same vineyard were identified as the obscure mealybug

(*Pseudococcus viburni*). Both grape and obscure mealybugs have the ability to transmit GLRaV-3 and have a wide host range that includes grapevines, figs, apples, and citrus crops (Millar et al. 2002). The Striped mealybugs are not known to transmit the viruses associated with GLD, but transmission of GLRaV-3 has been accomplished in small trials with the Gill's mealybug (*Chapter 3*). Both Striped and Gill's mealybugs have been found in vineyards as well as both pistachio and almond orchards in California, and are thought to be native to the southeastern United States (Gullan et al. 2003; Haviland et al. 2006). These two mealybugs also have a wide host range including a variety of woody plants and have three generations per year in California (Gullan et al. 2003; Haviland et al. 2006).

Based on our survey, the vine mealybug was not found in Virginia. The vine mealybug is the major concern in CA due to its wide host range (grapes, figs, apples, citrus, and many tropical crops such as dates, bananas, mangos, and avocados), ability to have many generations per year, effectiveness of transmission of GLRaV-3, and capability to cause direct damages on the grapevine (Millar et al. 2002). With the high incidence of both viruses and mealybugs in the state, it is important for growers to use appropriate control measures, such as systemic materials applied foliarly (Jones and Nita 2016).

In summary, this is the first report of expanded virus survey findings of GLRaV-1, GLRaV-4, GLRaV-4 s5, GLRaV-4 s9, GRSPaV, GVA, GVB, ToRSV, GRBaV from the state of Virginia, U.S.A. Along with GLRaV-2, GLRaV-3, and GFkV, ten major virus species and four mealybug species have now been found in the state. Co-infections of multiple grapevine viruses within the same grapevine have been found to be a common occurrence, especially because of the high percentage of GRSPaV-infected vines. Our data also show that the introduction of GRBaV probably happened prior to 1990, and has been masked by other diseases until its recent

discovery. Clean plant programs, insect vector management, and consistent grapevine virus testing are highly suggested in order for vineyards managers in the state of Virginia to appropriately manage their vines.

Table 2.1: Primers used for conventional and RT-PCR detection of grapevine viruses.

Virus	Primer name and Reference	PCR parameters (temp in °C)
GLRaV-1	HSP70-417/HSP70-737R (F. Osman et al. 2007)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GLRaV-1 Universal	dCP1-1/dCP1-2 (Esteves et al. 2013)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GLRaV-2	L2 F/U2 R (Bertazzon and Angelini 2004)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GLRaV-2 Universal	P19qtF4 and p24qtR (Beuve et al. 2007)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GLRaV-3	LC1 F/LC1 R (F. Osman and Rowhani 2006)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GLRaV-3 Universal	GEN-11112F/GEN-11233R (Chooi et al. 2013)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GLRaV-4	HSPV F/HSPC R (F. Osman et al. 2007)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GLRaV-4 Universal (strains 4, 5, 9)	LRAmp-F/LRAmp-R (Abou Ghanem-Sabanadzovic et al. 2012)	52°, 1h; 94, 2min; 40x (94, 30s; 50, 35s; 72,45s); 72, 7min
GLRaV-5	HSPV F/HSPC R (F. Osman et al. 2007)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GLRaV-9	LR9 F/LR9 R (Alkowni et al. 2004)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GRSPaV-1	RSP13/RSP14 (B. Meng et al. 1999)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GFkV	GFkV-585 F/GFkV-1117 R (R. A. Naidu and Mekuria 2010)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GVA	H587/C995 (Minafra et al. 1997)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GVB	C410/H28 (Minafra and Hadidi 1994)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GVCV	GVCV-F1/GVCV-R1 (Y. Zhang et al. 2011)	94°, 1min; 34x (94, 30s; 52, 40s; 72, 1min); 72, 7min
ToRSV	ToRSV5/ToRSV6 (Li et al. 2011)	50°, 30min; 94, 2min; 30x (94, 45s; 60, 45s; 68, 2min); 68, 5min
GRBaV	GVGF1/GVGR1 (Al Rwahnih et al. 2013)	94°, 2min; 35x (94, 30s; 60, 30s; 72, 1min); 72, 5min
GPgV	GPgV5619f/GPgV6668r (Giampetruzzi et al. 2012)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min

Table 2.2: Grapevine virus survey samples by virus species tested and number found.

Virus	Total Samples Positive	Total Samples Tested	Percent Positive
GLRaV-1	9	721	1.30
GLRaV-2	65	721	9.01
GLRaV-3	182	721	25.24
GLRaV-4	6	721	0.83
GLRaV-5	3	721	0.42
GLRaV-9	3	721	0.42
GRSPaV-1	389	721	53.95
GVA	31	721	4.30
GVB	15	721	2.08
GFkV	6	721	0.83
ToRSV	8	574	1.39
GPgV	0	574	-
GVCV	0	574	-
GRBaV	140	574	24.39

Table 2.3: Counts of the most common types of single and co-infections of all grapevine viruses found in this study.

Virus Positive Infection Type	Total
GRSPaV-1	192
Non-infected Vines	177
GLRaV-3, GRSPaV-1	66
GRBaV	63
GRBaV, GRSPaV-1	47
GLRaV-3	45
GLRaV-2, GRSPaV-1	16
GLRaV-3, GRSPaV-1, GVA	13
GLRaV-2	11
GLRaV-2, GLRaV-3, GRSPaV-1	11
GLRaV-2, GLRaV-3	10
GLRaV-3, GRSPaV-1, GRBaV	8
GLRaV-2, GRSPaV-1, GRBaV	5
GLRaV-3, GRBaV	4
GLRaV-3, GRSPaV-1, GVA, GVB	4
GLRaV-3, GVA, GVB	3
GLRaV-4, GLRaV-4 strain 9, GRSPaV-1	3
GVA, GRSPaV-1	3
GLRaV-1, GRBaV	2
GLRaV-1, GRSPaV-1	2
GLRaV-2, GRBaV	2
GLRaV-2, GRSPaV-1, GFkV	2
GLRaV-3, GFkV	2
GLRaV-3, GVB	2
GLRaV-3, GVB, GRBaV	2
GVB	2
RSPaV-1, ToRSV	2
GFkV	1
GLRaV-1, GLRaV-3	1
GLRaV-1, GLRaV-3, GRSPaV-1	1
GLRaV-1, GLRaV-3, GRSPaV-1, GVA	1
GLRaV-1, GRSPaV-1, GRBaV	1
GLRaV-1, GRSPaV-1, GVA	1
GLRaV-2, GLRaV-3, GLRaV-4, GLRaV-4 strain 5, RSPaV-1, GVA, GRBaV	1
GLRaV-2, GLRaV-3, GRBaV	1
GLRaV-2, GLRaV-3, GRSPaV-1, GFkV	1
GLRaV-2, GLRaV-3, GRSPaV-1, GRBaV	1
GLRaV-2, GLRaV-3, GRSPaV-1, GVB	1

GLRaV-2, GLRaV-3, GRSPaV-1, ToRSV	1
GLRaV-2, GLRaV-3, ToRSV	1
GLRaV-2, GRSPaV-1, ToRSV	1
GLRaV-3, GLRaV-4, GLRaV-4 strain 5, RSPaV-1, GVA, GRBaV	1
GLRaV-3, GRSPaV-1, GVB	1
GLRaV-4, GLRaV-5, GVB	1
GVA	1
GRSPaV-1, GVA, GRBaV	1
GRSPaV-1, GVA, ToRSV	1
GRSPaV-1, ToRSV, GRBaV	1
ToRSV	1
GLRaV-1	0
GLRaV-4	0
GLRaV-5	0
GLRaV-9	0

Table 2.4: Kendall's tau (upper right triangle) and number of pairs of co-infection (lower left triangle) values and *P*-values among six commonly found grapevine viruses, Virginia 2009-2014.

	GLRaV2[a]	GLRaV3[a]	GVA[a]	GVB[a]	RSPaV1[a]	GRBV[a]
GLRaV2		0.13**	-0.04	-0.01	-0.04	-0.01
		<0.001	0.25	0.75	0.34	0.79
GLRaV3	28		0.24**	0.21**	0.02	-0.14**
			<0.001	<0.001	0.57	<0.001
GVA	1	23		0.30**	0.13**	-0.06
				<0.001	<0.001	0.17
GVB	1	13	7		-0.04	0.00
					0.27	0.97
RSPaV1	40	111	26	6		-0.09*
						0.03
GRBV	10	18	3	2	66	

[a] The number on the top is either Kendall's Tau-b or Pearson's correlation coefficient, the number on the bottom is a *P*-value. The number with one and two asterisk(s) indicate significant association (or disassociation when the number is negative) with 95% and 99% confidence level, respectively.

Table 2.5: Differences in test statistics and estimated variances for the effect of region (*R*), vineyard within the region (*V*), and cultivar within the farm (*C*) on virus detection in the Virginia grapevine virus survey sample 2009-2014.

	Region (*R*)		Vineyard (*V*)		Cultivar (*C*)	
Virus	*F* statistics	*P*-value	Difference[a]	variance	Difference[a]	variance
GLRaV-2	0.67	0.67	-10.5	0.03	18.4*	0.03
GLRaV-3	0.88	0.51	6.7*	2.78	148.7*	2.78
GRSPaV-1	0.78	0.59	7.1*	6.2	176.7*	6.2
GVA	0.73	0.62	-10.1	0.03	45.0*	0.03
GVB	0.82	0.56	-17.9	< 0.01	4.3*	< 0.01
GRBaV	1.53	0.18	3.1	0.09	5.3*	0.14

[a] A difference in the two log likelihood ratio statistics between two models, where a number larger than 3.84 (indicated with an asterisk) is significantly better fit ($P \leq 0.05$) based on a χ^2 test.

Table 2.6: Effect of vine age (planting time period) on the percentage of viruses found in the Virginia grapevine virus survey sample 2009-2014.

Age[a]	*GLRaV-2*[b]		*GLRaV-3*[b]		*GRSPaV*[b]		*GVA*[b]		*GVB*[b]		*GRBaV*[b]	
Pre-1990	32.7% (0.04)	A	71.4% (0.06)	A	40.8% (0.07	B	12.3% (0.03)	A	8.2% (0.02)	A	42.9% (0.09)	A
1990's	9.5% (0.03)	B	43.1% (0.04)	B	52.6% (0.05)	AB	11.2% (0.02)	A	3.3% (0.01)	AB	19.4% (0.05)	B
2000's	6.8% (0.01)	B	17.5% (0.02)	C	55.4% (0.02)	A	2.2% (0.01)	B	1.3% (0.01)	B	24.3% (0.02)	AB

[a] Age of vine samples were grouped according to the planting time period. A total of 49, 116, and 556 samples were from the pre-1990, 1990s, and 2000s time periods, respectively.

[b] Least square mean of percentage of virus infected vines, the same letter after the number indicates that the numbers were not significantly different ($P \leq 0.05$) based on the Fisher's LSD (GLIMMIX, SAS 9.4) Standard error in parentheses below.

Table 2.7: Effect of elevation on the percentage of viruses found in the Virginia grapevine virus survey sample 2009-2014.

Elevation [a]	*GLRaV-2* [b]		*GLRaV-3* [b]		*GRSPaV* [b]		*GVA* [b]		*GVB* [b]		*GRBaV* [b]	
< 137m	10.4% (0.02)	A	15.4% (0.03)	C	46.1% (0.03)	B	1.3% (0.01)	B	1.7% (0.01)	A	27.7% (0.03)	A
137m-252m	9.4% (0.07)	A	34.6% (0.03)	A	58.3% (0.03)	A	10.8% (0.02)	A	3.3% (0.01)	A	26.7% (0.03)	AB
> 252m	6.8% (0.10)	A	25.8% (0.03)	B	57.5% (0.03)	A	0.8% (0.01)	B	1.3% (0.01)	A	18.7% (0.03)	B

[a] Elevation in meters are shown in three groups of the same sample size: <137m, 137m-252m, and > 252m included 241, 240, and 240 samples, respectively.

[b] Least square mean of percentage of virus infected vines, the same letter after the number indicates that the numbers were not significantly different ($P \leq 0.05$) based on the Fisher's LSD (GLIMMIX, SAS 9.4) Standard error in parentheses below.

Table 2.8: Probability of detecting viruses based on presence of mealybugs in the same vineyard.

Virus	**χ^2 value**[a]	***P*-value**[b]
GLRaV-2	0.97	0.32
GLRaV-3	73.19	< 0.001**
GRSPaV-1	1.63	0.20
GVA	4.53	0.03**
GVB	1.24	0.27
GRBaV	4.53	0.03**

[a] Pearson's χ^2 value from a logistic regression (JMP Pro 11)

[b] P-values to indicate significantly high probability of detecting each virus based on the presence of mealybugs in the same vineyard. One and two asterisks indicate 95% and 99% confidence, respectively.

Table 2.9: Number of Virginia isolates selected and used in sequence comparisons between other Virginia isolates as well as between publicly available GenBank isolates of the same virus species. Percent nucleotide identities are shown in parentheses beside the number of isolates used for the comparison.

Virus	**Among VA isolates**		**With GenBank isolates**	
GLRaV-1	4	(96-99%)	12	(90-99%)
GLRaV-4	3	(98-99%)	10	(77-99%)
GLRaV-5	3	(98-99%)	18	(80-100%)
GLRaV-9	1	n/a	6	(77-96%)
GRSPaV-1	7	(89-100%)	49	(87-100%)
GVA	13	(83-99%)	50	(81-99%)
GVB	5	(87-99%)	35	(81-99%)
GRBaV	44	(93-99%)	29	(92-99%)

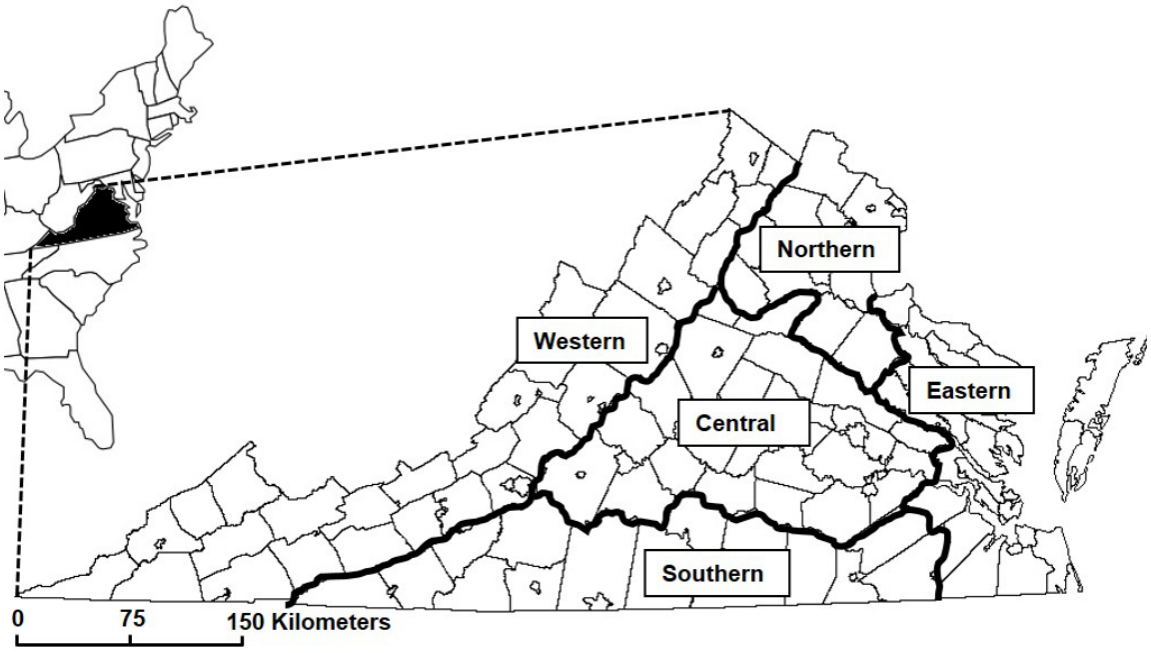


Figure 2.1: The state of Virginia, U.S. and its five "wine regions" divided on the map. The majority of vineyards and wineries can be found in the Northern and Central areas.

2.5 References

Agrios, G. N. (2005). *Plant Pathology*: Elsevier Academic Press.

Al Rwahnih, M., Dave, A., Anderson, M. M., Rowhani, A., Uyemoto, J. K., & Sudarshana, M. R. (2013). Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology, 103*(10), 1069-1076, doi:10.1094/PHYTO-10-12-0253-R.

Al Rwahnih, M., Rowhani, A., & Golino, D. A. (2015). First report of *Grapevine red blotch associated virus* in archival grapevine material from Sonoma County, California. *Plant Disease*, 150127132800003, doi:10.1094/PDIS-12-14-1252-PDN.

Al Rwahnih, M., Rowhani, A., Golino, D. A., Islas, C. M., Preece, J. E., & Sudarshana, M. R. (2015). Detection and genetic diversity of *grapevine red blotch-associated virus* isolates in table grape accessions in the National Clonal Germplasm Repository in California. *Canadian Journal of Plant Pathology, 37*(1), 130-135, doi:10.1080/07060661.2014.999705.

Alabi, O. J., Al Rwahnih, M., Karthikeyan, G., Poojari, S., Fuchs, M., Rowhani, A., Naidu, R.A. (2011). *Grapevine leafroll-associated virus 1* Occurs as Genetically Diverse Populations. *Phytopathology, 101*(12), 1446-1456, doi:10.1094/phyto-04-11-0114.

Alkowni, R., Rowhani, A., Daubert, S., & Golino, D. (2004). Partial characterization of a new ampelovirus associated with grapevine leafroll disease. *Journal of Plant Pathology*, 123-133.

Alley, L., & Golino, D. The origins of the grape program at foundation plant materials service. In *Proceedings of the ASEV 50th Anniversary Meeting, Seattle, WA, 2000* (pp. 222-230): American Society for Enology and Viticulture

Almeida, R. P. P., Daane, K. M., Bell, V. A., Blaisdell, G. K., Cooper, M. L., Herrbach, E., et al. (2013). Ecology and management of grapevine leafroll disease. *Frontiers in Microbiology, 4*, 13, doi:10.3389/fmicb.2013.00094.

Atallah, S. S., Gomez, M. I., Fuchs, M. F., & Martinson, T. E. (2012). Economic Impact of Grapevine Leafroll Disease on Vitis vinifera cv. Cabernet franc in Finger Lakes Vineyards of New York. *American Journal of Enology and Viticulture, 63*(1), 73-79, doi:10.5344/ajev.2011.11055.

Bertazzon, N., & Angelini, E. (2004). Advances in the detection of *Grapevine leafroll-associated virus 2* variants. *Journal of Plant Pathology, 86*(4, Special issue), 283-290.

Beuve, M., Candresse, T., Tannieres, M., & Lemaire, O. (2015). First Report of *Grapevine Pinot gris virus* (GPGV) in grapevine in France. *Plant Disease, 99*(2), 293-294, doi:10.1094/PDIS-10-14-1008-PDN.

Beuve, M., Sempe, L., & Lemaire, O. (2007). A sensitive one-step real-time RT-PCR method for detecting *Grapevine leafroll-associated virus 2* variants in grapevine. *Journal of Virological Methods, 141*(2), 117-124, doi:10.1016/j.jviromet.2006.11.042.

Boscia, D., Minafra, A., & Martelli, G. P. (1997). Filamentous viruses of grapevine: Putative trichoviruses and capilloviruses. In P. L. Monette (Ed.), *Filamentous viruses of woody plants*. (pp. 19-28): Trivandrum: Research Signpost.

Brady, S. G., Gadau, J., & Ward, P. S. (2000). Systematics of the ant genus *Camponotus* (Hymenoptera: Formicidae): a preliminary analysis using data from the mitochondrial gene cytochrome oxidase I. In A. D. Austin, & M. Dowton (Eds.), *Hymenoptera: evolution, biodiversity and biological control. Fourth International Hymenoptera*

Conference, held in Canberra, Australia, in January 1999. (pp. 131-139). Collingwood: CSIRO Publishing.

Braun, A., Palmiter, D., & Keplinger, J. (1966). Nematodes found in apple orchards in the Hudson Valley, 1956-1965. *Plant Dis. Reptr, 50*(10), 783-786.

Calvi, B. L. (2011). *Effects Of Red-leaf Disease On Cabernet Sauvignon At The Oakville Experimental Vineyard And Mitigation By Harvest Delay And Crop Adjustment*. ProQuest Dissertations Publishing,

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). *59*, 330-337.

Chooi, K. M., Cohen, D., & Pearson, M. N. (2013). Generic and sequence-variant specific molecular assays for the detection of the highly variable *Grapevine leafroll-associated virus 3*. *Journal of Virological Methods, 189*(1), 20-29, doi:10.1016/j.jviromet.2012.12.018.

Daane, K. M., Middleton, M. C., Sforza, R., Cooper, M. L., Walton, V. M., Walsh, D. B., et al. (2011). Development of a multiplex PCR for identification of vineyard mealybugs. *Environmental Entomology, 40*(6), 1595-1603, doi:10.1603/en11075.

Engelbrecht, D., & Kasdorf, G. (1987). Occurrence and transmission of *grapevine virus A* in South African grapevines. *South African Journal for Enology and Viticulture, 8*(1), 23-29.

Esteves, F., Teixeira Santos, M., Eiras-Dias, J. E., & Fonseca, F. (2013). Molecular data mining to improve antibody-based detection of *Grapevine leafroll-associated virus 1* (GLRaV-1). *Journal of Virological Methods, 194*(1-2), 258-270, doi:10.1016/j.jviromet.2013.09.004.

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, doi:10.1094/pdis-93-4-0395.

Ghanem-Sabanadzovic, N., Sabanadzovic, S., Gugerli, P., & Rowhani, A. (2012). Genome organization, serology and phylogeny of *Grapevine leafroll-associated viruses 4 and 6*: Taxonomic implications. *Virus Research, 163*(1), 120-128, doi:10.1016/j.virusres.2011.09.001.

Giampetruzzi, A., Roumi, V., Roberto, R., Malossini, U., Yoshikawa, N., La Notte, P., et al. (2012). A new grapevine virus discovered by deep sequencing of virus- and viroid-derived small RNAs in Cv Pinot gris. *Virus Research, 163*(1), 262-268, doi:10.1016/j.virusres.2011.10.010.

Glasa, M., Predajňa, L., Komínek, P., Nagyová, A., Candresse, T., & Olmos, A. (2014). Molecular characterization of divergent *grapevine Pinot gris virus* isolates and their detection in Slovak and Czech grapevines. *Archives of Virology, 159*(8), 2103-2107, doi:10.1007/s00705-014-2031-5.

Guidoni, S., Mannini, F., Ferrandino, A., Argamante, N., & Di Stefano, R. (1997). The effect of grapevine leafroll and rugose wood sanitation on agronomic performance and berry and leaf phenolic content of a Nebbiolo clone (Vitis vinifera L.). *American Journal of Enology and Viticulture, 48*(4), 438-442.

Gullan, P. J., Downie, D. A., & Steffan, S. A. (2003). A new pest species of the mealybug genus *Ferrisia fullaway* (Hemiptera: Pseudococcidae) from the United States. *Annals of the*

Entomological Society of America, 96(6), 723-737, doi:10.1603/0013-8746(2003)096[0723:anpsot]2.0.co;2.

Guo, Q., Honesty, S., Xu, M. L., Zhang, Y., Schoelz, J., & Qiu, W. P. (2014). Genetic diversity and tissue and host specificity of *Grapevine vein clearing virus*. *Phytopathology, 104*(5), 539-547, doi:10.1094/PHYTO-03-13-0075-R.

Haviland, D. R., Beede, R., Godfrey, K. E., & Daane, K. M. (2006). *Ferrisia gilli*: A new mealybug pest of pistachios and other deciduous crops. http://anrcatalog.ucdavis.edu/pdf/8207.pdf. Accessed November 2012.

Jones, T. J., & Nita, M. (2016). Spatio-temporal association of GLRaV-3-infected grapevines, and effect of insecticidal treatments on mealybug populations in Virginia vineyards. *European Journal of Plant Pathology*, 1-16, doi:10.1007/s10658-016-0878-x.

Jones, T. J., Rayapati, N., & Nita, M. (2015). Occurrence of *Grapevine leafroll associated virus-2, –3* and *Grapevine fleck virus* in Virginia, U.S.A., and factors affecting virus infected vines. *European Journal of Plant Pathology*, 1-14, doi:10.1007/s10658-015-0605-z.

Krenz, B., Thompson, J. R., Fuchs, M., & Perry, K. L. (2012). Complete genome sequence of a new circular DNA virus from grapevine. *Journal of Virology, 86*(14), 7715-7715.

Krenz, B., Thompson, J. R., McLane, H. L., Fuchs, M., & Perry, K. L. (2014). *Grapevine red blotch-associated virus* Is Widespread in the United States. *Phytopathology, 104*(11), 1232-1240, doi:10.1094/phyto-02-14-0053-r.

Li, R., Mock, R., Fuchs, M., Halbrendt, J., Howell, B., & Liu, Z. (2011). Characterization of the partial RNA1 and RNA2 3′ untranslated region of *Tomato ringspot virus* isolates from North America. *Canadian Journal of Plant Pathology, 33*(1), 94-99.

Littell, R. C., Milliken, G. A., Stroup, W. W., Wolffinger, R. D., & Schabenberger, O. (2006). *SAS for Mixed Models, Second Edition*. Cary, NC: SAS Institute, Inc.

Lunden, S., Meng, B., Avery, J., & Qiu, W. (2010). Association of Grapevine fanleaf virus, *Tomato ringspot virus* and *Grapevine rupestris stem pitting-associated virus* with a grapevine vein-clearing complex on var. Chardonnay. *European Journal of Plant Pathology, 126*(2), 135-144.

Malossini, U., Moscon, R., Ferrazza, M., Bianchedi, P., Varner, M., & Credi, R. Caratteristiche vegeto-produttive di viti Pinot grigio e Traminer aromatico affette da una nuova virosi segnalata in Trentino. In *IV Convegno Nazionale di Viticoltura (Conavi), 2012* (pp. 37 (032))

Mannini, F., Argamante, N., & Credi, R. (1996). Improvements in the quality of grapevine 'Nebbiolo' clones obtained by sanitation. In S. Poni, E. Peterlunger, F. Iacono, & C. Intrieri (Eds.), *First Ishs Workshop on Strategies to Optimize Wine Grape Quality* (pp. 319-324, Acta Horticulturae, Vol. 427). Leuven 1: International Society Horticultural Science.

Martelli, G. P. (2014). Directory of virus and virus-like diseases of the grapevine and their agents. *Journal of Plant Pathology, 96*(1), 1-136.

Martelli, G. P., Abou Ghanem-Sabanadzovic, N., Agranovsky, A. A., Al Rwahnih, M., Dolja, V. V., Dovas, C. I., et al. (2012). Taxonomic revision of the family Closteroviridae with special reference to the grapevine leafroll-associated members of the genus Ampelovirus and the putative species unassigned to the family. *Journal of Plant Pathology, 94*(1), 7-19.

Martelli, G. P., & Boudon-Padieu, E. (2006). *Directory of infectious diseases of grapevines*. (Vol. 55): International Centre for Advanced Mediterranean Agronomic Studies. Options Méditerranéennes, Ser. B, Studies and Research. 55: 59-75.

Martelli, G. P., Viruses, I. C. f. t. S. o., Grapevine, V. D. o. t., Food, & Nations, A. O. o. t. U. (1993). *Graft-transmissible Diseases of Grapevines: Handbook for Detection and Diagnosis*: Food and Agriculture Organization of the United Nations.

Martin, R. R., Eastwell, K. C., Wagner, A., Lamprecht, S., & Tzanetakis, I. E. (2005). Survey for viruses of grapevine in Oregon and Washington. *Plant Disease, 89*(7), 763-766, doi:10.1094/pd-89-0763.

Martin, R. R., James, D., & Lévesque, C. A. (2000). Impacts of molecular diagnostic technologies on plant disease management. *Annual Review of Phytopathology, 38*(1), 207-239.

Mekuria, Karasev, A. V., Martin, R. R., & Naidu, R. A. (2009a). First report of *Grapevine leafroll-associated virus-3* in six wine grape cultivars in Idaho. *Plant Disease, 93*(11), 1218, doi:10.1094/pdis-93-11-1218c.

Mekuria, Soule, M. J., Jarugula, S., & Naidu, R. A. (2009b). Current status of grapevine viruses in Washington State vineyards. *Phytopathology, 99*, S83.

Meng, B., Zhu, H. Y., & Gonsalves, D. (1999). *Rupestris stem pitting associated virus-1* consists of a family of sequence variants. *Archives of Virology, 144*(11), 2071-2085, doi:10.1007/s007050050623.

Milkus, B. N., & Goodman, R. N. (1999). A survey of Missouri vineyards for the presence of five grape viruses. *American Journal of Enology and Viticulture, 50*(1), 133-134.

Millar, J. G., Daane, K. M., McElfresh, J. S., Moreira, J. A., Malakar-Kuenen, R., Guillen, M., et al. (2002). Development and optimization of methods for using sex pheromone for monitoring the mealybug *Planococcus ficus* (Homoptera : Pseudococcidae) in California vineyards. *Journal of Economic Entomology, 95*(4), 706-714, doi:10.1603/0022-0493-95.4.706.

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, doi:10.1016/0166-0934(94)90076-0.

Minafra, A., Saldarelli, P., & Martelli, G. P. (1997). *Grapevine virus A*: nucleotide sequence, genome organization, and relationship in the Trichovirus genus. *Archives of Virology, 142*(2), 417-423, doi:10.1007/s007050050088.

Morelli, M., Catarino, A. D. M., Susca, L., Saldarelli, P., Gualandri, V., & Martelli, G. P. (2014). First report of *Grapevine Pinot gris Virus* from table grapes in Southern Italy. *Journal of Plant Pathology, 96*(2), 439-439.

Naidu, R., Rowhani, A., Fuchs, M., Golino, D., & Martelli, G. P. (2014). Grapevine Leafroll: A Complex Viral Disease Affecting a High-Value Fruit Crop. *Plant Disease, 98*(9), 1172-1185, doi:10.1094/PDIS-08-13-0880-FE.

Naidu, R. A., Maree, H. J., & Burger, J. T. (2015b). Grapevine Leafroll Disease and Associated Viruses: A Unique Pathosystem. *Annual Review of Phytopathology, 53*, 613-634, doi:10.1146/annurev-phyto-102313-045946.

Naidu, R. A., & Mekuria, T. A. (2010). First report of *Grapevine fleck virus* from Washington vineyards. *Plant Disease, 94*(6), 784, doi:10.1094/pdis-94-6-0784a.

Naidu, R. A., Soule, M. J., & Jarugula, S. (2006). Single and mixed infections of *Grapevine leafroll-associated viruses* in Washington State vineyards. *Phytopathology, 96*, S83.

Nita, M., Ellis, M. A., & Madden, L. V. (2008). Variation in disease incidence of Phomopsis cane and leaf spot of grape in commercial vineyards in Ohio. *Plant Disease, 92*(7), 1053-1061, doi:10.1094/pdis-92-7-1053.

Osman, F., Leutenegger, C., Golino, D., & Rowhani, A. (2007). Real-time RT-PCR (TaqMan®) assays for the detection of *Grapevine Leafroll associated viruses 1-5* and *9*. *Journal of Virological Methods, 141*(1), 22-29, doi:10.1016/j.jviromet.2006.11.035.

Osman, F., & Rowhani, A. (2006). Application of a spotting sample preparation technique for the detection of pathogens in woody plants by RT-PCR and real-time PCR (TaqMan). *Journal of Virological Methods, 133*(2), 130-136, doi:10.1016/j.jviromet.2005.11.005.

Pearson, R. C., & Goheen, A. C. (1988). *Compendium of grape diseases*: APS Press.

Plesko, I. M., Marn, M. V., & Zezlina, I. (2014). First Report of *Grapevine Pinot gris virus* Infecting Grapevine in Slovenia. *Plant Disease, 98*(7), 1014-1015, doi:10.1094/PDIS-11-13-1137-PDN.

Poojari, S., Alabi, O. J., Fofanov, V. Y., & Naidu, R. A. (2013). A leafhopper-transmissible DNA virus with novel evolutionary lineage in the family Geminiviridae implicated in grapevine redleaf disease by next-generation sequencing. *PLoS ONE, 8*(6), e64194.

Powell, C. A., Longenecker, J. L., & Forer, L. B. (1990). Incidence of *Tomato Ringspot Virus* and *Tobacco Ringspot Virus* in grapevines in Pennsylvania. *Plant Disease, 74*(9), 702-704, doi:10.1094/pd-74-0702.

Qiu, W., Avery, J., & Lunden, S. (2007). Characterization of a severe virus-like disease in Chardonnay grapevines in Missouri. *Plant Health Progress*.

Rayapati, N. (2012). Virus Diseases. http://wine.wsu.edu/research-extension/plant-health/virology/virus-diseases/. Accessed November 2012.

Rosa, C., Jimenez, J. F., Margaria, P., & Rowhani, A. (2011). Symptomatology and Effects of Viruses Associated with Rugose Wood Complex on the Growth of Four Different Rootstocks. *American Journal of Enology and Viticulture, 62*(2), 207-213, doi:10.5344/ajev.2011.10104.

Rosenberger, D., Harrison, M., & Gonsalves, D. (1983). Incidence of apple union necrosis and decline, *tomato ringspot virus*, and *Xiphinema* vector species in Hudson Valley orchards. *Plant Disease, 67*(4), 356-360.

Rosenberger, D., & Meyer, F. (1988). Control of dagger and lesion nematodes in apple and plum orchards with fenamiphos, carbofuran, and carbosulfan. *Plant Disease, 72*(6), 519-522.

Rowhani, A., Biardi, L., Johnson, R., Saldarelli, P., Zhang, Y. P., Chin, J., et al. Simplified sample preparation method and one-tube RT-PCR for grapevine viruses. In *Proceedings of XIII International Council for the Study of Viruses and Virus-Like Diseases of the Grapevine, Adelaide, 2000* (pp. 82)

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4*(4), 406-425.

Saldarelli, P., Giampetruzzi, A., Morelli, M., Malossini, U., Pirolo, C., Bianchedi, P., et al. (2015). Genetic Variability of *Grapevine Pinot gris virus* and Its Association with Grapevine Leaf Mottling and Deformation. *Phytopathology, 105*(4), 555-563, doi:10.1094/PHYTO-09-14-0241-R.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., & Flook, P. (1994). Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America, 87*(6), 651-701.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution, 28*(10), 2731-2739, doi:10.1093/molbev/msr121.

Thompson, J. R., Fuchs, M., & Perry, K. L. (2012). Genomic analysis of *Grapevine leafroll associated virus-5* and related viruses. *Virus Research, 163*(1), 19-27, doi:10.1016/j.virusres.2011.08.006.

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, doi:10.1094/phyto-100-8-0830.

Tuskan, R. G., & Tolin, S. A. (1984). Properties of grape and soybean isolates of Tomato Ringspot Virus. *Phytopathology, 74*(7), 808-808.

VWB (2012). The Economic Impact of Wine and Wine Grapes on the State of Virginia--2010. St. Helena, CA: A Frank, Rimerman + Co. LLP.

VWB (2016). Virginia 2015 Commercial Grape Report. Virginia Wine Marketing Office.

Wolf, T. K. (2008). *Wine grape production guide for eastern North America*. (NRAES-145). Ithaca, NY.: PALS Publishing (formerly NRAES).

Wolpert, J. A., & Vilas, E. P. (1992). Effect of mild leafroll disease on growth, yield, and fruit maturity indices of Riesling and Zinfandel. *American Journal of Enology and Viticulture, 43*(4), 367-369.

Zhang, Y., Singh, K., Kaur, R., & Qiu, W. P. (2011). Association of a novel DNA virus with the grapevine vein-clearing and vine decline syndrome. *Phytopathology, 101*(9), 1081-1090, doi:10.1094/PHYTO-02-11-0034.

Chapter 3

Grapevine red blotch associated virus and development of a real-time PCR assay.

3.1 Introduction

Among perennial crops worldwide, grapevines are affected by the greatest number of virus and virus-like agents (around 70) (Martelli 2014; Naidu et al. 2015a). Among these, only a few viruses are considered to be economically important pathogens (Martelli and Boudon-Padieu 2006; Rayapati 2012). Examples include the grapevine leafroll complex, rugose wood complex, degeneration/decline disease complex, and fleck disease complex (Martelli and Boudon-Padieu 2006). In addition to these, the recently discovered DNA virus *Grapevine red blotch-associated virus* (GRBaV) (Al Rwahnih et al. 2013), also known as *Grapevine Cabernet franc-associated virus* (Krenz et al. 2012), became a growing concern among grapevine growers in the United States. Its recent discovery in historic grapevine specimens in California (Al Rwahnih et al. 2015) suggests that this virus has had early routes in the wine industry yet has gone unnoticed until recently.

In 2011, several grapevines showing symptoms very similar to that of grapevine leafroll disease (GLD) continually tested negative for grapevine leafroll-associated viruses (GLRaVs) (Al Rwahnih et al. 2013; Calvi 2011; Krenz et al. 2012). Deep sequencing technology revealed the presence of a new virus of approximately 3.2kb in size (Krenz et al. 2012). GRBaV is a

single, circular ssDNA *Geminivirus* containing six open reading frames with bi-directional orientation (Fig. 3.1B) (Al Rwahnih et al. 2013; Al Rwahnih et al. 2015; Krenz et al. 2012; Sudarshana et al. 2015).

As noted, symptoms on GRBaV-infected vines are very similar to those of GLD. Red-fruited cultivars show red blotching of the leaves and leaf margins. Secondary and tertiary veins may also show reddening in some cultivars. White-fruited cultivars experience irregular chlorotic areas of the leaf blade (Sudarshana et al. 2015) (Fig. 3.1A). Sugar reduction in infected vines is approximately 2.4° (Calvi 2011) up to 5° Brix (Habili 2013).

The overall epidemiology of this virus is unknown, but a vector is thought to exist due to spatial-temporal increases of infected vines, consistent with short-distance spread compounded by patterns of aggregation (Sudarshana et al. 2015). In California, management options of infected vineyards have been limited to 1) removal of vineyards that are 4-10 years old with greater than 50% GRBaV incidence and experiencing a loss of revenue and fruit quality; or 2) improving the nutritional status of infected grapevines through nutrient application, fruit load adjustments, and delayed harvesting (Calvi 2011; Sudarshana et al. 2015). GRBaV is widespread in the U.S. and has been identified recently in California, New York, Virginia, Maryland, Pennsylvania, Texas, and Washington (Krenz et al. 2014b). Our recent survey efforts (Chapter 2) revealed that GRBaV is wide spread among Virginia vineyards. More than 24% of 574 total samples obtained were positive for GRBaV. When translated into number of vineyards surveyed in the state of Virginia, more than 46% of vineyards were affected. With this high rate of incidence for GRBaV in the state, a need to delve deeper into the GRBaV dataset and pursue better testing options arose.

Symptoms of virus diseases are often not the best indicator of infection (Agrios 2005; Jones et al. 2015); therefore molecular-based detection methods have been widely adapted (Jones et al. 2015; Martelli 2014; Osman et al. 2007). However, given the initial difficulty of finding GRBaV, a qPCR, which has been shown to be more sensitive than conventional methods of PCR for grapevine virus testing, such as the RT-PCR used in our previous study (Jones et al. 2015), could be a better candidate for GRBaV detection. For example, Al-Rwahnih et al. demonstrated that their conventional method of PCR for GLRaV-7 was inadequate, and developed a new qPCR method (Al Rwahnih et al. 2012b). The advantages of the qPCR procedures are higher sensitivity (Oberhansli et al. 2011) and capability to quantify the target DNA.

Therefore, in this study, an efficient qPCR method for GRBaV was compared with conventional PCR, which currently is the only available routine test (Al Rwahnih et al. 2013; Krenz et al. 2014b).

3.2 Materials and Methods

Virus samples. Vineyards were selected arbitrarily from across the major grape growing regions of Virginia (VA). Samples were collected throughout the 2011-2014. At each vineyard, multiple cultivars were randomly selected and sampled. Each sample contained 10 total petioles taken arbitrarily across the canopy from single symptomatic vines. This sampling method was used due to uneven virus distribution of grapevine viruses in grapevines (Charles et al. 2006). Sampled petioles were placed into a Ziploc bag, immediately stored in an iced cooler box, and taken back to the laboratory. Samples are then immediately processed into crude extract, or stored at -20°C

for a maximum of one week before being processed. In addition to the samples from Virginia, we obtained samples from Maryland, New Jersey, North Carolina, and Texas.

Crude extraction, Reverse Transcription PCR, and conventional PCR for virus samples. Samples were processed in the same manner as described previously for one tube one step RT-PCR (Jones et al. 2015; Rowhani et al. 2000). Petiole samples were removed from their Ziploc bags and 0.25 g of petiole sections, cut using sterile razor blades, were placed into grinding bags (BIOREBA, Switzerland) containing 5 ml of a filter-sterilized grapevine extraction buffer (1.59 g/liter Na_2CO_3, 2.93 g/liter $NaHCO_3$, 2% Polyvinylpyrrolidone-40, 0.2% Bovine Serum Albumin, and 0.05% Tween 20) (Sigma-Aldrich Co. LLC, St. Louis, MO) (Rowhani et al. 2000). The samples were then homogenized using a mechanical grinder (BIOREBA, Switzerland, Homex 6 [115V]) and crude extracts were transferred into 1.5-ml microcentrifuge tubes. Samples were stored at -80°C until used in future molecular diagnostic assays. This extraction method will be referred to as the "grinding bag extraction method".

For routine virus detection using conventional and reverse transcription PCR, 4 μl crude extract was added to 50 μl GES denaturing buffer (0.1 M glycine, pH 9.0; 50 mM NaCl; 1 mM EDTA; 0.5% Triton X-100) in a 0.5ml microcentrifuge tube. Following incubation at 95°C for 10 minutes, samples were placed on ice for a minimum of 5 minutes before adding 2 μl of the GES denatured homogenate to PCR tubes containing: 13.4 µl nuclease-free H2O, 2.5 µl 10X PCR buffer containing (New England Biolabs, Ipswitch, MA), 2.5 µl sucrose/cresol red (20% w/v sucrose, 1 mM cresol red) (Sigma-Aldrich Co. LLC, St. Louis, MO), 1.25 µl virus specific forward primer (20 µM), 1.25 µl virus specific reverse primer (20 µM), 1.25 µl 100mM dithiothreitol (Sigma-Aldrich Co. LLC, St. Louis, MO), 0.5 µl dNTPs (10 mM) (Invitrogen, Grand Island, NY), 0.1 µl RnaseOUT (40 U/µl) (Invitrogen), 0.035 µl Superscript III RTase (200

U/µl)(Invitrogen), and 0.25 µl Taq DNA polymerase (5 U/µl) (New England Biolabs) (Naidu et al. 2006; Osman et al. 2007; Rowhani et al. 2000). For conventional PCR reactions, an additional 0.135 µl H20 was used to substitute the RTase or RNaseOUT.

The PCR tubes were placed in a thermal cycler (Bio-Rad C1000 thermocycler, Hercules, CA) and subjected to the following cycle parameters for primer set GVGF1/GVGR1 (Al Rwahnih et al. 2013) to amplify a 446bp portion of the V2 gene: 94°, 2min; 35x (94, 30s; 60, 30s; 72, 1min); 72, 5min. Following amplification, gel electrophoresis using a 1.2% agarose gel resolved PCR products, and DNA bands were visualized using a Fotodyne digital imaging system (Fotodyne, Hartland, WI, U.S.). Positive and negative controls were used to validate test results. Amplified V2 gene segments of DNA from 43 randomly selected PCR-positive samples for each virus was purified using the QIAquick PCR Purification kit (Qiagen Inc USA, CA) using the manufacturer's protocol, and then sequenced to confirm identity and technique at the Virginia Biocomplexity Institute of Virginia Tech (VBI) (Blacksburg, VA, U.S.) using an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA).

Clustal X (Conway Institute, UK) was used to perform multiple sequence alignments for the V2 gene of GRBaV under default parameters. The V2 gene was used in this study as it is conserved, and also has been used as a measure of relatedness within the species as two clades have emerged from recent genetic analysis. Corresponding sequences from GenBank were used in these alignments and comparisons of nucleotide identity were inferred. MEGA 5 software was used to perform evolutionary analysis and evolutionary histories were inferred using the Neighbor-Joining method with evolutionary distances computed using the Maximum Composite Likelihood method (Saitou and Nei 1987; Tamura et al. 2011).

Taqman qPCR development for Red Blotch. To create candidate primers, nucleotide data for the V2 portion of the GRBaV genome were obtained from 48 red blotch positive grapevine isolates from the survey. Multiple sequence alignment was achieved with these 48 partial sequences along with 17 corresponding sequences from GenBank using Clustal X (Conway Institute, UK) under default parameters. Using this alignment, a consensus sequence was generated and used to create three primer-probe sets using Primer3 software (Untergasser et al. 2012) for potential detection under qPCR methodology:

Primer-probe set RB1 (190bp product)

Forward: 5'-ATGGGTTAGGGGATGAGGCT-3',

Reverse: 5'-GGCAGTAATTCTTTCCTCAAACA,

Probe: 5'-6FAM-ACGCTGGATCCGTTTATTCGTCGTCA-TAMRA-3');

Primer-probe set RB2 (99bp product),

Forward: 5'-GGTATATCCGAGAAACAGACTGT-3',

Reverse: 5'-CTCAAACCACTCCTCGCACT-3',

Probe: 5'-6FAM-GCGACAGCTGATTAGGCGTGTCGC-TAMRA-3'); and

Primer-probe set RB3 (133bp product)

Forward: 5'-CGACAGCTGATTAGGCGTG-3',

Reverse: 5'-TCTTCTCTGCTTCCGTTG-GC-3',

Probe: 5'-6FAM-ACAACCCTCAAACCACTCCTCGCA-TAMRA-3').

All three primer sets were first tested with a conventional PCR against confirmed positive samples to validate sensitivity and specificity of the primer sets. The *Vitis vinifera* resveratrol synthase gene was selected for use as an endogenous control due to its reliable copy number within the genome (Velasco et al. 2007). The primer-probe set of ResF, ResR, and ResP (Saito et

al. 2013; Valsesia et al. 2005) were used alongside all reactions as an endogenous control in qPCR detection and comparison.

A standard run mode protocol for Real Time PCR of 2 min @ 50°C, 10 min @ 95°C, with 40 cycles of 15 sec @ 95°C followed by 1 min @ 60°C was used as the starting point for method development. A standard concentration of 900nM forward and reverse primers and a 250nM concentration of probe was used in a 25µl final reaction volume according to the TaqMan Gene Expression Master Mix and ABI Step-One Plus real-time PCR System applications (Applied Biosystems, Foster City, CA). Using one sample, known to be infected with GRBaV, the optimum annealing temperature was first tested using temperature gradients (between 56°C and 61°C) while maintaining a forward and reverse primer concentration of 900nM, a probe concentration of 250nM, and a template volume of 2µl (Table 3.1). Once an optimal temperature was found for primer-probe sets RB1, RB2, and RB3, the optimal volume of template was tested with varying amounts of template of the same sample added into the 25µl total volume reaction: 0.5, 1, 1.5, or 2µl (Table 3.2).

Optimal primer concentration for RB1, RB2, and RB3 was determined using varying combinations of forward and reverse primer concentrations (ranging from 300nM to 900nM) while using the optimum temperature and sample template for each primer set (Table 3.3). Probe concentration was left at 250nM to avoid probe limitation. Standard curves were generated using one reference sample of unknown copy number. This sample was assigned an arbitrary copy number of 10 to its highest concentration, and assigned copy numbers thereafter to other dilutions of that reference sample based on the dilution factors used (both 1:5 and 1:10 dilution series were used) (Mehle et al. 2013). A standard curve was generated with this sample and the slope and R^2 value of the log-linear section of the curve was used to determine amplification

efficiency for all three primer sets for red blotch as well as confirm the endogenous control's efficiency (Nita, *unpublished data*).

In order to maximize the results of the qPCR protocol, the efficacy of two extraction methods were compared after the best combinations of primer sets and qPCR conditions were determined. The grinding bag extraction method stated above and used in our previous studies (Jones et al. 2015; Rowhani et al. 2000) was tested against the standard protocol for the Isolate II Plant DNA Kit (Bioline, Taunton, MA).

All qPCR data was recorded using the ABI Step-One Plus real-time PCR System (Applied Biosystems, Foster City, CA). Samples were run in triplicate to ensure a proper ΔCt value. As a result of a lack of known copy number standards, relative comparisons with the comparative C_t method of $2^{-\Delta Ct}$ was used to compare gene expression levels among samples (Livak and Schmittgen 2001; Schmittgen and Livak 2008). The ΔCt value is a calculation of the Ct value of the gene of interest minus the Ct value of the internal control. In this case, ΔCt values are calculated by averaging Ct values from three repetitions of the target gene (GRBaV V2 in this case) and subtracting this value from the averaged Ct values of three repetitions of the endogenous control gene (the *Vitis vinifera* resveratrol synthase gene). This helps normalize the detected amount of GRBaV V2 gene based on the endogenous reference to account for different input amounts of the initial DNA (Livak and Schmittgen 2001; Schmittgen and Livak 2008). The $2^{-\Delta Ct}$ measurement yields a linear form value that can be used in comparative analysis to determine if individual "treatments" (= sample in our case) might have an effect on Ct values (Livak and Schmittgen 2001; Schmittgen and Livak 2008). Calculations of $2^{-\Delta Ct}$ values were made for each positive sample using the average C_t value for red blotch and endogenous control triplicates. These $2^{-\Delta Ct}$ values were examined further to determine the potential effect of region

(within VA and other states), vineyard within a state, cultivar within a vineyard, and month of sample collection. A generalized linear mixed model (PROC GLIMMIX, SAS 9.4, Cary, NC) was used for the analysis of variance.

3.3 Results

Extended Virus Survey. Between 2011 and 2014, a total of 574 grapevines from 94 separate vineyard locations from Virginia (85), Maryland (1), New Jersey (6), North Carolina (1), and Texas (1) were sampled and tested via conventional PCR for GRBaV (Al Rwahnih et al. 2013) (Chapter 2). Table 3.4 shows a breakdown of samples taken for GRBaV by region, samples, and positive cultivars. Overall, 140 (or 23%) of grapevine samples were positive for GRBaV, which translates into 54% of vineyards sampled from all regions in this study containing at least one GRBaV-infected grapevine (Table 3.4). Multiple cultivars were found to be positive for GRBaV, including those belonging to *Vitis vinifera,* French-American intra-specific hybrids, *V. labrusca* and other native American cultivars, and table grapes (Table 3.4). All regions examined had cases of GRBaV except for the three samples taken from a single vineyard in Maryland.

Phylogenetic tree analysis of the V2 gene of GRBaV samples from Virginia and other parts of the U.S. resulted in two distinct clades, both containing multiple Virginia isolates (Fig. 4.2). These two clades were similar to the previously reported two clades of GRBaV (Al-Rwahnih et al. 2015), and 13 and 30 isolates from our study fit into clades 1 and 2, respectively.

GRBaV qPCR development. For validation of the GRBaV qPCR procedure using a conventional simplex PCR, primer-probe sets RB1, RB2, and RB3 were validated. All primer-

probe sets successfully amplified a limited number of red blotch positive samples using the following conditions: 2 µl template, 20 µM forward and reverse primer, 25 µl final volume at 94°, 2min; 35x (94, 30s; 60, 30s; 72, 1min); 72, 5min. The PCR products were sequenced and identity of the products were confirmed as the targeted V2 gene.

Among the tested temperature between 56°C and 61°C (Table 3), the optimal annealing/extending temperatures for RB1, RB2, and RB3 were found to be 58°C, 59°C, and 59°C, respectively (Table 3.1). The optimal starting template and primer concentration volume was found to be 2 µl and 900 nM (for both forward and reverse primers) based on Ct value comparison for all three primer-probe sets (Table 3.2, Table 3.3).

Using the best combinations of temperature and volumes of starting template and primer concentration, all three primer sets were examined to detect 50 known (previously amplified and sequenced) GRBaV-positive samples in triplicate and Ct values were compared. Primer-probe sets RB1 and RB2 successfully amplified 40 and 35 samples, respectively. On the other hand, primer-probe set RB3 successfully detected all 50 samples. Thus, Primer-probe set RB3 was then selected to use in standard curve analysis.

When two extraction methods were compared with the C_t slopes, the grinding bag extraction method resulted in an efficiency of 69.3% for the GRBaV primer-probe set RB3 and 69.4% for the endogenous control (Fig. 3.2, A and B). On the other hand, the Bioline kit resulted in an efficiency of 93.8% with RB3 and the endogenous control efficiency was at 89.8% (Fig. 3.2, C and D). The better amplification efficiency with the Bioline kit most likely was achieved with better purification of the sample and elimination of potential inhibitors for the GRBaV qPCR. Thus, in summary, the optimal reaction conditions for the GRBaV qPCR were achieved

using the Bioline Isolate II plant DNA kit for extraction and primer-probe set RB3 with 900nM forward/reverse primers, 250nM probe, a 59C annealing temperature, and 2 µl of template.

Using the Bioline kit for the extraction, a total of 574 samples, which contained 140 GRBaV-positive samples (from Chapter 2), were re-tested with the GRBaV qPCR procedure. The GRBaV qPCR with the Bioline kit extraction detected all the 140 known positive samples, and there were no additional GRBaV positive samples detected. Thus, in terms of GRBaV detection, there was no difference between two methods. Therefore, if the sole purpose is for detection of GRBaV from a grapevine, the grinding bag extraction method followed by a simplex PCR is most likely sufficient.

Results from the GRBaV qPCR ($2^{-\Delta Ct}$ values) were examined further to determine the potential effect of region (within Virginia and other states), vineyard within a state, cultivar within a vineyard, and month of sample collection (Fig. 3.4). Results from the generalized linear mixed model showed significant region and cultivar (within a vineyard) effects (Table 3.5) on the mean $2^{-\Delta Ct}$ values. When the dataset was broken down by state, the mean $2^{-\Delta Ct}$ values for Texas samples was significantly higher than other regions, with an exception of North Carolina (Table 3.6). However, this result needs to be treated with caution as both Texas and North Carolina samples consisted of relatively small number of high mean $2^{-\Delta Ct}$ value samples (Table 3.4, Fig. 3.4).

Furthermore, when the results from regions with significant effect of cultivar within a vineyard were examined closely, there were very large differences in the mean $2^{-\Delta Ct}$ values among samples, rather than cultivars. For example, in Northern VA vineyard I, there were two samples taken from Cabernet Sauvignon, and the mean $2^{-\Delta Ct}$ values from these two samples were significantly different from each other (Table 3.7). The lack of pure cultivar effect could

also be shown when the same cultivar from the different regions were compared. For example, Merlot from vineyard A in New Jersey had the mean $2^{-\Delta Ct}$ value of 298 while Merlot from vineyard A in Northern VA had 3.3.

3.4 Discussion

With over half of the visited vineyards having an infected vine, it is clear GRBaV is widespread in the state of Virginia. In addition, we found GRBaV in New Jersey, North Carolina, and Texas. To our understanding, this is the first report of GRBaV in the states of North Carolina and Texas. These results are also interesting in that no grapevine species are resistant to this virus as *Vitis vinifera,* French-American Hybrids, native American cultivars, and table grapes cultivars were shown here to be infected.

A TaqMan® qPCR assay was developed for GRBaV in order to reduce cross-contaminations from conventional PCR methods, eliminate the need for gel electrophoresis and UV visualization, provide clear data to help in genetic comparisons between samples, and potentially detect samples not normally detected with conventional PCR methods. The developed assay was able to successfully detect all GRBaV samples that were previously detected using a conventional PCR assay (Al Rwahnih et al. 2013) for the V2 gene of this virus. Previously negative samples remained negative with this method, suggesting that the conventional PCR assay is just as sensitive as qPCR. This is generally not the case with many qPCR assays as they typically improve the sensitivity of detection for grapevine viruses (Osman et al. 2007; Fatima Osman et al. 2008).

Although the optimal template volume was 2 µl, there were minimal differences with using 1.5 or 1 µl of template. Adding additional template greater than 2 µl to the real-time

reaction tubes reduced detection efficiency and consistently increased Ct values. This is most likely due to many phenolic and polysaccharide compounds present in grapevine tissues (Demeke and Adams 1992; Newbury and Possingham 1977; Osman et al. 2007; Osman et al. 2008; Osman and Rowhani 2006; Rezaian and Krake 1987). These compounds can inhibit the qPCR reaction, as was discussed by the authors who developed multiple TaqMan® qPCRs for GLRaVs (Osman et al. 2007). Probably for the same reason, the use of crude extract from the grinding bag extraction method as template in the GRBaV qPCR reactions showed higher Ct values compared to extraction of template using the Bioline kit. Purified samples from the Bioline kit reduces inhibitors and result in cleaner reactions (Osman et al. 2007).

Significant effects of the effect of the region and cultivar within a vineyard on the normalized Ct values ($2^{-\Delta Ct}$) of GRBaV were identified. In general, $2^{-\Delta Ct}$ values had extensive range within cultivars, regions, and dates of sampling. It was initially hypothesized that late-season samples might have a significantly different $2^{-\Delta Ct}$ value as was shown with GLRaVs (Monis and Bestwick 1996). This was not the case in this study because each month contained samples with high $2^{-\Delta Ct}$ values; however, our samples were collected at different months in different regions. Our dataset is a series of snapshots from different regions; thus, to monitor the changes in GRBaV titer in grapevines, we may need to sample from the same set of grapevines over the course of a season or seasons. Similarly, the significant regional effects were also most likely driven by a few samples with high $2^{-\Delta Ct}$ values, such as the ones found with Texas and North Carolina samples. New Jersey, Northern VA, and Texas were the three regions where effect of cultivar within a vineyard was significant to $2^{-\Delta Ct}$ values. Indeed, there was a wide range of differences among different cultivars within the same vineyard. However, some cases (e.g., Merlot in New Jersey and both Cabernet Sauvignon and Merlot in Northern VA) depicted

scenarios where the same cultivar from the same vineyard displayed a wide range in $2^{-\Delta Ct}$ values. Moreover, the same cultivar from different regions showed widely differing $2^{-\Delta Ct}$ values. Thus, the difference was actually due to the difference among sampling sites, rather than a true effect of cultivar.

A phylogenetic analysis revealed that VA samples fall into two different clades, which fit the previously reported GRBaV clades. This evidence of genetic diversity among Virginia samples, high levels of infection found among our samples, and also the evidence of GRBaV-positive vines from vines planted prior to 1990's (Chapter 2), indicates that GRBaV is not necessarily a new issue, but rather an old issue that has just emerged due to the pre-existing masking effects of grapevine leafroll disease. Sample JR8 (Clade 2) in this study was an American table grape, thus helping to provide a second report, and thus confirmation, of table grapes being infected with GRBaV (Al Rwahnih et al. 2015).

In this study, a GRBaV qPCR was developed, and utilized to examine 576 samples we obtained from the previous survey study (Chapter 2), and a few others collected from Maryland, North Carolina, Pennsylvania, and Texas. GRBaV was confirmed in all the states with an exception of Maryland, which provided only three samples. In addition to rapid diagnosis with the GRBaV qPCR, it will allow us to use comparative methods to monitor GRBaV titer in grapevines. With this detection ability we will be able to recommend to growers at what point they should be getting their vines tested for red blotch.

Table 3.1: The mean Ct values for primer-probe sets RB1, RB2, and RB3 among different annealing temperatures with a constant template and primer volume for all primer-probe set.

					Primer-Probe Pair		
Forward Primer (nM)	**Reverse Primer (nM)**	**Probe (nM)**	**Template Volume (µl)**	**Temperature ° C**	**RB1 mean Ct**	**RB2 mean Ct**	**RB3 mean Ct**
900	900	250	2	56	22.62	25.89	21.7
900	900	250	2	57	22.63	25.8	21.7
900	900	250	2	58	22.52	25.81	21.73
900	900	250	2	59	22.96	25.73	21.64
900	900	250	2	60	23.01	26.12	22.27
900	900	250	2	61	23.12	26.5	22.25

Table 3.2: The mean Ct values for primer-probe sets RB1, RB2, and RB3 among different template volumes with a constant primer volume and temperatures for each primer-probe set.

					Primer/Probe Pair		
Forward Primer (nM)	**Reverse Primer (nM)**	**Probe (nM)**	**Template Volume (µl)**	**Temperature ° C**	**RB1 mean Ct**	**RB2 mean Ct**	**RB3 mean Ct**
900	900	250	0.5	58 (or 59)	22.81	26.11	22.56
900	900	250	1	58 (or 59)	22.88	26.01	22.34
900	900	250	1.5	58 (or 59)	22.5	25.97	21.69
900	900	250	2	58 (or 59)	22.5	25.71	21.65

Table 3.3: The mean Ct values for primer-probe sets RB1, RB2, and RB3 among different primer concentrations with a constant template volume and temperatures for each primer-probe set.

					Primer/Probe Pair		
Forward Primer (nM)	**Reverse Primer (nM)**	**Probe (nM)**	**Template Volume (µl)**	**Temperature ° C**	**RB1 Mean Ct**	**RB2 Mean Ct**	**RB3 Mean Ct**
300	300	250	2	58 (or 59)	26.74	28.13	24.58
300	600	250	2	58 (or 59)	24.77	27.65	23.62
600	300	250	2	58 (or 59)	24.7	27.23	23.67
600	600	250	2	58 (or 59)	23.79	26.44	22.54
600	900	250	2	58 (or 59)	23.61	26.1	22.23
900	600	250	2	58 (or 59)	23.65	26.31	22.12
900	900	250	2	58 (or 59)	22.52	25.76	21.69

Table 3.4: Summary of sample collected for GRBaV survey, 2011-2014.

Region	Number of vineyards positive for GRBaV infection	Total Number of vineyards visited	Number of samples collected	Number of GRBaV-positive samples	Cultivars positive for GRBaV
Central VA	15	25	151	38	American Table Grape Cabernet franc Cabernet Sauvignon Chambourcin Chardonnay Merlot Norton Petit Verdot Sauvignon blanc Syrah Vidal blanc Viognier
Eastern VA	8	21	100	26	Cabernet franc Cabernet Sauvignon Chardonel Chardonnay Malbec Merlot Petit Verdot Scuppernong
Maryland	1	1	3	0	N/A
New Jersey	6	6	38	12	Cabernet franc Cabernet Sauvignon Merlot Syrah
North Carolina	1	1	5	3	Cabernet franc
Northern VA	10	25	144	23	Cabernet franc Cabernet Sauvignon Merlot Norton Petit Manseng Petit Verdot Sauvignon blanc Viognier
Southern VA	2	6	30	3	Cabernet Sauvignon Merlot
Texas	1	1	19	19	Blanc Du Bois Lenoir
Western VA	6	8	84	16	Cabernet Sauvignon Cabernet franc Chardonel Concord Pinot noir Riesling
Total	51	94	574	140	

Table 3.5: ANOVA table for examination of the effect of region, vineyard within a region, cultivar within a vineyard (~sampling point), and sampling timing (month of the year) to the $2^{-\Delta Ct}$ values, GRBaV survey 2011-2014.

Source of variation[a]	Num DF[b]	Den DF[b]	F[c]	Pr > F
Region	7	49	6.28	<0.001**
Vineyard(Region)	40	49	0.43	0.99
Cultivar(Region x Vineyard)	25	49	1.74	0.05*
Timing	9	49	0.14	0.99

[a] Effect to be examined using a generalized linear model (PROC MIXED, SAS 9.4), a variable followed by a variable(s) in parentheses indicate the nesting of variables

[b] Numerator and denominator degree of freedom

[c] F statistics and *P*-values. A *P*-value followed by one and two asterisks indicate a significance at 95% and 99% confidence level, respectively.

Table 3.6: The effect of region on the mean $2^{-\Delta Ct}$ values, GRBaV survey 2011-2014.

Region	LS Means[a]	SE[a]		
Central VA	203.56	40.75	B	
Eastern VA	44.07	57.45		C
New Jersey/Rutgers	33.67	65.12		C
North Carolina	334.02	124.06	B	A
Northern VA	87.22	52.4	B	C
Southern VA	33.92	131.59		C
Texas	484.22	53.03		A
Western VA	28.54	64.15		C

[a] LS Means = least square mean estimate from the generalized linear model, SE = standard error, the same letter after the number indicates that the numbers were not significantly different ($P \leq 0.05$) based on the Fisher's LSD (GLIMMIX, SAS 9.4).

Table 3.7: Effect of cultivar within a vineyard (~ sampling point) on the mean $2^{-\Delta Ct}$ values, GRBaV survey, 2011-14.

Region	Vineyard	Cultivar	LS Means[a]	SE[a]	
New Jersey	A	Merlot	298.17	0.01	A
	A	Cabernet Sauvignon	128.00	0.01	B
	B	Cabernet franc	0.02	0.01	D
	B	Cabernet Sauvignon	0.01	0.01	D
	C	Merlot	44.94	0.01	C
	C	Syrah	0.02	0.01	D
	D	Merlot	0.01	0.01	D
	E	Unknown	0.02	0.01	D
	F	Syrah	0.03	0.01	D
	F	Merlot	0.02	0.01	D
	F	Cabernet Sauvignon	0.01	0.01	D
Northern VA	A	Merlot	3.27	53.77	C
	A	Petit Verdot	0.01	38.02	C
	B	Unknown	256.00	53.77	A
	C	Cabernet franc	192.82	26.89	AB
	C	Cabernet Sauvignon	56.89	53.77	BC
	D	Viognier	0.03	38.02	C
	E	Merlot	184.32	38.02	AB
	F	Norton	9.99	53.77	C
	F	Sauvignon blanc	0.06	53.77	C
	G	Cabernet franc	310.83	53.77	A
	G	Merlot	0.01	53.77	C
	H	Petit Manseng	0.02	53.77	C
	I	Cabernet Sauvignon	212.31	53.77	AB
	I	Cabernet Sauvignon	0.02	53.77	C
	J	Merlot	0.02	53.77	C
	J	Petit Verdot	203.95	38.02	AB
Texas	A	Blanc Du Bois	839.43	136.19	A
	A	Lenoir	129.00	92.52	B

[a] LS Means = least square mean estimate from the generalized linear model, SE = standard error, the same letter after the number indicates that the numbers were not significantly different ($P \leq 0.05$) based on the Fisher's LSD (GLIMMIX, SAS 9.4).

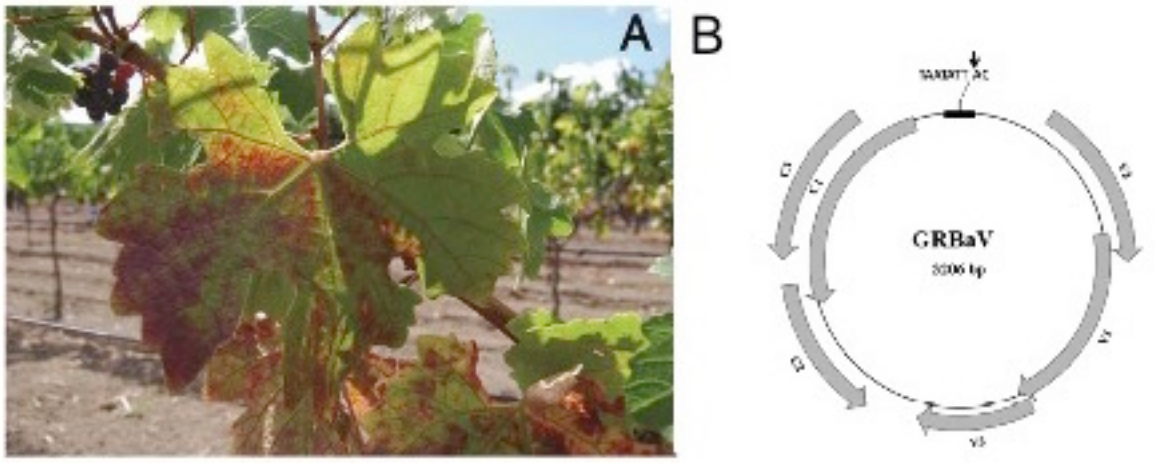


Figure 3.1: Symptoms and genome of GRBaV(Al Rwahnih et al. 2013). A. Symptoms of GRBaV are very similar to grapevine leafroll disease leaf symptoms except reddening is not limited to between veins (or interveinal). B. 3206bp Circular genome of GRBaV containing 6 ORFs.

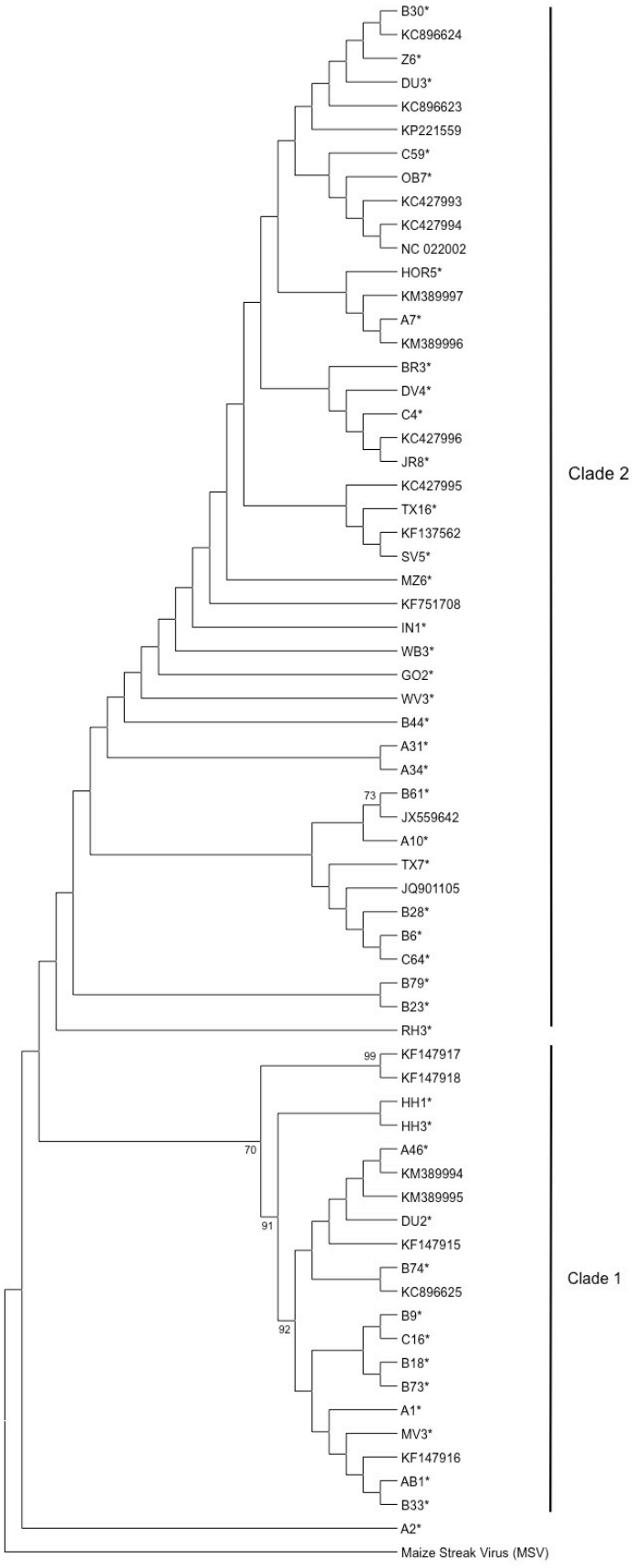

B30*
KC896624
Z6*
DU3*
KC896623
KP221559
C59*
OB7*
KC427993
KC427994
NC 022002
HOR5*
KM389997
A7*
KM389996
BR3*
DV4*
C4*
KC427996
JR8*
KC427995
TX16*
KF137562
SV5*
MZ6*
KF751708
IN1*
WB3*
GO2*
WV3*
B44*
A31*
A34*
73
B61*
JX559642
A10*
TX7*
JQ901105
B28*
B6*
C64*
B79*
B23*
RH3*
99
KF147917
KF147918
HH1*
HH3*
70
A46*
KM389994
KM389995
91
DU2*
KF147915
B74*
KC896625
92
B9*
C16*
B18*
B73*
A1*
MV3*
KF147916
AB1*
B33*
A2*
Maize Streak Virus (MSV)
Clade 2
Clade 1

Figure 3.2: The evolutionary history was inferred for GRBaV V2 gene isolates (rooted with Maize streak virus) from VA and GenBank using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches however bootstrap values corresponding to partitions reproduced in less than 70% bootstrap replicates are not shown. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 66 nucleotide sequences. There were a total of 427 positions in the final dataset. VA isolates used in this analysis are denoted with an asterisk *.

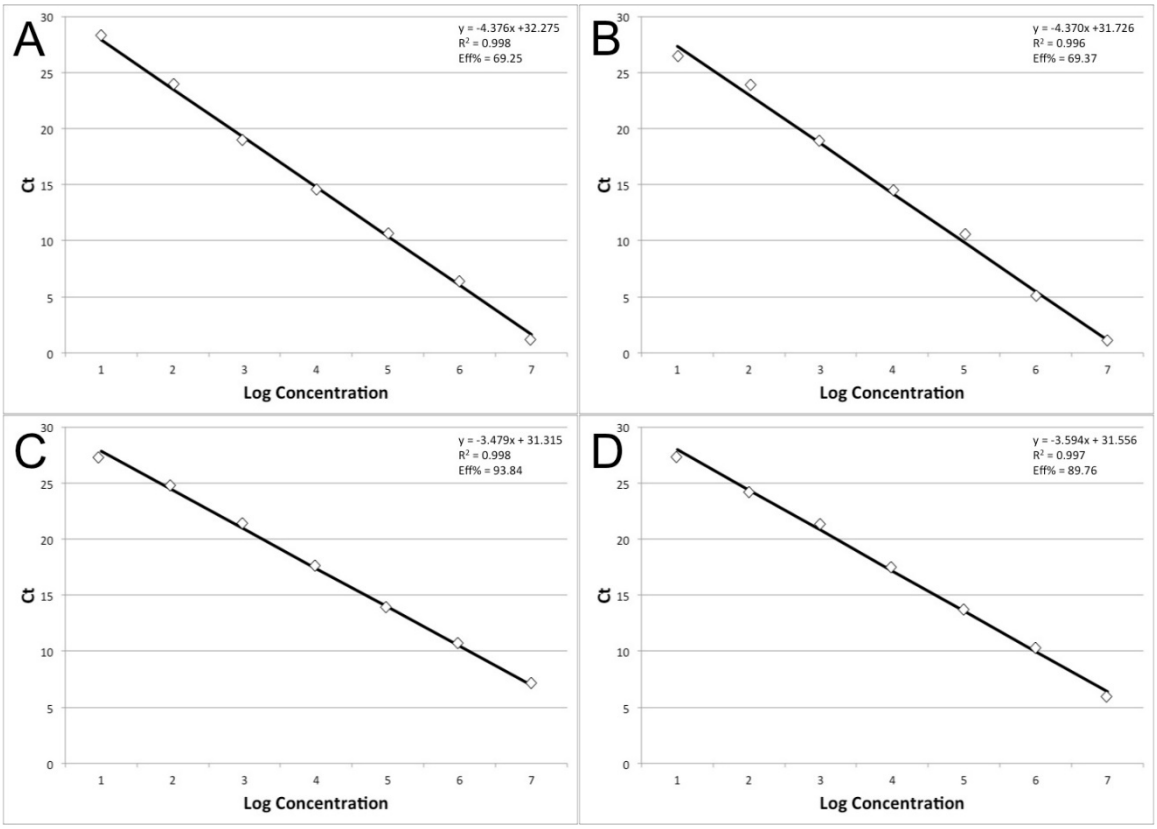


Figure 3.3: Determination of efficiency of qPCR assays using standard curve analysis and C_t slope method with seven concentrations covering a 6-log dilution range for two extraction methods of the sample for Primer-probe set RB3 and the endogenous control. Equation, R^2 value, and efficiency percentage are shown. A: Primer-probe set 3 for red blotch using GES extraction method, B: Endogenous control using GES extraction method, C: Primer probe set RB3 for red blotch using Bioline kit extraction method, D: Endogenous control using Bioline kit extraction method.

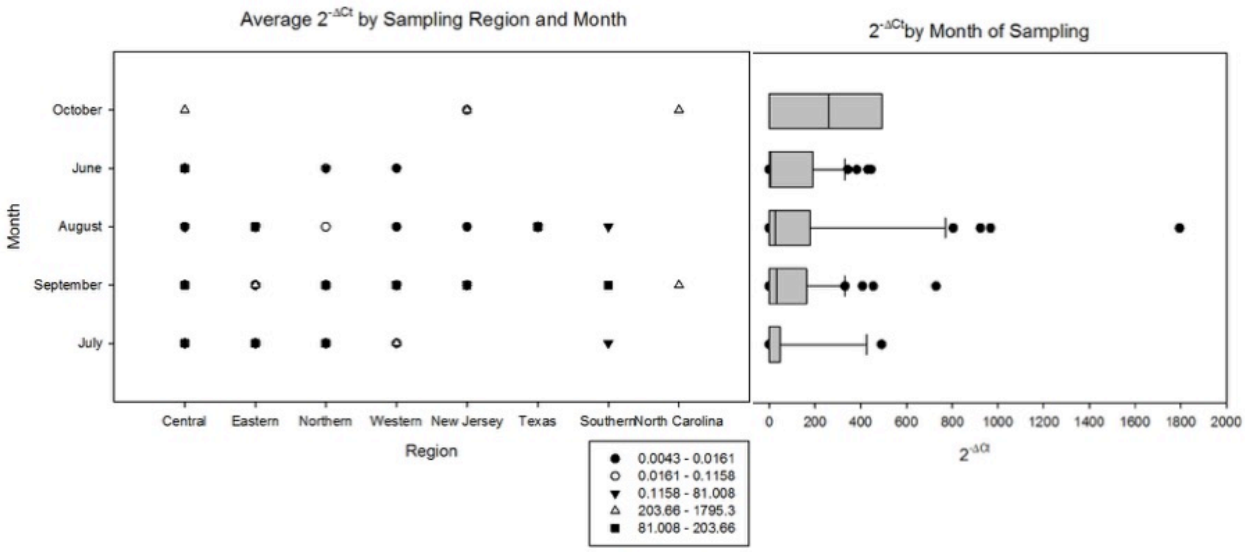


Figure 3.4: Non-significant general trend of $2^{-\Delta Ct}$ values of GRBaV positive grapevines by month and region of sampling.

3.5 References:

Agrios, G. N. (2005). *Plant Pathology*: Elsevier Academic Press.

Al Rwahnih, M., Dave, A., Anderson, M. M., Rowhani, A., Uyemoto, J. K., & Sudarshana, M. R. (2013). Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology, 103*(10), 1069-1076, doi:10.1094/PHYTO-10-12-0253-R.

Al Rwahnih, M., Osman, F., Sudarshana, M., Uyemoto, J., Minafra, A., Saldarelli, P., et al. (2012). Detection of *Grapevine leafroll-associated virus 7* using real time qRT-PCR and conventional RT-PCR. *Journal of Virological Methods, 179*(2), 383-389, doi:10.1016/j.jviromet.2011.11.026.

Al Rwahnih, M., Rowhani, A., & Golino, D. A. (2015). First report of *Grapevine red blotch associated virus* in archival grapevine material from Sonoma County, California. *Plant Disease*, 150127132800003, doi:10.1094/PDIS-12-14-1252-PDN.

Al Rwahnih, M., Rowhani, A., Golino, D. A., Islas, C. M., Preece, J. E., & Sudarshana, M. R. (2015). Detection and genetic diversity of *grapevine red blotch-associated virus* isolates in table grape accessions in the National Clonal Germplasm Repository in California. *Canadian Journal of Plant Pathology, 37*(1), 130-135, doi:10.1080/07060661.2014.999705.

Calvi, B. L. (2011). *Effects Of Red-leaf Disease On Cabernet Sauvignon At The Oakville Experimental Vineyard And Mitigation By Harvest Delay And Crop Adjustment*. ProQuest Dissertations Publishing,

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). *59*, 330-337.

Demeke, T., & Adams, R. P. (1992). The effects of plant polysaccharides and buffer additives on PCR. *BioTechniques, 12*(3), 332-332.

Habili, N. (2013). Highlights of a newly-emerging grapevine virus: Grapevine red blotch-associated virus.

Jones, T. J., Rayapati, N., & Nita, M. (2015). Occurrence of *Grapevine leafroll associated virus-2, −3* and *Grapevine fleck virus* in Virginia, U.S.A., and factors affecting virus infected vines. *European Journal of Plant Pathology*, 1-14, doi:10.1007/s10658-015-0605-z.

Krenz, B., Thompson, J. R., Fuchs, M., & Perry, K. L. (2012). Complete genome sequence of a new circular DNA virus from grapevine. *Journal of Virology, 86*(14), 7715-7715.

Krenz, B., Thompson, J. R., McLane, H. L., Fuchs, M., & Perry, K. L. (2014). *Grapevine red blotch-associated virus* Is Widespread in the United States. *Phytopathology, 104*(11), 1232-1240, doi:10.1094/phyto-02-14-0053-r.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *methods, 25*(4), 402-408.

Martelli, G. P. (2014). Directory of virus and virus-like diseases of the grapevine and their agents. *Journal of Plant Pathology, 96*(1), 1-136.

Martelli, G. P., & Boudon-Padieu, E. (2006). *Directory of infectious diseases of grapevines*. (Vol. 55): International Centre for Advanced Mediterranean Agronomic Studies. Options Méditerranéennes, Ser. B, Studies and Research. 55: 59-75.

Mehle, N., Prezelj, N., Hren, M., Boben, J., Gruden, K., Ravnikar, M., et al. (2013). A Real-Time PCR Detection System for the Bois Noir and Flavescence Dorée Phytoplasmas and

Quantification of the Target DNA. In M. Dickinson, & J. Hodgetts (Eds.), *Phytoplasma* (Vol. 938, pp. 253-268, Methods in Molecular Biology): Humana Press.

Monis, J., & Bestwick, R. K. (1996). Detection and localization of grapevine leafroll associated closteroviruses in greenhouse and tissue culture grown plants. *American Journal of Enology and Viticulture, 47*(2), 199-205.

Naidu, R. A., Maree, H. J., & Burger, J. T. (2015). Grapevine Leafroll Disease and Associated Viruses: A Unique Pathosystem. *Annual Review of Phytopathology, 53*, 613-634.

Naidu, R. A., Soule, M. J., & Jarugula, S. (2006). Single and mixed infections of *Grapevine leafroll-associated viruses* in Washington State vineyards. *Phytopathology, 96*, S83.

Newbury, H. J., & Possingham, J. V. (1977). Factors Affecting the Extraction of Intact Ribonucleic Acid from Plant Tissues Containing Interfering Phenolic Compounds. *Plant Physiology, 60*(4), 543-547, doi:10.1104/pp.60.4.543.

Oberhansli, T., Altenbach, D., & Bitterlin, W. (2011). Development of a duplex TaqMan real-time PCR for the general detection of phytoplasmas and 18S rRNA host genes in fruit trees and other plants. *Bulletin of Insectology, 64*, S37-S38.

Osman, F., Leutenegger, C., Golino, D., & Rowhani, A. (2007). Real-time RT-PCR (TaqMan®) assays for the detection of *Grapevine Leafroll associated viruses 1-5* and *9*. *Journal of Virological Methods, 141*(1), 22-29, doi:10.1016/j.jviromet.2006.11.035.

Osman, F., Leutenegger, C., Golino, D., & Rowhani, A. (2008). Comparison of low-density arrays, RT-PCR and real-time TaqMan ® RT-PCR in detection of grapevine viruses. *Journal of Virological Methods, 149*(2), 292-299, doi:10.1016/j.jviromet.2008.01.012.

Osman, F., & Rowhani, A. (2006). Application of a spotting sample preparation technique for the detection of pathogens in woody plants by RT-PCR and real-time PCR (TaqMan). *Journal of Virological Methods, 133*(2), 130-136, doi:10.1016/j.jviromet.2005.11.005.

Rayapati, N. (2012). Virus Diseases. http://wine.wsu.edu/research-extension/plant-health/virology/virus-diseases/. Accessed November 2012.

Rezaian, M. A., & Krake, L. R. (1987). Nucleic acid extraction and virus detection in grapevine. *Journal of Virological Methods, 17*(3), 277-285, doi:10.1016/0166-0934(87)90137-6.

Rowhani, A., Biardi, L., Johnson, R., Saldarelli, P., Zhang, Y. P., Chin, J., et al. Simplified sample preparation method and one-tube RT-PCR for grapevine viruses. In *Proceedings of XIII International Council for the Study of Viruses and Virus-Like Diseases of the Grapevine, Adelaide, 2000* (pp. 82)

Saito, S., Dunne, K. J., Evans, K. J., Barry, K., Cadle-Davidson, L., & Wilcox, W. F. (2013). Optimisation of techniques for quantification of Botrytis cinerea in grape berries and receptacles by quantitative polymerase chain reaction. *Australian Journal of Grape and Wine Research, 19*(1), 68-73, doi:10.1111/ajgw.12011.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4*(4), 406-425.

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nature protocols, 3*(6), 1101-1108.

Sudarshana, M., Perry, K. L., & Fuchs, M. (2015). *Grapevine red blotch-associated virus*, an emerging threat to the grapevine industry. *Phytopathology*, doi:10.1094/phyto-12-14-0369-fi.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary

distance, and maximum parsimony methods. *Molecular Biology and Evolution, 28*(10), 2731-2739, doi:10.1093/molbev/msr121.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., et al. (2012). Primer3—new capabilities and interfaces. *Nucleic Acids Research, 40*(15), e115-e115, doi:10.1093/nar/gks596.

Valsesia, G., Gobbin, D., Patocchi, A., Vecchione, A., Pertot, I., & Gessler, C. (2005). Development of a high-throughput method for quantification of Plasmopara viticola DNA in grapevine leaves by means of quantitative real-time polymerase chain reaction. *Phytopathology, 95*(6), 672-678, doi:10.1094/PHYTO-95-0672.

Velasco, R., Zharkikh, A., Troggio, M., Cartwright, D. A., Cestaro, A., Pruss, D., et al. (2007). A high quality draft consensus sequence of the genome of a heterozygous grapevine variety. *PLoS ONE, 2*(12), e1326.

Chapter 4

Transmission of GLRaV-3 by *Ferrisia gilli*, evaluation of insecticidal treatments, and spatio-temporal association of GLRaV-3 in Virginia Vineyards

4.1 Introduction

Grapevine leafroll disease (GLD) is a viral disease present in all grapevine-growing regions of the world (Fuchs et al. 2009b). GLD can significantly affect vine vigor along with both fruit yield and grape quality (Alley et al. 1963; Cabaleiro et al. 1999; Credi and Babini 1997; Goheen and Cook 1959; Goheen et al. 1958; Kliewer and Lider 1976; Kovacs et al. 2001; Lider et al. 1975; Wolpert and Vilas 1992; Woodham et al. 1984). In severely infected vineyards, direct crop losses in terms of yield can be as high as 40% (Wolpert and Vilas 1992), and indirect crop losses can be caused in the form of decreased wine quality caused by uneven ripening of berries, reduced sugar levels, changes in pH and TA, and poor berry color due to reduced anthocyanin pigments in red-fruited cultivars (Cabaleiro et al. 1999; Fuchs et al. 2009b; Rayapati et al. 2008).

Mealybugs and scale insects (order Hemiptera, suborder Sternorrhyncha, superfamily Coccoidea, families Coccidea (scale) and Pseudococcidae (soft scale/mealybug), have been

reported as vectors of grapevine-infecting viruses, most importantly the ampeloviruses (family *Closteroviridae*) associated with grapevine leafroll disease (GLD), *grapevine leafroll associated virus-1* (GLRaV-1) and GLRaV-3 (Tsai et al. 2010). GLRaV-3 is more widespread across grapevine-growing regions than other GLRaVs (Rayapati et al. 2008).

Based on transmission studies with mealybugs, GLRaV-3 is transmitted in a semi-persistent manner. Acquisition of the virus takes approximately 0.25 to 12 h and the virus can be retained within the vector for 12 h to 5 days (Charles et al. 2006). First instars of mealybugs are considered as the most efficient phase of transmission (Petersen and Charles 1997). The first mealybug documented to successfully transmit GLRaV-3 to a healthy grapevine in a laboratory setting was *Planococcus ficus* (vine mealybug) (Engelbrecht and Kasdorf 1990; Rosciglione and Gugerli 1989; Tanne et al. 1989). Recent studies have demonstrated that the grape (*Pseudococcus maritimus*), longtailed (*Pseudococcus longispinus*), citrophilus (*Pseudococcus calceolariae*), citrus (*Planococcus citri*), and obscure (*Pseudococcus viburni*) mealybugs are all capable of transmitting GLRaV-3 (Tsai et al. 2010; Tsai et al. 2008).

Both *Pseudococcus maritimus* and Gill's mealybugs (*Ferrisia gilli*) were commonly found in VA vineyards (Jones et al. 2015). There were also isolated cases of the striped mealybug (*Ferrisia virgata*) (Jones et al. 2015), and the obscure mealybug (*Pseudococcus viburni*) (Chapter 2) found in VA vineyards. The grape mealybug is commonly associated with GLRaV-3 and has a wide host range including grapevines, figs, apples, and citrus crops (Millar et al. 2002). There is no knowledge yet about the ability of either the striped or Gill's mealybugs to transmit the viruses associated with GLD.

In addition to transmitting GLRaV-1 and -3, mealybugs can transmit other viruses such as GVA and GVB (Tsai et al. 2010; Boscia et al. 1997). Furthermore, they can cause damage

from excretion of honeydew (Triplehorn et al. 2005), which is not only a major attractant to ants (Geiger and Daane 2001; Phillips and Sherk 1991), but also promotes the formation of sooty mold. Some species (e.g., *Pl. ficus*) even cause direct feeding injury (Flaherty et al. 1992).

Naturally occurring biological control is the primary means of mealybug control in vineyards; however, chemical control is the primary means of mealybug management once the natural controls have been disrupted in some manner. Previous studies in Virginia have shown the effectiveness of chemical control on mealybug management; however, it was not enough to prevent the spread of GLRaV-3 (Jones and Nita 2016). The difficulty of management arises from different factors: some insecticide treatments can increase mealybug populations by eliminating beneficial insects (Jones and Nita 2016); mealybugs tend to hide under bark tissues; and multiple overlapping generations per year makes targeted insecticide applications difficult (Geiger and Daane 2001).

In this study, we examine the ability of the Gill's mealybug to transmit GLRaV-3, describe the movement of GLRaV-3 throughout vector-infested fields, and further confirm the effectiveness of foliarly applied mealybug chemical management under Virginia environmental conditions.

4.2 Materials and Methods

Transmission of GLRaV-3 to healthy plants by *Ferissia gilli*

Validation of viruliferous nature: Prior to the experiment, a study was conducted to validate the viruliferous nature of *F. gilli* with respect to GLRaV-3, where we determined whether adult females pass on GLRaV-3 to their offspring or not. A small group of *F. gilli* was

established on a GLRaV-3 infected plant in 2014. Females were closely observed, and as soon as 1st instars emerged, both the adult female and 1st instars were tested for GLRaV-3. A total of eleven adult females and eleven groups of 1st instars were tested. Mealybug nucleic acid extraction was conducted with the QIAGEN DNeasy Blood and Tissue kit, and a one-tube one-step RT-PCR method (hereafter referred as the LR3 RT-PCR) was used for GLRaV-3 detection (Jones et al. 2015; Osman et al. 2007; Rowhani et al. 2000).

The extracts from the kit were added directly to PCR tubes containing: 13.4 µl nuclease-free H2O, 2.5 µl 10X PCR buffer containing (New England Biolabs, Ipswich, MA), 2.5 µl sucrose/cresol red (20% w/v sucrose, 1 mM cresol red) (Sigma-Aldrich Co. LLC, St. Louis, MO), 1.25 µl virus specific forward primer (20 µM), 1.25 µl virus specific reverse primer (20 µM), 1.25 µl 100 mM dithiothreitol (Sigma-Aldrich Co. LLC, St. Louis, MO), 0.5 µl dNTPs (10 mM) (Invitrogen, Grand Island, NY), 0.1 µl RnaseOUT (40 U/µl) (Invitrogen), 0.035 µl Superscript III RTase (200 U/µl) (Invitrogen), and 0.25 µl Taq DNA polymerase (5 U/µl) (New England Biolabs) (Naidu et al. 2006; Osman et al. 2007; Rowhani et al. 2000) for a total reaction volume of 25 µl.

Reaction cycle for GLRaV-3 PCR using primer set LC1 F/LC1 R (Osman and Rowhani 2006) was: 52°C for 1h followed by 35 cycles of 94°C for 30s; 54°C for 45s; 72°C for 1min, with a final extension at 72°C for 2min. A 1.2% agarose gel was used to visualize products under a Fotodyne digital imaging system (Fotodyne, Hartland, WI, U.S.) to confirm presence of the target nucleic acid within a sample.

Grapevine preparation: Vines used in the transmission study were harvested from a section of a 'Cabernet franc' vineyard at the AHS AREC, Winchester, VA. Vines were tested for GLRaV-3 in early August and again in late September of 2014 to confirm they were virus free. A

total of 100 cuttings were taken in early spring 2015 from these virus-tested vines, to propagate virus-free vines in 1.5-gallon pots. Cuttings were trimmed to three internodes in length, and the bottom end was dipped into rooting hormone (Green Light, A Valent U.S.A. Company, San Antonio, TX). These cuttings were grown in vermiculite for 4-5 weeks in a greenhouse, then transferred to 1.5-gallon pots. Similarly, canes from GLRaV-3-positive Cabernet franc vines in another vineyard were used to create 50 propagated vines in 1.5-gallon pots. These vines were grown in two separate locations to reduce the risk of spread of GLRaV-3 to the virus-free vines. All vines used in this study were trained to single shoots to ensure that mealybugs would be feeding on an infected area of the plant, since grapevine viruses are not evenly distributed throughout all shoots (Charles et al. 2006).

Three ultra-fine-mesh cages (1.83m x 1.83m x 1.83m in dimension, BioQuip Products Inc., Rancho Dominguez, CA) were used in the experiment to avoid contact with other insects and placed in a greenhouse. The clean, virus free potted vines were grown inside of cage #1 in a greenhouse for 8 weeks prior to the initiation of the experiment. On 14 June, 2015, all propagated Cabernet franc vines (from both the infected and virus-free fields) were tested for GLRaV-3 using the LR3 RT-PCR (Jones et al. 2015; Rowhani et al. 2000) to confirm these vines as negative or positive for GLRaV-3. After testing, one single-shooted GLRaV-3 positive vine was selected as the "acquisition vine", and placed in the cage #2 by itself.

F. gilli *rearing*. For the transmission assay, a *F. gilli* colony was started with 54 adult female *F. gilli* collected from a vineyard in Orange County, VA in May 2015. Using a fine point paintbrush, mealybugs were gently transferred to sprouted potatoes in the field, transported back to the greenhouse, and gently transferred again to a large, multiple-shoot virus-free Cabernet

franc vine, which was grown in a 18.93 L pot in one of the aforementioned insect cages (cage #3).

GLRaV-3 transmission assay with F. gilli. On 15 June 2015, a total of 72 *F. gilli* 1st instars from the mealybug-rearing vine in the cage #3 were placed onto the acquisition vine in the cage #2 and allowed to feed for 24 hours in the cage. This feeding time was determined based on previous research that indicated grape mealybug transmission abilities peak at a 24-hour feeding period for GLRaV-3 (Tsai et al. 2008). Following the acquisition period, the *F. gilli* instars were transferred to virus-free vines to determine their ability to transmit GLRaV-3.

Twenty-four virus-free propagated Cabernet franc vines in cage #1 were separated into six groups of four potted vines. The grouping was for different feeding/transmission times of 1h, 2h, 6h, 12h, 24h, and 48h. Each feeding/transmission time group of four vines was further subdivided to have different numbers of mealybugs on each vine. Within each time group, two vines received five mealybugs each while the other two received one mealybug each.

First instars from the acquisition vine in the cage #2 were sampled promptly after their emergence on 16 June, 2015, then placed onto leaves of virus-free vines that had been brought over from the cage #1. Vines were grouped according to each feeding/transmission time length, and feeding/transmission for each time length were conducted separately from each other to avoid crowding in the cage #2. After the feeding/transmission times had expired, mealybugs were removed from the vines by hand. These removed mealybugs from each vine were placed into 0.5-ml tubes containing 95% ethanol, and stored at -20°C.

In addition, there were ten mealybugs that were transferred to the acquisition vine, allowed to feed for 24 hours (positive control), and another ten mealybugs from the healthy vine that were not subjected to any treatment (negative control). Both were also transferred to 0.5-ml

microcentrifuge tubes containing 95% ethanol and placed in the freezer at -20°C. After the feeding/transmission time, grapevines were transferred to the cage #3 (which became empty after removing the virus-free vine that was used for rearing of *F. gilli*), watered regularly, and managed for powdery mildew twice using foliarly applied potassium bicarbonate (Kaligreen applied at 3.4 kg/ha, Toagosei Co., LTD, Tokyo, Japan).

All insects from this study were then ground in liquid nitrogen and subjected to nucleic acid extraction using a standard protocol for the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). These samples were then tested for GLRaV-3 using the LR3 R-PCR (Jones et al. 2015; Rowhani et al. 2000), Each group of mealybugs, or single mealybug, per plant was tested as one sample and the ten positive and negative control samples were tested separately. In addition, species confirmation was conducted using a vineyard mealybug multiplex PCR assay (Daane et al. 2011; Jones et al. 2015).

Vines subjected to transmission/feeding were tested for GLRaV-3 using the LR3 RT-PCR. Five petioles were arbitrarily collected in August and October for testing. The time for the assessments was based on a study (Tsai et al. 2008) where the authors reported that two months following transmission, GLRaV-3 is detectable using RT-PCR. The RNA extraction was conducted as follows. Petiole samples were cut using a sterile razor blade into 0.25 g of petiole sections and placed into grinding bags (BIOREBA, Switzerland) containing 5 ml of a filter-sterilized grapevine extraction buffer (1.59 g/liter Na_2CO_3, 2.93 g/liter $NaHCO_3$, 2% polyvinylpyrrolidone-40, 0.2% bovine serum albumin, and 0.05% Tween 20) (Sigma-Aldrich Co. LLC, St. Louis, MO) (Rowhani et al. 2000), and homogenized using a mechanical grinder (BIOREBA, Switzerland, Homex 6 [115V]). Crude extract was transferred to 1.5-ml

microcentrifuge tubes and stored at -80°C. Then the extract was subjected to the LR3 RT-PCR procedure described in the previous section (Jones et al. 2015; Rowhani et al. 2000).

GLRaV-3 movement within Chardonnay, AHS AREC 2013-2015.

The spatio-temporal patterns associated with the spread of GLRaV-3 was examined within a 10 row x 10 vine section of 'Chardonnay' vines infested with grape and Gill's mealybugs at the AHS-AREC, Winchester VA (Fig. 4.1). This was a three-year continuation of the previous study in this same location (Jones and Nita 2016). During the previous study, 37 of 100 total vines were infected with GLRaV-3, and showing significant evidence of aggregation (SADIE's index of aggregation $I_a = 2.1$ (Jones and Nita 2016)) by the end of third season in 2012.

The same 100 vines were intensively sampled in September in 2013, 2014, and 2015. Fifteen petioles were sampled from each vine, taken randomly across the canopy to maximize chances of detecting GLRaV-3 (Charles et al. 2006). Sampled petioles were placed into a Ziploc bag, stored in a cooler on ice, and immediately processed into crude extract to be used in the LR3 RT-PCR procedure (Jones et al. 2015; Rowhani et al. 2000).

Insecticide trials

Cabernet Sauvignon AHS AREC interplanting trial: A three-year study was implemented in 2012 at an experimental vineyard at the AHS AREC in Winchester VA. Cabernet Sauvignon vines in this vineyard were originally planted in 1990, trained in the Lyre system (i.e., divided canopy with vertical shoot growth), and there were three rows, and each row contained 13 panels of vines. In this trial, each panel (the three vines between line posts) was used as a sub-plot. One

vine in each sub-plot was an old Cabernet Sauvignon, known to be infected with GLRaV-3 and infested by overwintering female grape and Gill's mealybugs. In 2012, two certified Cabernet franc grapevines (hereafter referred as young vines) were planted at 5 feet and 10 feet from the old vine (Fig. 4.2).

The experimental design was a randomized complete block design with six replications. Each plot had three replicated sub-plots of three treatments, and each of the sub-plot was separated by at least one empty panel (6.3 m) (Fig. 4.2). The position of the young vines in relation to the infected, mealybug-harboring old vine was reversed in three plots in order to examine effects from the westerly-prevailing wind direction (i.e., the block effect was relative direction to the old vine). Under-trellis cover crops were managed using herbicides to remove any potential mealybug reservoirs on the ground.

There were three foliarly applied insecticide treatments applied to each sub-plot within a plot: 1) two applications of a tetramic-acid derivative (spirotetramat, Movento, 0.439 L/ha, Bayer CropScience LP); 2) two applications of a pyrethroid (ß-cyfluthrin, Baythroid XL, 0.219 L/ha, Bayer CropScience LP), and 3) no insecticide spray as a control. Applications of treatments were performed twice each year at 1-2 inch shoot growth (about a week after bud-break) and then again at bloom: 19 May and June 14 2012; 23 May and June 12, 2013; 22 May and 14 June 2014. Assignment of treatments to sub-plots within a plot was random, and the same treatment was applied to the same sub-plot for the three years. Fungal diseases were controlled by a standard fungicide application program (mancozeb and sulfur, both applied at 3.4 kg/ha at a 14-day interval) that should not affect mealybug activities.

Petioles on these vines were sampled arbitrarily throughout the canopy and then subjected to the LR3 RT-PCR detection (Jones et al. 2015; Rowhani et al. 2000) for detection of

GLRaV-3 annually in mid-September. Throughout the seasons, mealybugs were counted every one to two weeks by a rater who spent a total of 2.5 minutes per side of a Lyre-trained vine visually counting mealybugs (Geiger and Daane 2001). The rater examined shoots, leaves, cordons, trunks, and underneath the bark for the counting. There were eight, nine, and fourteen total counting dates between 18 May and 27 July, 2012, 22 May and 19 August, 2013, and 13 May and 8 September, 2014, respectively (Fig. 4.3). In the spring of 2015, all vines were excavated and root systems were visually inspected for mealybugs.

Merlot AHS AREC trial: A three-year field trial was implemented in 2012 at a 'Merlot' vineyard in the AHS AREC in Winchester, VA. Prior to the start of the experiment, mealybugs had not been found in this vineyard and all vines were tested with the LR3 RT-PCR to confirm that these vines were GLRaV-3-free. However, a neighboring (3 m away) Chardonnay vineyard block had multiple confirmations of vines infected with GLRaV-3, and our record indicated that the number of GLRaV-3-positive vines had been increasing rapidly between 2009-2011 (Jones and Nita 2016). Merlot vines were trained in a Vertical Shoot Positioning (VSP) system. A portion of the vineyard with four rows containing 25 vines per row was used. The experimental design was a randomized block design where each row was a block, and within a row there were five treatments that were applied to five consecutive vines. Locations of the treatments were assigned randomly within a block.

Foliarly applied treatments were: 1) the tetramic acid derivative spirotetramat, (Movento, 0.439 L/ha, Bayer CropScience LP); 2) the pyrethroid ß-cyfluthrin (Baythroid XL, 0.219 L/ha, Bayer CropScience LP); 3) low rate of chlorpyrifos (Lorsban, 1.4 L/ha, Dow AgroSciences LLC, Indianapolis, IN); 4) high rate of organophosphate chlorpyrifos (Lorsban, 1.6 L/ha, Dow

AgroSciences LLC, Indianapolis, IN); and 5) water as a control. Treatments were applied twice per season, at 1- to 2-inch growth (approximately 7 to 10 days after bud break) and at bloom, with an exception of the high rate of chlorpyrifos that was applied only once at bud swell stage (~ 7 to 5 days prior to bud break). Treatment applications were made on the following dates: 12 April and 31 May, 2012; 12 April (for chlorpyrifos high rate) then 17 May and 12 June, 2013; 22 April (for chlorpyrifos high rate) then 12 May and 18 June 2014. Fungal diseases were controlled by the fungicide program described above.

Twenty petioles were taken from each panel (across all five vines) and pooled for GLRaV-3 testing using the LR3 RT-PCR procedure annually at the end of September. Throughout the seasons, mealybugs were visually counted every one to two weeks by a rater spending a total of 5 minutes per treatment plot (= a panel of 5 vines). The dates of counting were the same as for the Cabernet Sauvignon AHS AREC interplanting trial mentioned above.

Chardonnay Orange County, VA trial: A field trial during the 2013 and 2014 seasons was implemented at a commercial vineyard in Orange County, VA. A single row of Chardonnay (planted in 1989, Lyre system trained, 2.1 m between vines) with a previous history of mealybug infestation was used. A randomized complete block design with four blocks was implemented. Each block contained six consecutive vines, and treatments were applied on every other vine; thus, there was an untreated buffer vine between treated vines. Vines that received treatments within a block were randomly assigned. The three treatments examined were: 1) acetamiprid (Assail, 0.182 L/ha, United Phosphorus, Inc. [UPI], King of Prussia, PA); 2) insecticidal soap (M-Pede, 18.7 L/ha Gowan Co., Yuma, AZ); and 3) water (negative control). Treatments were

applied twice per season at bloom and two weeks post-bloom (7 June and 19 June, 2013 and 2 June and 16 June, 2014).

Throughout the seasons, mealybugs were counted every one to two weeks by a rater spending a total of 5 minutes per treated vine, 2.5 minutes per side. There were seven counting dates between 12 June and 20 August, 2013 and eleven between 2 June and 9 September, 2014. A final, early-season count was made in May 2015 to examine potential effects from previous-year treatments. Fungal diseases were controlled by a standard fungicide application program developed by the grower that should not affect mealybug activities. Other insecticides were regularly used in this vineyard, but not on the trial row and adjacent rows.

Rkatsiteli Orange County, VA trial: A field trial was implemented between 2013 and 2014 in a vineyard of Lyre-trained cultivar Rkatsiteli in another commercial vineyard in Orange County, VA. Vines were trained to a Lyre system and spacing between rows and between vines within a row was 3.6 m and 2.1 m, respectively. Two adjacent rows in this block were selected due to high mealybug populations observed in these rows. Foliar treatments were applied in a randomized complete block design with six repetitions spread across the two rows. Each block contained 12 consecutive vines, and every other vine received a treatment.

Six treatments tested were: 1) the neonicotinoid dinotefuran (Scorpion, 0.292 L/ha, Gowan Company, Yuma, AZ), 2) the tetramic acid derivative spirotetramat (Movento, 0.439 L/ha, Bayer CropScience LP), 3) the pyrethroid ß-cyfluthrin (Baythroid XL, 0.219 L/ha, Bayer CropScience LP), 4) a low rate of organophosphate chlorpyrifos (Lorsban, 1.4 L/ha Dow AgroSciences LLC, Indianapolis, IN), 5) a high rate of chlorpyrifos (Lorsban, 1.6 L/ha Dow AgroSciences LLC, Indianapolis, IN), and 6) no spray as a control. Treatments were assigned

randomly to vines within a block. In 2012, high rate of chlorpyrifos was applied 23 March, at dormant stage, the all other treatments were applied on 22 April for post-bud break and 16 May 2012 for bloom spray. For 2013, high rate of chlorpyrifos applied on 15 April, and the other treatments were applied on both 15 April and 19 June. For 2014, high rate of chlorpyrifos was applied on 7 May, and the other treatments were applied on both 7 May and 16 June.

Mealybugs were counted every one to two weeks throughout the season by a rater spending a total of 5 minutes per treated vine, 2.5 minutes per side. There were eight, seven, and eleven total counting dates between 30 April – 28 August, 2012, 28 May – 20 August, 2013 and 2 June – 9 September, 2014, respectively. A final, early season count was made on 20 May, 2015 to examine potential effects from previous year treatments. Fungal diseases were controlled by a standard fungicide application program developed by the grower that should not affect mealybug activities. Other insecticides were regularly used in this vineyard, but not on the trial rows and rows directly adjacent to the trial row.

Statistical Methods:

Spatial and spatio-temporal analyses on intensive sampling data were conducted using a software package of Spatial Analysis by Distance IndicEs, or SADIE (Perry 1998; Perry et al. 1996; Perry et al. 1999). SADIE uses a distance to regularity (D_a) method that, in this case, utilizes coordinates and counts of sampled grapevines. From the sampled location, these infected vines were virtually moved to create uniform distribution of virus infected vines in the map, then the shortest total distance individuals have to move is called a distance to regularity (D_a) (Nita et al. 2012). The SADIE package then randomizes the location of the virus infected vines, and recalculates the distance to regularity for the number of user-defined permutations. For our

assays, 9,999 permutations were assigned. Then, the average distance to regularity from permutations (E_a) is used to determine the index of aggregation (I_a) where $I_a=D_a/E_a$, where a value >1.5 indicates an aggregated spatial pattern of the virus infected vines (Nita et al. 2012).

In addition, spatio-temporal associations of one virus over two years can be examined using a cluster analysis in the SADIE package. Based on the level of aggregaion of virus infected vines between two years, cluster index (χ) for each vine in the dataset can classified into two groups, patch (higher in aggregation) and gap (lower in aggreagation) (Madden et al. 2007; Perry et al. 1999). The average cluster index (X) describes spatio-temporal association (or disassociation) among virus infected vines (Perry et al. 1999; Winder et al. 2001).

The interplanted Cabernet Sauvignon AREC vineyard's data were analyzed using a linear mixed model (PROC MIXED, SAS ver. 9.4, SAS institute, Cary, NC) for ANOVA, where the relative direction of the old vine, date, treatment, and interaction between date and treatment effects were considered as fixed factors, and repetition was considered as a random factor. The dependent variables examined were the mean number of mealbugs and the probability of detecting GLRaV-3. A generalized linear mixed model (PROC GLIMMIX, SAS ver. 9.4) was used for ANOVA for the other three vineyard's data. Date, treatment, and their interaction were considered fixed factors, and block was considered as a random factor. For the Merlot AHS AREC trial, dependent variables examined were the mean count of mealbugs and the probability of detecting GLRaV-3. For the two trials at Orange County, the dependent variable was the mean number of mealybugs per treatment. For the mean count of mealybugs and the probability of detecting GLRaV-3, Poisson and binomial distribution was assumed, respectively. When there was a significant effect of factor(s), Fisher's least significant difference (LSD) was used for examination of difference among treatments.

4.3 Results

Transmission of GLRaV-3 to healthy plants by *Ferissia gilli*

Validation of viruliferous nature: Initial testing for trans-ovarial passing of GLRaV-3 resulted in nine of eleven adult females positive for GLRaV-3 while all eleven 1st instar progeny were negative for GLRaV-3. These insects were also subjected for species identification and confirmed as *F. gilli*. With the initial acquisition assay, ten mealybugs were subjected to feeding on the acquisition vine for 24 hours, and then sampled to determine species ID and evidence of GLRaV-3 acquisition. All ten mealybugs were *F. gilli* according to multiplex PCR, and four out of ten were positive for GLRaV-3. Ten control mealybugs from the virus-free vine harbored no GLRaV-3.

GLRaV-3 transmission assay with F. gilli. All mealybugs used in the transmission assay (72 total), were confirmed to be *F. gilli* with the multiplex PCR following the study. When examining the presence of GLRaV-3 in these insects following transmission times, 1, 8, and 7 out of 12 mealybugs/time period were positive for GLRaV-3 for time periods 12, 24, and 48h respectively (Table 4.1). Although the sample size was small, these results demonstrated *F. gilli* was able to acquire GLRaV-3 after 12 hours of feeding, and suggest an approximately 67% rate of acquisition per mealybug after 24 hours of feeding period.

Three vines tested positive for GLRaV-3 at the end of August 2015, which was 2.5 months post-transmission (Table 4.2). One vine with the 24-hour feeding period was GLRaV-3 positive and two with the 48-hour feeding period (Table 4.2). All three vines that were positive were from vines that had 5 mealybugs/vine. At the end of October 2015, 4.5 months post-

transmission assay was conducted. The same three vines were positive for GLRaV-3 (Table 4.2). No vines were positive following 12 or fewer hours of feeding time, and no vines were positive for any time periods where only 1 mealybug was used for transmission, most likely due to the fact many mealybugs used in this study did not acquire GLRaV-3 (Table 4.1 and 4.2).

GLRaV-3 movement within Chardonnay, AHS AREC 2013-2015

The spread of GLRaV-3 occurred in all three years, with 4, 5, and 11 new vine infections in 2013, 2014, and 2015 (Fig. 4.1). Percent increase in infected vines from 2012-2013, 2013-2014, and 2014-15 were 10.8%, 12.2%, 23.9%, respectively. SADIE analysis of Index of Aggregation (I_a) revealed that in all three years, significant levels of aggregation were present, with values increasing each year (Table 4.3), suggesting a strong clustering pattern of infected vines. Association of clustering between years using SADIE's overall measure of spatio-temporal association chi (X) was X =0.95 ($P < 0.001$) for 2013-2014 and X =0.86 ($P < 0.001$) for 2014-2015, suggesting that between years, previously infected vines significantly influence the chance of a nearby vine being infected. These results indicate the spread of GLRaV-3 vines was driven by relatively short distance movement of mealybugs in this vineyard.

Insecticide trials

Cabernet Sauvignon AHS AREC interplanting trial: In 2012, fewer than one mealybugs were found on old vines and no mealybugs were found on young vines (Fig. 4.3 A) In 2013, there was still less than one mealybug found per old vine; however, a mealybug found on a young, ß-cyfluthrin treated vine (Fig. 4.3 B). In 2014 mealybug population increased compared with the

previous two years (Fig. 4.3 C). The actual mealybug counts per vine throughout the season ranged from 0 to 36. Multiple mealybugs were found on newly planted vines.

In 2014, ß-cyfluthrin treated vines generally maintained higher populations of mealybugs than the spirotetramat treated vines (Fig. 4.3). Treatment effects were not significant ($P > 0.05$) in all three years; however, there was a significant date and treatment interaction in 2014, indicating the effect of treatment varied by dates (Table 4.4). There were two peaks of the mean mealybug counts, occurring around early July and early August (Fig 4.3 C). Untreated control and ß-cyfluthrin were not significantly different from each other ($P > 0.05$), but five out of 13 count dates showed significant difference between ß-cyfluthrin and spirotetramat treatment (Fig. 4.3 C). Since spirotetramat treatment was not significantly different from the untreated control, it only indicated that ß-cyfluthrin treatment resulted in higher mean mealybug counts than spirotetramat.

GLRaV-3 did not spread from old vines to young vines in the first year of this study. However, in 2013, two cases of GLRaV-3 found to spread on two adjacent vines within the same ß-cyfluthrin treatment panel (Fig. 4.4). In 2014, increased spread of GLRaV-3 was observed in many sub-plots regardless of the treatment. Eight, six, and one vines were newly infected with GLRaV-3 following the 2014 season for the untreated control, ß-cyfluthrin, and spirotetramat treated vines respectively (Fig. 4.4). When the ANOVA was conducted on the effect of treatment on the probability of detecting GLRaV-3 positive vines, the treatment effect was significant ($F_{2, 33} = 3.4$, $P = 0.03$). Least square mean comparison based on LSD showed the probability of detecting GLRaV-3 from spirotetramat treated vines was significantly lower than ß-cyfluthrin or untreated treated vines, while ß-cyfluthrin and untreated were not significantly different from each other. At the beginning of 2015, only a handful of mealybugs were found on

untreated control and ß-cyfluthrin treated old vines. When the ANOVA was conducted on the effect of treatment from 2014 on the probability of finding mealybugs early in 2015, the treatment effect was not significant ($F_{2, 46} = 1.86$, $P = 0.17$). Excavated roots following the study did not harbor mealybugs, which confirmed the results from our previous study (Jones and Nita 2016), indicating that both grape and Gill's mealybugs are less likely to overwinter on the root system of grapevines.

Merlot AHS AREC trial: In all three years, no significant differences were found between treatments, as mealybug numbers were consistently low (Fig. 4.5). No mealybugs were found in 2012, but 2013 and 2014 had actual counts ranging from 0 to 1 mealybugs per vine. However, by the end of the third year, GLRaV-3 was detected in two out of four panels for both chlorpyrifos treatments (low and high rates) and spirotetramat. GLRaV-3 was also detected in three out of four panels for the ß-cyfluthrin and water control treatments.

GLRaV-3 was not detected in 2012 and 2013 in this plot; however, at the end of 2014, GLRaV-3 was detected in two out of four panels for both chlorpyrifos treatments (low and high rates) and spirotetramat, and three out of four panels for the ß-cyfluthrin and control treatments. The effect of treatments on the probability of detecting GLRaV-3 per sub-plot was examined. The results from the generalized linear mixed model showed there was no significant effect of treatment ($F_{4, 15} = 0.3$, $P = 0.87$). In addition, mealybugs on these vines were counted again at the beginning of 2015, one mealybug was found on one panel of both the water control and ß-cyfluthrin treated vines. These counts were so low that we were not able to run the analysis to show the effect of treatment in 2014 to mealybug counts at the beginning of the year 2015.

Chardonnay Orange County, VA trial: In 2013 mealybugs were only found at this location, mostly in July and August. This location also saw a mealybug population increase in 2014 with highest numbers of mealybugs present as the season progressed (the actual mealybug counts per vine throughout the season ranged from 0 to 16.). In both years, no significant treatment effect ($P \leq 0.05$) was found (Table 4.4), in spite of higher mealybug counts at this location than other trial locations (Fig. 4.6). In 2014, the treatment effect was marginally significant ($P = 0.07$), where acetamiprid resulted in numerically lower mealybug counts than M-Pede or the water control. At the beginning of 2015, only a handful of mealybugs were found on all three treatments. When the ANOVA was conducted on the effect of treatment from 2014 on the probability of finding mealybugs early in 2015, the treatment effect was not significant ($F_{2, 8} = 1.43$, $P = 0.30$).

Rkatsiteli Orange County, VA trial: During 2012 and 2013, low numbers of mealybugs per vine were recorded (Fig. 4.7 A and B). This location experienced a similar increase in 2014 mealybug population as other trial locations. The actual mealybug counts per vine throughout the season ranged from 0 to 29. The majority of mealybugs were found in mid-June, mid-July, and late-August.

In both 2012 and 2013, neither date nor treatment was significant (Table 4.4). In 2014, the effects of date, treatment, and interaction between two were significant on the count of mealybugs (Fig. 4.7). Early in 2014, the mean counts of mealybugs on ß-cyfluthrin, spirotetramat, and high rates of chlorpyrifos treatment were often significantly lower than untreated control (e.g., 10 June in Fig. 4.7 C). On 1 July, 2014 and again on 15 July to 12 August, 2014 the overall mealybug counts became low (Fig. 4.7 C). This was most likely due to mealybug seasonal biology, which is reflective of adult female mealybug movement back under

bark tissues to give birth to a new generation (Geiger and Daane 2001). Then, as the season progressed, the mean number of mealybugs on ß-cyfluthrin became relatively higher, while that of both spirotetramat, and high rates of chlorpyrifos treatments remained low (Fig. 4.7 C). At the beginning of 2015, mealybugs were found on vines of all treatments. However, when the ANOVA was conducted on the effect of treatment from 2014 on the probability of finding mealybugs in early 2015, the treatment effect was not significant ($F_{2, 29} = 1.83$, $P = 0.14$).

4.4 Discussion:

This is the first documented report of *Ferissia gilli* as a vector of GLRaV-3. Other than recent unpublished data ability to acquire and transmit GLRaV-3 by this species was not proven in the past as cited by (Wunderlich et al. 2013). Acquisition results suggest although *F. gilli* was able to acquire GLRaV-3 after 6 hours of feeding, the rate of acquisition was very low (1/12 or 8.3%) with shorter feeding time. An approximately 67% rate of virus acquisition by *F. gilli* following 24 hours of feeding period was shown, and results from 48 hours of feeding did not differ greatly (58%). The vine mealybug, which is considered to be the most efficient at transmission of GLRaV-3 (Tsai et al. 2010) was shown to successfully acquire the virus after 1 hour of plant feeding with the success rate steadily increasing up to the maximum rate at 24 (around 60-70%) (Tsai et al. 2008). Thus, our results suggested that the maximum rate of acquisition between two species could be similar.

The transmission study showed one plant (out of two) following a 24-hour transmission period and two plants (i.e. all plants) at a 48-hour period were confirmed with GLRaV-3 after a 3-month incubation period. Due to the small sample size, we were not able to determine the effect of acquisition time to the rate of transmission, but the results may suggest higher rate of

transmission with longer acquisition time. With the vine mealybugs, a significant effect of longer acquisition period on better probability of successful transmission was confirmed (C. W. Tsai et al. 2008). The results of our transmission assays supported the semi-persistent nature of mealybug transmission of closteroviruses (Martelli et al. 2002a; Ng and Falk 2006). *F. gilli* is able to acquire GLRaV-3 within 24 hours and transmit GLRaV-3 within 24 hours. Trans-ovarial passing of GLRaV-3 was not found in *F. gilli* consistent with the results from vine mealybug testing (Tsai et al. 2008).

The Gill's mealybug is a hypothesized native to the southeastern United States (Gullan et al. 2003) and has been recently described as a pest of pistachios, almonds, and grapes in California (Gullan et al. 2003; Haviland et al. 2006). This specific vector can cause damage to fruit clusters directly (Wunderlich et al. 2013) but is not known to transmit any other viruses at this moment; however, its ability to transmit GLRaV-1 and members of the rugose wood complex should be investigated as other common vineyard mealybugs have this capability (e.g. vine and grape mealybugs) (Tsai et al. 2010; Boscia et al. 1997).

Gill's mealybugs are reported to have two in-season generations in California (1st instars appearing in June and late-August/early-September) with second and third instars as the overwintering stages found under bark along the cordon and trunks (Wunderlich et al. 2013). A partial third generation can occur with this insect that is dependent on vineyard location and seasonal temperatures (Wunderlich et al. 2013). The Gill's mealybug is polyphagous, feeding on many different plants including many woody shrubs and trees (both evergreen and deciduous) as well as areca palms and barnyard grasses (Gullan et al. 2003). Morphological identification of *Ferrisia* species has been historically difficult (Gullan et al. 2003), thus molecular techniques should be utilized for accurate identification. With it's wide host ranges and ability to transmit

GLRaV-3, vineyard managers need to be aware of this mealybugs presence in their fields and take actions to help manage this pest.

The spatial and insecticidal portion of this study was a follow up to our previous work (Jones and Nita 2016) to aid in understanding the spread of GLRaV-3 and management of mealybug population by the use of systemic materials so that the spread of GLRaV-3 can be minimized. The spatial analysis of GLRaV-3 in AHS AREC Chardonnay vineyard, showed consistent clustered/aggregated pattern in 2013, 2014, and 2015. Each year aggregation levels of infected vines increased, as was the case in 2010-2012 (Jones and Nita 2016) and also in multiple seasons in Spain (Cabaleiro et al. 2008). An observed spread between years of 10.8%, 12.2%, 23.9% shows how rapid GLRaV-3 can spread to clean vines with multiple vectors (*Pseudococcus maritimus* and *Ferissia gilli*) present in the field. Rates of spread of GLRaV-3 have been found to differ among studies. A study in California observed an average rate of spread of 10% per year for GLRaV-3 (Golino et al. 2008), observations at a vineyard in Spain showed a slower spread with approximately 3% per year (Cabaleiro et al. 2008), and several years of observation in New Zealand demonstrated a much higher rate of almost doubling in a year following several years of slow spread (Habili and Nutter 1997).

Considering very fast rate of GLRaV-3 spread, the acquisition and transmission efficiency under field conditions might increase compared to what we have observed in the lab, or we may have larger number of mealybugs in our field than were observed. Transmission efficiency can vary even within virus and vector species for plant viruses that are vector borne (Burrows et al. 2007; Gray et al. 2007; Yokomi and DeBorde 2005). Also, biological and environmental factors are important in vector transmission success, where proven vectors sometimes will not transmit the respective virus given various conditions. Greenhouse/potted

plant conditions are different than field conditions in terms of weather, natural predators, management strategies, and pesticide drift. It has also been shown that genetic differences in virus isolates being used as well as different biotypes of the same insect species can negatively affect transmission test results (Hogenhout et al. 2008; Ng and Falk 2006).

In addition to the evidence of aggregation, a strong spatio-temporal association with GLRaV-3 positive vines between 2013-2014 and 2014-2015. This association between newly infected and previously infected vines suggested relatively short distance movement of mealybugs (Cabaleiro et al. 2008; Geiger and Daane 2001; Jones and Nita 2016). As it was shown in our acquisition and transmission assays, 1st instars of *F. gilli*, which are widely present in the AHS AREC Chardonnay vineyard, are capable of infecting healthy grapevines. These 1st instars are very small and more mobile than other life stages (Tsai et al. 2010; Tsai et al. 2008). In the field, they can walk short distances to adjacent vines, or be blown to nearby vines through natural and man-made (sprayer equipment) air currents, or even transferred by vineyard workers through clothing, clippings/cutting, or pruning equipment. In our case, strong eastward spread of the GLRaV-3 along the row direction suggested that wind and movement by vineyard workers were the primary mode of movement.

Two trials at the AHS AREC aimed to prevent the movement of mealybugs and GLRaV-3 to newly planted grapevines. The Cabernet Sauvignon AHS AREC interplanting trial portrayed the case when growers replace infected vines with new virus-tested vines within a vineyard. The field trial at the Merlot AHS AREC plot was used to examine the effects of five treatments (with an untreated control) on preventing the entry of mealybugs and GLRaV-3 into a healthy planting, which was located adjacent to an infected vineyard block with mealybugs. In both studies, mealybug populations were consistently very low and caused treatment effect to only be

significant in 2014 (Cabernet Sauvignon AHS AREC interplanting trial), and never significant (Merlot AHS AREC trial). However, regardless of the treatment effect or lack of mealybugs, results of both trials showed the spread of GLRaV-3 to newly planted vines (Cabernet Sauvignon AHS AREC interplanting trial) and new blocks (Merlot AHS AREC trial).

Two field trials at Orange County, VA were aimed to reduce the existing mealybug population using insecticides with different modes of action. Mealybug populations in the Chardonnay vineyard were generally higher than other trial locations; however, treatment effects were not significant, even with the use of a systemic insecticide, acetamiprid. The mean mealybug counts for acetamiprid in 2014 was numerically lower than that of an insecticidal soap (M-pede) or water sprayed check; however, it was marginally significantly ($P = 0.07$). These results and also the lack of its efficacy from our previous trial (Jones and Nita 2016) may indicate relatively low efficacy of acetamiprid to mealybug population in VA.

At the Rkatsiteli Orange County, VA trial, the mean mealybug counts for ß-cyfluthrin treatment was lower at the beginning of the season, showing its effectiveness as a contact insecticide; however, as the season progressed, the mean mealybug counts increased, as was observed in the Cabernet Sauvignon AHS AREC interplanting trial. This result strengthens our concern on the potential negative effect caused by a broad-spectrum contact insecticide. In VA, ß-cyfluthrin is not currently recommended for use on grape with the speficific intention of mealybug and virus management (Pfeiffer et al. 2016) and should be used later in the season for the control of spotted-wing drosphila. This chemicals broad spectrum abilities and has been found to disrupt populations of natural enemies of mealybugs (Daane et al. 2012; Mgocheki and Addison 2009; Walton and Pringle 2001; Pfeiffer et al. 2016) resulting in increased mealybug population (Jones and Nita 2016) and a decrease in natural predators.

Foliar applications of dinotefuran, spirotetramat, and chlorpyrifos (low rate) all resulted in significantly low counts of mealybugs, especially late in the season. This is promising results that at least we have several modes of action group that can be used for mealybug control. chlorpyrifos is a broad spectrum contact insecticide, sharing the organophosphate mechanism affecting the nervous system by inhibiting acetylcholine breakdown causing overstimulation while will lead to neurotoxicity and death (Smegal). Dinotefuran is a neonicotinoid with one-way systemic activity that selectively binds to, and thus blocks, insect nicotinic acetylcholine receptor sites causing paralysis and death (Wakita et al. 2003).

Based in results from this study, our previous study (Jones and Nita 2016), and the current pest management guide for Virginia (Pfeiffer et al. 2016), spirotetratmat appeared to be the best option as it provided the significant reduction in the GLRaV-3 spread with Cabernet Sauvignon AHS AREC interplanting trial., as it was shown in another study in New York (Wallingford et al. 2015). It also outperformed other chemicals in regards to reduction of mealybug population at Cabernet Sauvignon AHS AREC interplanting and Rkatsiteli Orange County, VA trials. Spirotetratmat's primary targets are sucking insect pests (Bell et al. 2008; Brück et al. 2009) and has been shown to provie a 70% decrease in mealybug populations when applied twice in the season (prior to bloom and 30 days post bloom) (Loeb 2012).

Spirotetramat is a tetramic acid derivative that has a two-way systemic action [also called ambimobility or two-way systemicity (Bangels et al. 2011; Bell et al. 2008; Brück et al. 2009; Nauen et al. 2008)], allowing the chemical to move both up and down within a plant via both xylem and phloem. Spirotetramat does not interact with acetylcholine like dinotefuran or chlorpyrifos do. Instead, this ketoenole inhibits lipogenesis in insects which will decrease lipid synthesis, inhibit young insect growth, and reduce the ability of adult insects to reproduce

(Bangels et al. 2011; Bell et al. 2008; Brück et al. 2009; Nauen et al. 2008). For phloem feeding insects, like mealybugs, systemic activities work better at reducing the overall populations. One might expect a more significant effect from spirotetramat than dinotefuran or chlorpyrifos as spirotetramt has two-way systemic activity (Bangels et al. 2011; Bell et al. 2008; Brück et al. 2009; Nauen et al. 2008) that allows the chemical movement through both the phloem and xylem of the plant whereas dinotefuran or chlorpyrifos have only one way (phloem) system action.

Additionally, a high rate of chlorpyrifos, which was applied to bark tissues on cordons and the main trunk only once before bud break, consistently resulted in low mealybug counts throughout the season in 2014. This result showed the effect of delayed dormant application of chlorpyrifos against overwintering population of mealybugs. However, it should be noted that although tested in this trial and also at Merlot AHS AREC trial, the US label of chlorpyrifos (tradename Lorsban, Bayer Crop Science) currently does not list mealybugs on grapes as target insects and this material will be phased out over the next few years.

This research supports our previous findings that foliar applications of insecticides can work as a suitable option for application of control methods for mealybugs on grapevine. It has been shown elsewhere that drenching, chemigation, and soil injection applications could perform better than foliar application. For example, in California one group determined irrigation systems applying buprofezin (Applaud, Nichino America, Wilmington, DE) better controlled the vine mealybug when compared to foliar applications of either imidacloprid (Admire, Bayer Crop Science, Research Triangle Park, NC) or chlorpyrifos (Lorsban) (Daane et al. 2006). On the other hand, foliar application is very common in VA and other part of grape growing regions since it does not require any special equipment other than a foliar sprayer which growers already own.

A consistently found significant effect of date on count of mealybugs at three of the four trial plots throughout two to three years of trial duration suggested seasonal change in mealybug numbers. Figure 4.3 and Figure 4.6A, which were from two separate locations in VA showed two clear peaks in mealybug numbers (early July and late July; mid-June and mid-August). These two peaks are most likely time points when young mealybugs hatched earlier in the season became large enough to be easily visible. The similar trends with two seasonal peaks were observed in our previous study (Jones and Nita 2016). Figure 4.7 shows three peaks (early-June, mid-July, and late-August), which suggested potentially three overlapping generations under VA environmental conditions for a combination of *F. gilli* and *Ps. Maritimus*. This data suggests that not only can multiple species of mealybugs co-exist on the same vines, but they also seem to have two to three in-season generations in Virginia.

In our previous study (Jones and Nita 2016), we conculded that application of a systemic insecticides soon after bud break was not approprate for mealybug management under Virginia conditions. Thus, applications on this study were timed around late spring/early summer to target emergence of 1st instars. This application timing has shown good results in other studies (Daane et al. 2006; Haviland et al. 2010a, 2010b, 2011; Jones and Nita 2016; Wallingford et al. 2015). Indeed, spirotetratmat, dinotefuran, and chlorpyrifos were able to reduce the mealybug population compared to untreated vines or broad-spectrum insecticides; however, these treatments were still not enough to completely stop the spread of GLRaV-3.

The lack of efficacy against the spread of GLRaV-3 poses a large issue not only when growers interplant new virus-tested vines among older vines (which was shown in the Cabernet Sauvignon AHS AREC interplanting trial.) but also when growers are willing to replant an entire vineyard with new virus-tested vines. In order to avoid loss in production, growers are most

likely to plant new vineyards 2-3 years prior to removal of old infested vineyard so that young vines will be in production by the time of rouging. However, as demonstrated in Merlot AHS AREC trial, even when very low mealybug populations were present, GLRaV-3 was detected among panels of vines regardless of treatments. Our results from the Cabernet Sauvignon AHS AREC interplanting trial showed significant reduction in the probability of detection of GLRaV-3 with spirotetramat, but we probably need more sample sizes to confirm the efficacy under different conditions. Thus, further studies to address both suppression of mealybug populations in an existing vineyard and protection of newly planted vineyard would be beneficial for growers.

In summary, this study demonstrated GLRaV-3 acquisition and transmission by *F. gilli*, and determined effective chemical groups (spirotetramat, dinotefuran, and chlorpyrifos) to control mealybug populations. However, even the best material targeting times of emergence of 1st instars early in the season was not able to completely prevent the spread of GLRaV-3. Our study also revealed that the rate of spread of GLRaV-3 under Virginia condition can be very rapid.

Table 4.1: Numbers of successful GLRaV-3 acquisition by *F. gilli* after feeding on GLRaV-3 infected grapevine for the designated time periods.

	Acquisition time (in hours)					
	1	**2**	**6**	**12**	**24**	**48**
GLRaV-3-positive *F. gilli*[a]	0/12	0/12	0/12	1/12	8/12	7/12

[a] (number of GLRaV-3-positive insect)/(number of GLRaV-3-negative insect)

Table 4.2: Numbers of GLRaV-3-positive *F. gilli* immediately following time of transmission, and grapevines after 2.5 and 4.5 months post-transmission.

			Transmission time (in hours)					
Month after feeding[a]	**Number of *F. gilli* per vine[b]**	**GLRaV-3-positive[c]**	**1**	**2**	**6**	**12**	**24**	**48**
2.5	1	*F. gilli*[d]	0	0	0	0	1	1
		grapevine	0	0	0	0	0	0
	5	*F. gilli*[e]	0	0	0	1	7	6
		grapevine	0	0	0	0	1	2
4.5	1	grapevine	0	0	0	0	0	0
	5	grapevine	0	0	0	0	1	2

[a] The same vines were assessed at 2.5 and 4.5 months after feeding.

[b] The number of *F. gilli* placed onto each potted virus-free Cabernet franc vines. For each acquisition time and the number of *F. gilli* combination, two vines were used. (Note that numbers are per mealybug or per group of 5)

[c] Detection of GLRaV-3 was conducted immediately after feeding

[d] Two vines used for each transition time period = two total *F. gilli* per time period

[e] Two vines used for each transition time period = ten total *F. gilli* per time period

Table 4.3: Percentage of GLRaV-3-positive vines, index of aggregation (I_a), and overall index of clustering (X) from SADIE outputs based on observation with a 10x10 sampling grid at the Chardonnay AHS AREC vineyard 2013-2015.

Year	GLRaV-3 (%)[a]	I_a[b]	X[c]
2013	41	2.2*	N/A
2014	46	2.4*	0.95**
2015	57	2.6*	0.86**

[a] Percentage (%) of virus-positive vines based on RT-PCR results

[b] Index of aggregation values (I_a) of GLRaV-3-positive vines from SADIE shell nonparametric analysis. Significant levels of aggregation ($P \leq 0.05$) are denoted by an asterisk.

[c] Overall index of clustering of GLRaV-3-positive vines between two years from SADIE. Significant level of spatio-temporal association ($P \leq 0.01$) was denoted by two asterisks.

Table 4.4: ANOVA tables for the Cabernet Sauvignon AHS AREC interplantings, Merlot AHS AREC, Chardonnay Orange, and Rkatsiteli Orange trials to examine the effects of relative direction of older vine (only at Cabernet Sauvignon AHS AREC interplanting trial.), date of mealybug count, treatment, and date and treatment interaction.

AHS AREC Interplant						
	2012		**2013**		**2014**	
Effect	*F*[a]	*P*-value[a]	*F*[a]	*P*-value[a]	*F*[a]	*P*-value[a]
Direction	0.95	0.33	1.25	0.26	1.30	0.25
Date	0.69	0.68	1.52	0.15	3.55	<0.001**
Treatment	2.76	0.10	6.82	0.01**	2.50	0.12
Date*Treatment	0.90	0.55	0.67	0.83	1.81	0.01**
AHS AREC Merlot						
	2012		**2013**		**2014**	
Effect	*F*[a]	*P*-value[a]	*F*[a]	*P*-value[a]	*F*[a]	*P*-value[a]
Date	0.00	1.00	0.00	1.00	0.00	1.00
Treatment	0.00	1.00	0.00	1.00	0.00	1.00
Date*Treatment	0.00	1.00	0.00	1.00	0.00	1.00
Orange Chardonnay						
			2013		**2014**	
Effect			*F*[a]	*P*-value[a]	*F*[a]	*P*-value[a]
Date			5.11	<0.001**	7.47	<0.001**
Treatment			0.00	1.00	2.76	0.07
Date*Treatment			0.72	0.73	0.86	0.64
Orange Rkatsiteli						
	2012		**2013**		**2014**	
Effect	*F*[a]	*P*-value[a]	*F*[a]	*P*-value[a]	*F*[a]	*P*-value[a]
Date	0.03	1.00	0.00	1.00	9.59	<0.001**
Treatment	0.00	1.00	0.00	1.00	19.87	<0.001**
Date*Treatment	0.10	1.00	0.23	1.00	1.81	<0.001**

[a] *F* statistic from a generalized linear mixed model (PROC GLIMMIX, SAS 9.4), *P*-value is to indicate significantly high treatment effect. One and Two asterisks indicate 95% and 99% confidence, respectively.

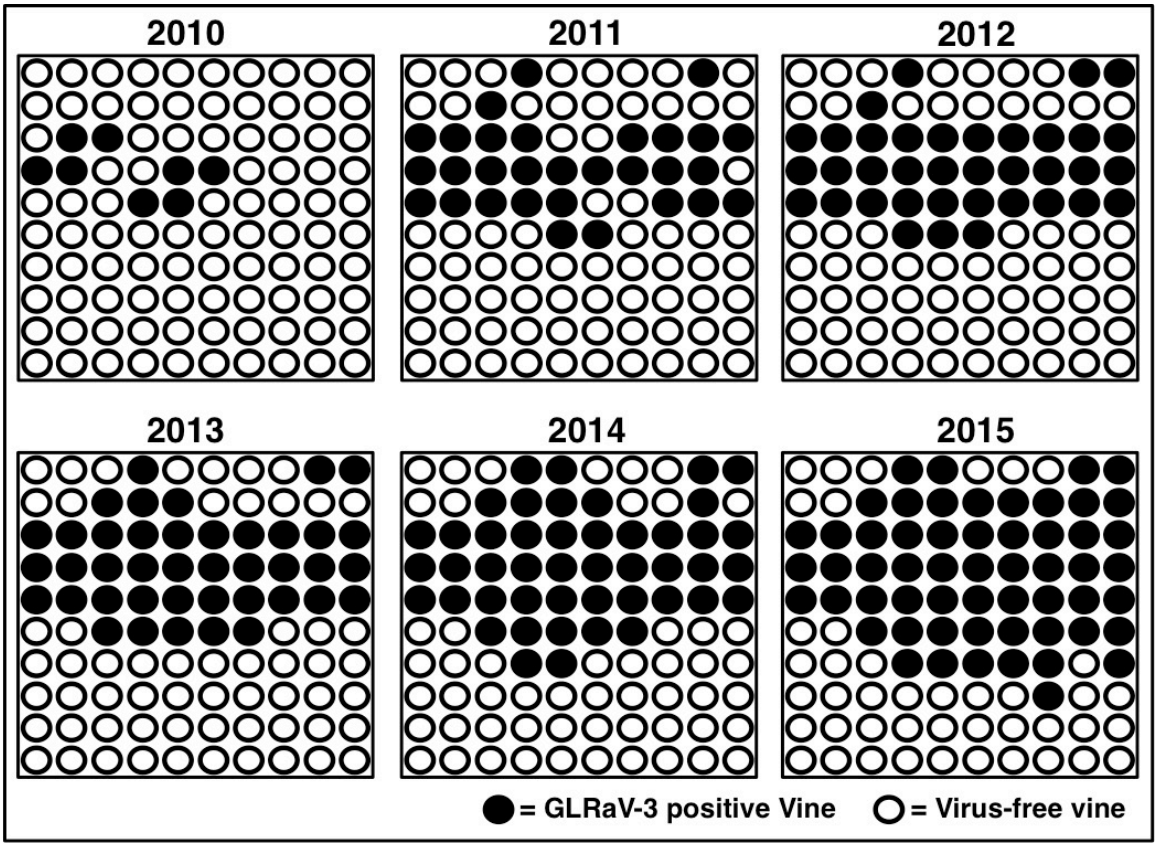


Figure 4.1: Spread of GLRaV-3 through 10 rows x 10 vine field of cultivar Chardonnay. Directly above the plot is a Cabernet Sauvignon vineyard with a history of GLRaV-3 and mealybugs. GLRaV-3 positive vines as discovered through RT-PCR at end of season are shown as filled in (black, darkened) circles. The top three panels (2010-2012) were copied from the previously reported study (Jones and Nita 2016).

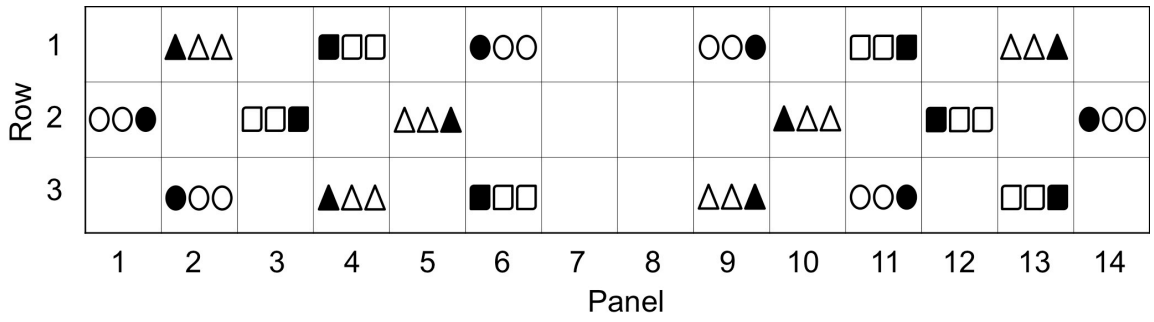
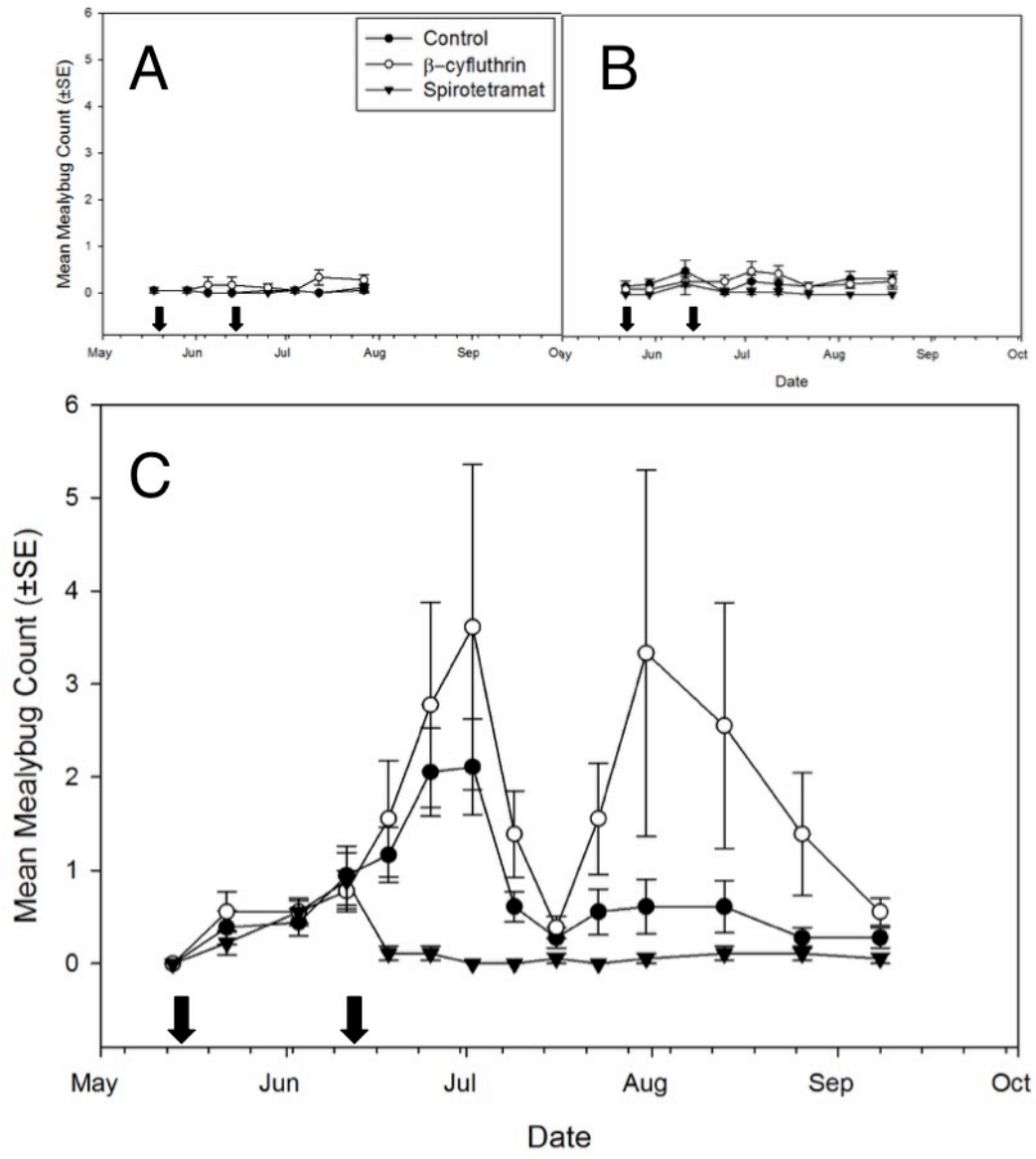


Figure 4.2: Cabernet Sauvignon AHS AREC interplanting trial diagram: Cabernet Sauvignon inter-planted vineyard layout at the Winchester, AHS AREC, VA, USA. Each symbol represents one vine, with blackened symbols representing old, GLRaV-3 infected Cabernet Sauvignon vines and open symbols representing the inter-planted, healthy young Cabernet franc vines planted at 1.5 and 3 meters from each old vine within treatment. Symbols represent three treatments: Circle (Control), Triangle (ß-cyfluthrin, Baythroid XL, 0.219 L/ha), Square (spirotetramat, Movento, 0.439 L/ha). Blank spaces were panels that contained no grapevines, or gaps.



2014	5/22	6/03	6/11	6/18	6/25	7/02	7/09	7/16	7/23	7/31	8/13	8/26	9/08
● Control	A	A	A	AB	AB	A	AB	AB	A	A	A	A	AB
○ β-cyfluthrin	A	A	A	A	A	A	A	A	A	A	A	A	A
▼ Spirotetramat	A	A	A	B	B	A	B	B	A	A	A	A	B

Figure 4.3: The mean and standard error of mealybug counts per treatment per date from the Cabernet Sauvignon AHS AREC interplanting trial in Winchester, VA, USA in 2012 (A), 2013 (B), and 2014 (C). There were eight total counting dates between 18 May and 27 July, 2012.

there were nine total counting dates between 22 May and 19 August, 2013. There were thirteen total counting dates between 22 May and 8 September, 2014. Table below the figure panel C depicts the specific significant differences among treatments by date for 2014. Different letters within dates show significant differences ($P \leq 0.05$) between treatments using Fisher's LSD. Treatment application dates are marked on figure with arrows: 19 May and June 14 2012; 23 May and June 12, 2013; 22 May and 14 June 2014.

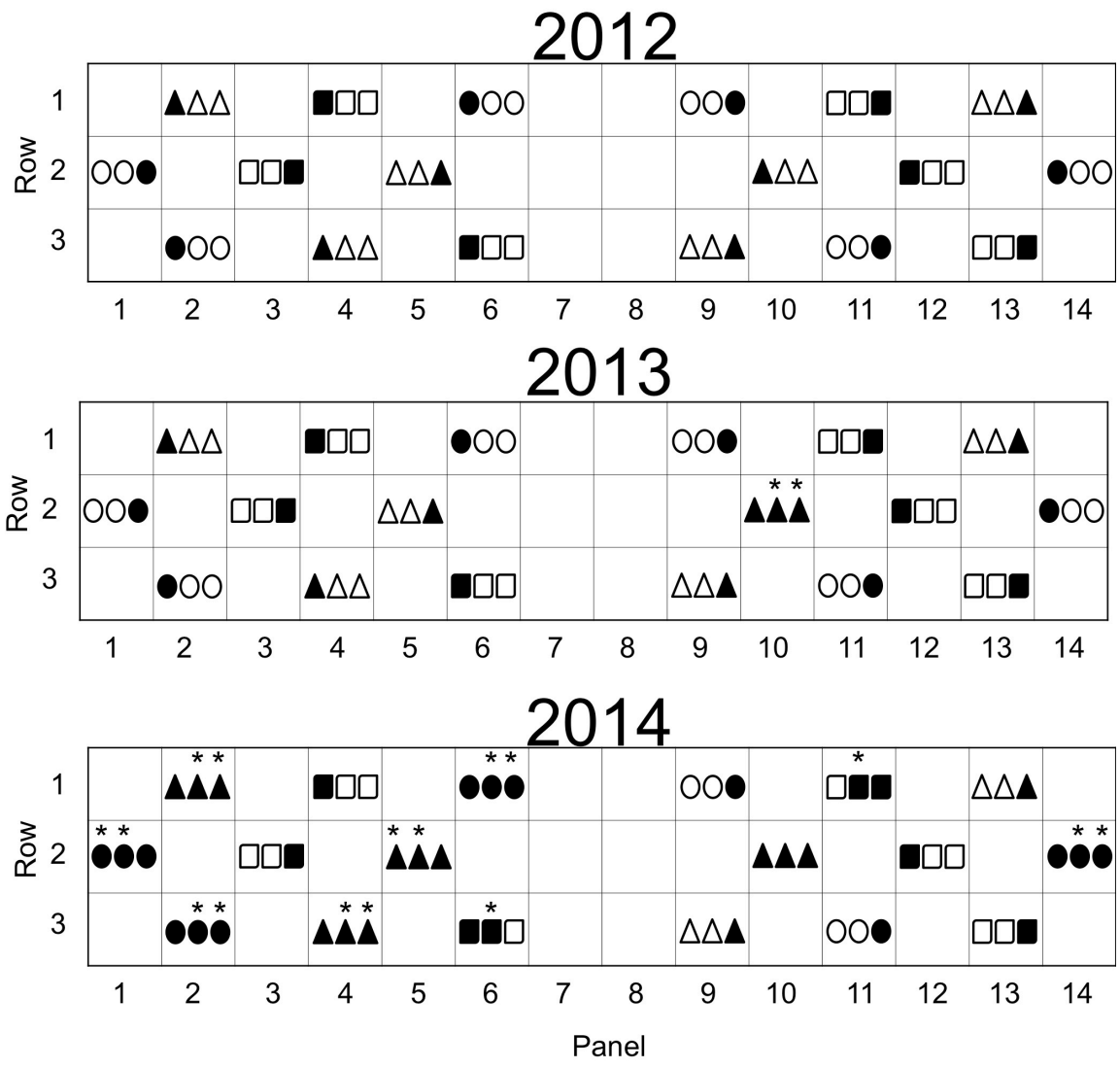


Figure 4.4: Spread of GLRaV-3 through the vineyard at the Cabernet Sauvignon AHS AREC interplanting trial. Each symbol represents one vine, with blackened symbols representing old, GLRaV-3 infected Cabernet Sauvignon vines and open symbols representing the inter-planted, virus-free young Cabernet franc vines planted at 1.5 and 3 meters from each old vine within treatment. Symbols represent three treatments: Circle (Control), Triangle (ß-cyfluthrin, Baythroid XL, 0.219 L/ha), Square (spirotetramat, Movento, 0.439 L/ha). Blank spaces were panels that contained no grapevines, or gaps. New infections by year are filled in and marked with an asterisk (*).

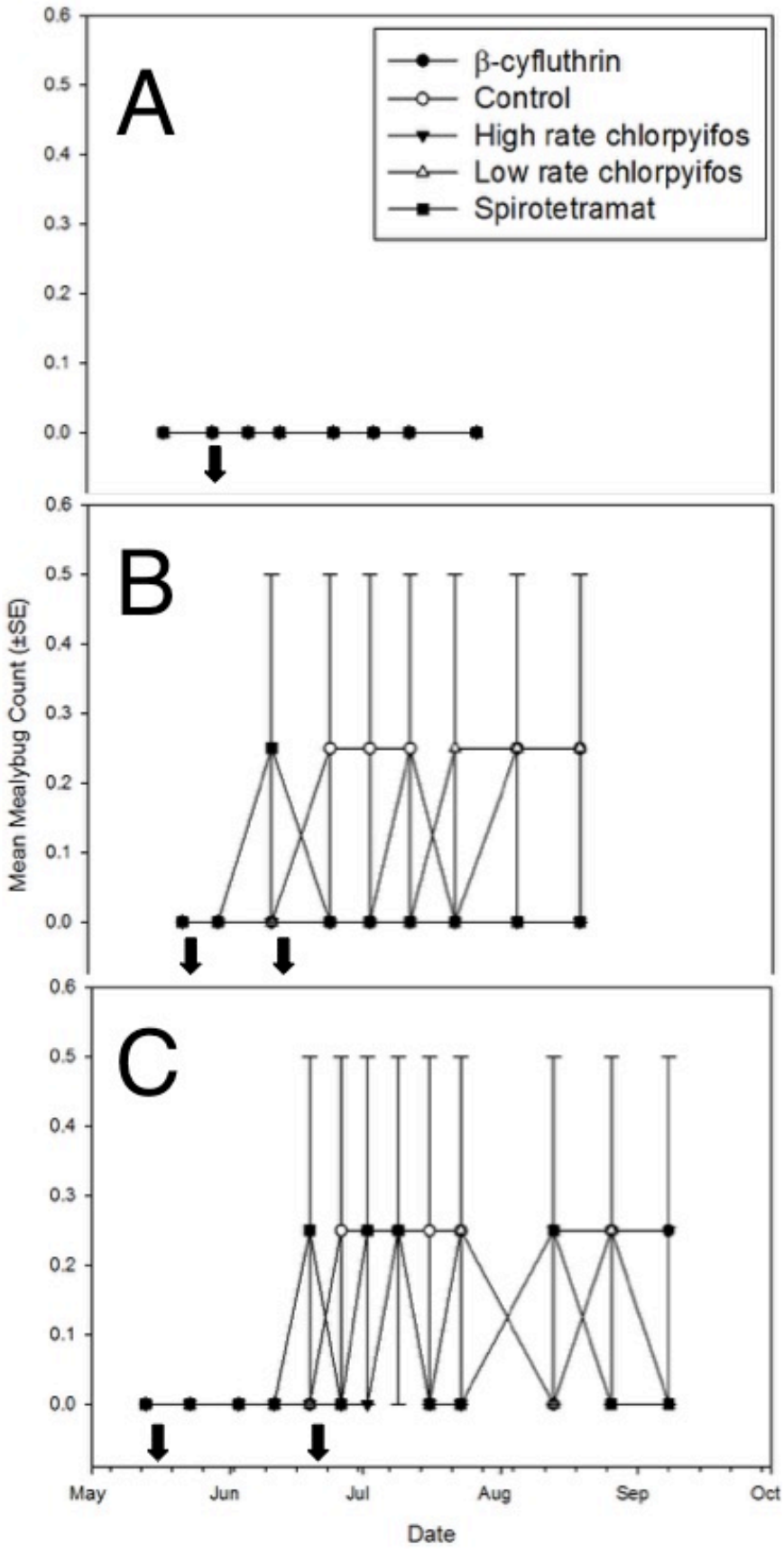

A
B
C
β-cyfluthrin
Control
High rate chlorpyifos
Low rate chlorpyifos
Spirotetramat
Mean Mealybug Count (±SE)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
May
Jun
Jul
Aug
Sep
Oct
Date

Figure 4.5: The mean and standard error of mealybug count per treatment per date from the Merlot AHS AREC trial in Winchester, VA, USA throughout time in 2012 (A), 2013 (B), and 2014 (C). There were eight total counting dates between 18 May and 27 July, 2012. There were nine total counting dates between 22 May and 19 August, 2013. There were thirteen total counting dates between 22 May and 8 September 2014. Treatment application dates are marked on figure with arrows: 12 April and 31 May, 2012; 12 April (for chlorpyrifos high rate) then 17 May and 12 June, 2013; 22 April (for chlorpyrifos high rate) then 12 May and 18 June 2014.

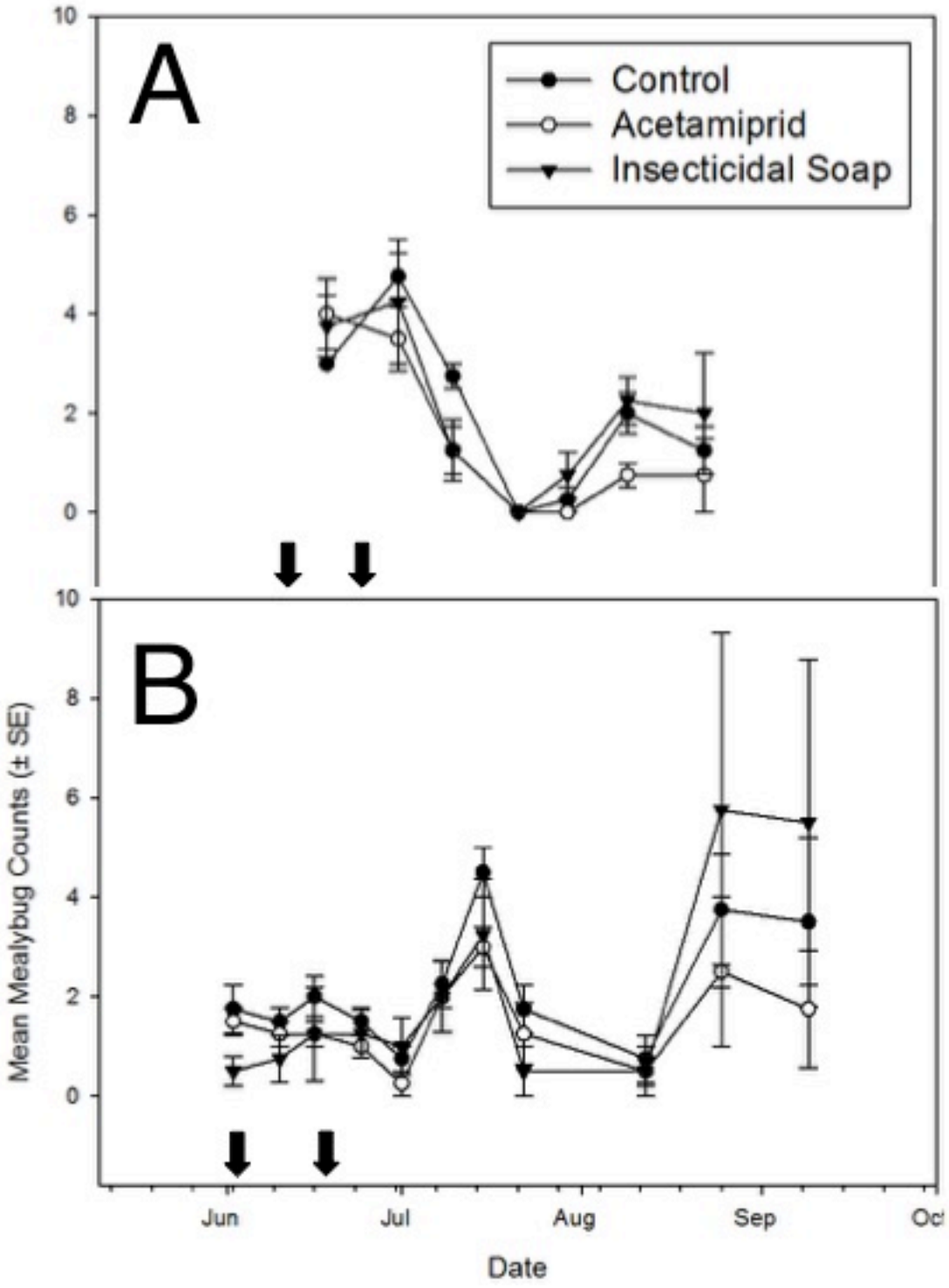
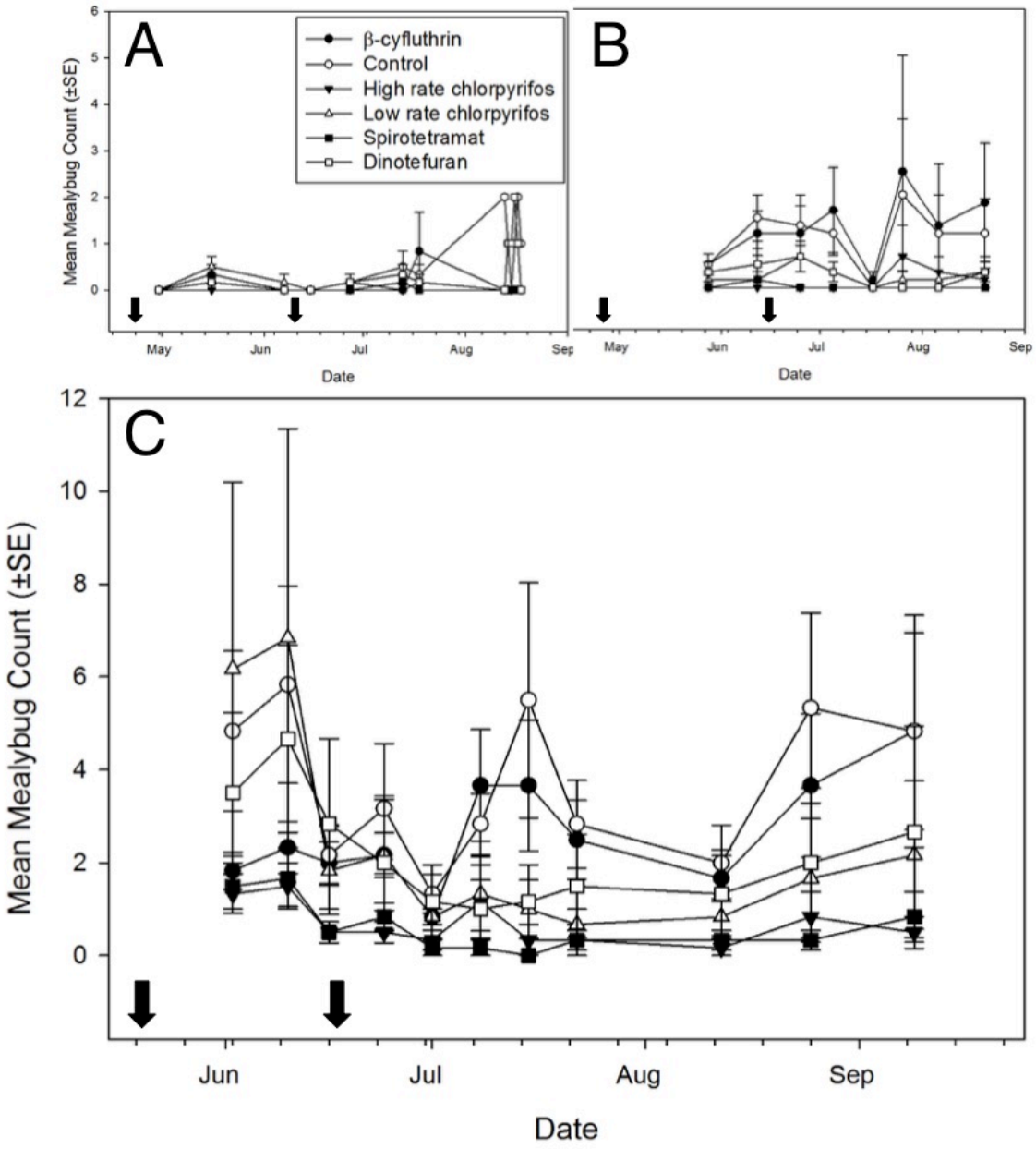


Figure 4.6: The mean and standard error of mealybug count per treatment per date from the Chardonnay Orange County, CA trial in 2013 and 2014 seasons. There were seven total counting dates between 12 June and 20 August, 2013. There were eleven total counting dates between 2

June and 9 September, 2014. Treatment application dates are marked on figure with arrows: 7 June and 19 June, 2013 and 2 June and 16 June, 2014



2014	6/02	6/10	6/16	6/24	7/01	7/08	7/15	7/22	8/12	8/25	9/9
● β-cyfluthrin	CD	B	A	AB	A	A	A	A	A	AB	A
○ Control	AB	A	A	A	A	AB	A	A	A	A	A
▼ High rate chlorpyrifos	D	B	B	C	A	BC	B	B	B	CD	D
△ Low rate chlorpyrifos	A	A	AB	AB	A	BC	B	B	AB	C	BC
■ Spirotetramat	D	B	B	BC	A	C	AB	B	B	D	CD
□ Dinotefuran	BC	A	A	AB	A	C	B	AB	AB	BC	AB

Figure 4.7: The mean and standard error of mealybugs per treatment per date from the Rkatsiteli Orange County, VA trial in Orange, VA, USA in 2012 (A), 2013 (B), and 2014 (C). There were eight total counting dates between 30 April and 27 July, 2014. There were eight total counting dates between 28 May and 20 August 2013. There were eleven total counting dates between 2 June and 9 September 2014. Treatment application dates are marked on figure with arrows: High rate chlorpyrifos 19 April, other materials on 14 June 2012. High rate of chlorpyrifos 25 April, 2013, all other materials on 19 June 2013. For 2014, High rate of chlorpyrifos 7 May, 2014 all other treatments were applied 16 June 2014. Table below the figure panel C depicts the specific significant differences among treatments by date for 2014. Different letters within dates show significant differences ($P \leq 0.05$) between treatments using Fisher's LSD.

4.5 References

Alley, C., Goheen, A., Olmo, H., & Koyama, A. (1963). The effect of virus infections on vines, fruit and wines of Ruby Cabernet. *American Journal of Enology and Viticulture, 14*(3), 164-170.

Bangels, E., Peusens, G., Belien, T., & Gobin, B. (2011). Compatibility of spirotetramat (Movento) in integrated pome fruit production. *Acta Hort., 917*, 77-83.

Bell, J., Krueger, S., & Steffens, R. (2008). Development of MoventoTM for sucking pest control on annual and perennial crops in the United States. *Bayer CropScience Journal, 61*(2), 307-328.

Boscia, D., Minafra, A., & Martelli, G. P. (1997). Filamentous viruses of grapevine: Putative trichoviruses and capilloviruses. In P. L. Monette (Ed.), *Filamentous viruses of woody plants*. (pp. 19-28): Trivandrum: Research Signpost.

Brück, E., Elbert, A., Fischer, R., Krueger, S., Kühnhold, J., Klueken, A. M., et al. (2009). Movento< sup>®</sup>, an innovative ambimobile insecticide for sucking insect pest control in agriculture: Biological profile and field performance. *Crop Protection, 28*(10), 838-844.

Burrows, M. E., Caillaud, M. C., Smith, D. M., & Gray, S. M. (2007). Biometrical genetic analysis of luteovirus transmission in the aphid *Schizaphis graminum*. *Heredity, 98*(2), 106-113, doi:10.1038/sj.hdy.6800909.

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, doi:10.1007/s10658-007-9254-1.

Cabaleiro, C., Segura, A., & Garcia-Berrios, J. J. (1999). Effects of *grapevine leafroll-associated virus 3* on the physiology and must of Vitis vinifera L. cv. Albarino 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). *59*, 330-337.

Credi, R., & Babini, A. R. (1997). Effects of virus and virus-like infections on growth, yield, and fruit quality of Albana and Trebbiano Romagnolo grapevines. *American Journal of Enology and Viticulture, 48*(1), 7-12.

Daane, K. M., Almeida, R. P., Bell, V. A., Walker, J. T., Botton, M., Fallahzadeh, M., et al. (2012). Biology and management of mealybugs in vineyards. In *Arthropod Management in Vineyards:* (pp. 271-307): Springer.

Daane, K. M., Bentley, W. J., Walton, V. M., Malakar-Kuenen, R., Millar, J. G., Ingels, C. A., et al. (2006). New controls investigated for vine mealybug. *California Agriculture, 60*(1), 31-38, doi:10.3733/ca.v060n01p31.

Daane, K. M., Middleton, M. C., Sforza, R., Cooper, M. L., Walton, V. M., Walsh, D. B., et al. (2011). Development of a multiplex PCR for identification of vineyard mealybugs. *Environmental Entomology, 40*(6), 1595-1603, doi:10.1603/en11075.

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.

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.

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, doi:10.1094/pdis-93-4-0395.

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, doi:10.1603/0022-0493-94.1.291.

Goheen, A. C., & Cook, J. A. (1959). Leafroll (red-leaf or rougeau) and its effects on vine growth, fruit quality, and yields. *Amer Jour Enol and Viticulture, 10*((4)), 173-181.

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. A., Weber, E., Sim, S., & Rowhani, A. (2008). Leafroll disease is spreading rapidly in a Napa Valley vineyard. *California Agriculture, 62*(4), 156-160, doi:10.3733/ca.v062n04p156.

Gray, S. M., Caillaud, M. C., Burrows, M., & Smith, D. M. (2007). Transmission of Two Viruses that Cause Barley Yellow Dwarf is Controlled by Different Loci in the Aphid, *Schizaphis graminum*. *Journal of insect science, 7*(25), 1-15, doi:10.1673/031.007.2501.

Gullan, P. J., Downie, D. A., & Steffan, S. A. (2003). A new pest species of the mealybug genus *Ferrisia fullaway* (Hemiptera : Pseudococcidae) from the United States. *Annals of the Entomological Society of America, 96*(6), 723-737, doi:10.1603/0013-8746(2003)096[0723:anpsot]2.0.co;2.

Habili, N., & Nutter, F. W. (1997). Temporal and spatial analysis of *grapevine leafroll-associated virus 3* in Pinot Noir grapevines in Australia. *Plant Disease, 81*(6), 625-628, doi:10.1094/pdis.1997.81.6.625.

Haviland, D. R., Buckey, J. H., & Rill, S. M. (2010a). *In-season control of vine mealybug in 'Autumn Royal' table grapes in Kern County, 2009* (Vol. 35, Vol. 1).

Haviland, D. R., Buckey, J. H., & Rill, S. M. (2010b). *In-season control of vine mealybug in 'Flame Seedless' table grapes in Kern County, 2008* (Vol. 35, Vol. 1).

Haviland, D. R., Hashim-Buckey, J., & Rill, S. M. (2011). *In-season control of vine mealybug in 'Red Globe' table grapes in Kern County, 2010* (Vol. 36, Vol. 1).

Hogenhout, S. A., Ammar, E. D., Whitfield, A. E., & Redinbaugh, M. G. (2008). Insect vector interactions with persistently transmitted viruses. *Annual Review of Phytopathology, 46*(1), 327-359, doi:10.1146/annurev.phyto.022508.092135.

Jones, T. J., & Nita, M. (2016). Spatio-temporal association of GLRaV-3-infected grapevines, and effect of insecticidal treatments on mealybug populations in Virginia vineyards. *European Journal of Plant Pathology*, 1-16, doi:10.1007/s10658-016-0878-x.

Jones, T. J., Rayapati, N., & Nita, M. (2015). Occurrence of Grapevine leafroll associated virus-2, –3 and Grapevine fleck virus in Virginia, U.S.A., and factors affecting virus infected vines. *European Journal of Plant Pathology*, 1-14, doi:10.1007/s10658-015-0605-z.

Kliewer, W. M., & Lider, L. (1976). Influence of leafroll virus on composition of Burger fruits. *American Journal of Enology and Viticulture, 27*(3), 118-124.

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.
Lider, L., Goheen, A., & Ferrari, N. (1975). A comparison between healthy and leafroll-affected grapevine planting stocks. *American Journal of Enology and Viticulture, 26*(3), 144-147.
Loeb, G. M. (2012). Grape Insect and mite pests-2012 field season. http://www.fruit.cornell.edu/grape/pdfs/Loeb-Grape%20Insect%20Mite%20Pests%202012.pdf. Accessed November 2012.
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., Agranovsky, A. A., Bar-Joseph, M., Boscia, D., Candresse, T., Coutts, R. H. A., et al. (2002). The family Closteroviridae revised. *Archives of Virology, 147*(10), 2039-2044, doi:10.1007/s007050200048.
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.
Millar, J. G., Daane, K. M., McElfresh, J. S., Moreira, J. A., Malakar-Kuenen, R., Guillen, M., et al. (2002). Development and optimization of methods for using sex pheromone for monitoring the mealybug *Planococcus ficus* (Homoptera : Pseudococcidae) in California vineyards. *Journal of Economic Entomology, 95*(4), 706-714, doi:10.1603/0022-0493-95.4.706.
Naidu, R. A., Soule, M. J., & Jarugula, S. (2006). Single and mixed infections of *Grapevine leafroll-associated viruses* in Washington State vineyards. *Phytopathology, 96*, S83.
Nauen, R., Reckmann, U., Thomzik, J., & Thielert, W. (2008). Biological profile of spirotetramat (Movento®) - a new two-way systemic (ambimobile) insecticide against sucking pest species. *Bayer CropScience Journal, 61*(2), 245-278.
Ng, J. C. K., & Falk, B. W. (2006). Virus-vector interactions mediating nonpersistent and semipersistent transmission of plant viruses. *Annual Review of Phytopathology, 44*(1), 183-212, doi:10.1146/annurev.phyto.44.070505.143325.
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, doi:10.1111/j.1439-0434.2011.01857.x.
Osman, F., Leutenegger, C., Golino, D., & Rowhani, A. (2007). Real-time RT-PCR (TaqMan®) assays for the detection of *Grapevine Leafroll associated viruses 1-5* and *9*. *Journal of Virological Methods, 141*(1), 22-29, doi:10.1016/j.jviromet.2006.11.035.
Osman, F., & Rowhani, A. (2006). Application of a spotting sample preparation technique for the detection of pathogens in woody plants by RT-PCR and real-time PCR (TaqMan). *Journal of Virological Methods, 133*(2), 130-136, doi:10.1016/j.jviromet.2005.11.005.
Perry, J. N. (1998). Measures of spatial pattern for counts. *Ecology, 79*(3), 1008-1017, doi:10.1890/0012-9658(1998)079[1008:mospfc]2.0.co;2.
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.

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, doi:10.1046/j.1365-3059.1997.d01-44.x.

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. http://pubs.wsu.edu. Accessed 2012 April.

Rosciglione, B., & Gugerli, P. Transmission of grapevine leafroll disease and an associated closterovirus to healthy grapevine by the mealybug *Planococcus ficus* Signoret. In *Proceedings 9th Meeting of ICVG, Kiryat Anavim, 1989* (pp. 1987, 1967-1969)

Rowhani, A., Biardi, L., Johnson, R., Saldarelli, P., Zhang, Y. P., Chin, J., et al. Simplified sample preparation method and one-tube RT-PCR for grapevine viruses. In *Proceedings of XIII International Council for the Study of Viruses and Virus-Like Diseases of the Grapevine, Adelaide, 2000* (pp. 82)

Schooley, N., Baudoin, A. B., Derr, J. F., Pfeiffer, D. G., Weaver, M. J., & Wolf, T. K. (2010). Crop profile for grapes in Virginia. http://www.ipmcenters.org/cropprofiles/docs/vagrapes.pdf.

Smegal, D. C. Human Health Risk Assessment Chlorpyrifos. In O. o. P. U.S. Environmental Protection Agency, Pesticides and Toxic Substances, Office of Pesticide Programs, Health Effects Division, U.S. Government Printing Office: Washington, DC, 2000; pp 1-131 (Ed.).

Tanne, E., Ben-Dov, Y., & Raccah, B. (1989). Transmission of closterolike particles by mealybugs (Pseudococcidae) in Israel. *Phytoparasitica, 17*(64).

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, doi:10.1094/phyto-100-8-0830.

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, doi:10.1094/phyto-98-10-1093.

Wakita, T., Kinoshita, K., Yamada, E., Yasui, N., Kawahara, N., Naoi, A., et al. (2003). The discovery of dinotefuran: a novel neonicotinoid. *Pest Management Science, 59*(9), 1016-1022, doi:10.1002/ps.727.

Wallingford, A. K., Fuchs, M. F., Martinson, T., Hesler, S., & Loeb, G. M. (2015). Slowing the Spread of Grapevine Leafroll-Associated Viruses in Commercial Vineyards With Insecticide Control of the Vector, *Pseudococcus maritimus* (Hemiptera: Pseudococcidae). *Journal of insect science, 15*(1).

Walton, V. M., & Pringle, K. L. (2001). Effects of pesticides and fungicides used on grapevines on the mealybug predatory beetle Nephus 'boschianus' (Coccinellidae, Scymnini). *South African Journal for Enology and Viticulture, 22*(2), 107-110.

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.

Wolpert, J. A., & Vilas, E. P. (1992). Effect of mild leafroll disease on growth, yield, and fruit maturity indices of Riesling and Zinfandel. *American Journal of Enology and Viticulture, 43*(4), 367-369.

Woodham, R., Antcliff, A., Krake, L., & Taylor, R. (1984). Yield differences between Sultana clones related to virus status and genetic factors. *Vitis, 23*(2), 73-83.

Wunderlich, L. R., Cooper, M. L., & Daane, K. M. (2013). Seasonal Biology of Ferrisia gilli (Hemiptera: Pseudococcidae) in California Sierra Foothill Vineyards. *Journal of Economic Entomology, 106*(4), 1716-1725, doi:10.1603/EC13046.

Yokomi, R. K., & DeBorde, R. L. (2005). Incidence, transmissibility, and genotype analysis of Citrus tristeza virus (CTV) isolates from CTV eradicative and noneradicative districts in central California. *Plant Disease, 89*(8), 859-866, doi:10.1094/PD-89-0859.

Chapter 5

Nitro-pure nitrocellulose membranes as an effective storage and testing medium for grapevine viruses

5.1 Introduction

Among perennial crop worldwide, the most number of viruses (> 70) have been detected from grapevines (Martelli 2014; Naidu et al. 2015a). A majority of these viruses are considered as minor threats to production, because of low economic significance or limited geographical distribution. However, there are several viruses that are considered a major threat due to high economic importance (Martelli and Boudon-Padieu 2006; Rayapati 2012). Examples are the grapevine leafroll complex, rugose wood complex, and degeneration/decline disease complex (Martelli and Boudon-Padieu 2006).

Grapevine leafroll disease (GLD) is present in all grapevine-growing regions of the world (Fuchs et al. 2009b). Based on studies conducted in several countries, GLD significantly affects vine vigor, fruit yield, and grape quality (Alley et al. 1963; Cabaleiro et al. 1999; Credi and Babini 1997; Goheen and Cook 1959; Goheen et al. 1958; Kliewer and Lider 1976; Kovacs et al. 2001; Lider et al. 1975; Wolpert and Vilas 1992; Woodham et al. 1984). In severely infected vineyards with a susceptible cultivar, direct crop losses can range from 10% to 40% (Wolpert and Vilas 1992). In addition, uneven ripening of berries and reduced sugar level are commonly associated with the infection. Also, poor berry color due to reduced anthocyanin pigments are

associated with infected red-fruited wine grape cultivars (Cabaleiro et al. 1999; Fuchs et al. 2009b; Rayapati et al. 2008). These traits can result in indirect crop loss in the form of revenue losses due to poor wine quality (Cabaleiro et al. 1999; Fuchs et al. 2009b; Rayapati et al. 2008). Moreover, the vector for these viruses, mealybugs and scale insects, are widely present in many grape growing regions (Daane et al. 2008; Daane et al. 2011; Tsai et al. 2010; Wallingford et al. 2015).

Viruses associated with GLD are called grapevine leafroll-associated viruses (GLRaVs) and named as GLRaV-1, -2, -3, -4, and -7 (Fuchs 2007; Fuchs et al. 2009b). These viruses belong to the family *Closteroviridae*. GLRaV-2 was assigned to the genus *Closterovirus*, GLRaV-7 was classified into to the newly created genus *Velarivirus,* and GLRaV-1, -3, and -4 and its strains were grouped in the genus *Ampelovirus*. GLRaV-4, -5, -6, -9, -De, -Pr, and -Car were originally classified as separate species; however, GLRaV-4 is now considered as a consolidation of these divergent strains (Ghanem-Sabanadzovic et al. 2012; Martelli et al. 2012; Thompson et al. 2012). Currently, these viruses are now referred to as GLRaV-4 strains 5, 6, 9, De, Pr, and Car (Ghanem-Sabanadzovic et al. 2012; Martelli et al. 2012; Thompson et al. 2012).

The rugose wood disease complex is a group of viruses (mostly belong to the family *Flexiviridae* genera *Vitivirus or Foveavirus*) that primarily affects the vascular system of grapevines (Martelli et al. 1993; Pearson and Goheen 1988; Rosa et al. 2011). This complex includes Grapevine virus A (GVA), Grapevine virus B (GVB), and Grapevine Rupestris stem pitting associated virus (GRSPaV). These viruses are widespread and cause a slow decline of vines, stem grooving (especially with GVA), corky bark (GVB), and graft incompatibility (Rosa et al. 2011). Canes of severely infected vines will ripen irregularly or not at all (Martelli et al. 1993). Symptoms caused by these viruses develop slowly over the course of multiple years;

however, symptoms may become more severe when multiple viruses are found within a vine (Guidoni et al. 1997; Mannini et al. 1996). Although grapevines commonly can live for 50-100 years, in the Finger Lakes region of the US, vineyards tend to have an average of 25 years (Atallah et al. 2012) before growers replant. The decision of replanting is often due to lower production by older vines (Wolf 2008), and viruses in the rugose wood disease complex can play a role in the decline of production. Moreover, a recent survey from our group (Chapter 2) revealed that GRSPaV is wide spread (>53% of our sample of 721) among Virginia vineyards. Due to the high initial investment on vineyards and relatively slow return on investment, it is critical for growers to have long, productive years before the needs for replanting arise.

The newly discovered DNA virus *Grapevine red blotch-associated virus* (GRBaV) (family *Geminiviridae*) (Al Rwahnih et al. 2013), which is also known as *Grapevine Cabernet franc-associated virus* (Krenz et al. 2012) and *Grapevine redleaf disease* (Poojari et al. 2013), is a developing concern among growers since this virus can cause sugar reduction. One study showed that red blotch-infected vines exhibit a reduction of up to 5° Brix (Habili 2013). Other symptoms of GRBaV-infected vines are very similar to that of GLD. Much is still unknown about cultivar, rootstock, and yield effects. GRBaV is widespread in the U.S. and has been identified recently in California, New York, Virginia, Maryland, Pennsylvania, Texas, and Washington (Krenz et al. 2014a).

Recognizing the impacts of virus diseases, many grapevine-growing regions of the United States have implemented virus surveys to document the presence of viruses in vineyards. Surveys conducted in California, Idaho, Missouri, New York, Oregon, Virginia Washington State have shown the presence of GLRaV-1, -2, -3, -4, -7, and GLRaV-4 strains -5, -9, and -Car, GVA, RSPaV, *Grapevine fanleaf virus* (GFLV), *Grapevine fleck virus* (GFkV),

Arabis mosaic virus (ArMV), *Tomato ringspot virus* (ToRSV), GRBaV, and *Grapevine vein clearing virus* (GVCV) (Fuchs 2007; Martin et al. 2005; Mekuria et al. 2009a; Mekuria et al. 2009b; Milkus and Goodman 1999; Naidu and Mekuria 2010; Naidu et al. 2006; Al Rwahnih et al. 2013; Zhang et al. 2011; Poojari et al. 2013; Jones et al. 2015). Our recent Virginia survey (Chapter 2) has shown that GLRaV-3, RSPaV-1, and GRBaV are the three most common viruses found in the state.

These survey methods most commonly rely on in-season or winter field sampling of grapevine tissues followed by nucleic acid extraction and a molecular-based detection method, such as PCR, RT-PCR, or qPCR (Fuchs 2007; Martin et al. 2005; Mekuria et al. 2009a; Mekuria et al. 2009b; Milkus and Goodman 1999; Naidu and Mekuria 2010; Naidu et al. 2006; Al Rwahnih et al. 2013; Zhang et al. 2011; Poojari et al. 2013; Jones et al. 2015). Sampling generally consists of coordinating with growers about site visits, driving to vineyards, sampling petioles of suspected grapevines, placing those petioles into Ziploc bags, and placing those bags on ice. Once on ice, the samples must be transported to cold storage (4°C or -20°C) where they can be stored for a short period of time because nucleic acid extraction and freezing needs to occur before samples lose their viability.

The use of various membranes (such as FTA cards and Nylon and Nitropure Nitrocellulose (NPN) membranes) has been suggested as a simpler and robust virus sampling and testing method. Plant sap can be blotted onto these membranes prior to use in multiple assays such as PCR, RT-PCR, qPCR, and TBIA (tissue blot immunoassay) (Chang et al. 2011; Chang and Tolin 2008, 2010; Osman and Rowhani 2006). These membranes are capable of binding to nucleic acid and keeping virus integrity at room temperature for long periods of time (Chang et al. 2011; Chang and Tolin 2008, 2010; La Notte et al. 1997b; Osman and Rowhani

2006). FTA cards and Hybond N^+ Nylon membrane spotting techniques have been shown to be effective for use to detect grapevine viruses and virus-like agents (including, but not limited to GLRaV-1, -3, -5, -9, GVA, GVB, GRSPaV, GFkV, *Xylella fastidiosa*, and phytoplasmas) (La Notte et al. 1997b; Osman and Rowhani 2006) and FTA cards and NPN membranes have been shown to be effective for cucumoviruses and potyviruses (Chang et al. 2011).

In this study, we examined several procedures for membrane-based sampling and testing for ten grapevine viruses, and developed a membrane-based sampling kit for GLRaV-3 and GRBaV. This membrane-based method not only has significant application to be used in the field by growers, but also helps advance the field of grapevine virus detection.

5.2 Materials and Methods:

Samples and membranes: Grapevine virus samples used in this experiment included previously extracted nucleic acid stored at -80C from our previous study (Jones et al. 2015) and current survey (Chapter 2). In addition, a total of 150 fresh grapevine petiole samples were collected from three vineyards with known various virus infections. Two vineyards from Orange Co. VA, and one vineyard located at the research center, AHS Jr. AREC, Winchester, VA were used. For each sample, fifteen fresh grapevine petiole tissue were sampled arbitrarily from a canopy of vines that were previously identified with virus infections.

Two different membranes were examined. The first was FTA classic cards (Whatman, GE Healthcare Bio-Sciences, Pittsburgh, PA), which is known for successful tissue-blot and RT-PCR/ qPCR on multiple grapevine viruses (Osman and Rowhani 2006). The second membrane was a 0.45 μm pore size NitroPure Nitrocellulose (NPN) membrane (Maine Manufacturing LLC, Sanford, ME), which is known for successful tissue-blot and RT-PCR on cucumoviruses and

potyviruses (Chang et al. 2011; Chang and Tolin 2008, 2010); however this has not used for grapevine viruses in previous studies.

Initial testing of NPN membranes, confirmation of FTA cards, and sample viability. An experiment was conducted using pre-extracted field samples collected from 2011 and 2014 (samples from Chapter 2) in order to validate functions of FTA and NPN membranes and also determine viability of the stored samples. Each sample contained 10 total petioles taken arbitrarily across the canopy from single, symptomatic vines. This sampling method was used due to uneven virus distribution of GLRaV-3 in grapevines (Charles et al. 2006). Sampled petioles were placed into a Ziploc bag, immediately stored in an iced cooler box, and taken back to the laboratory. Petiole samples were cut using sterile razor blades down to 0.25 g of petiole sections, placed into grinding bags (BIOREBA, Switzerland) containing 5 ml of a filter-sterilized grapevine extraction buffer (GEB) (1.59 g/liter Na_2CO_3, 2.93 g/liter $NaHCO_3$, 2% Polyvinylpyrrolidone-40, 0.2% Bovine Serum Albumin, and 0.05% Tween 20) (Sigma-Aldrich Co. LLC, St. Louis, MO) (Rowhani et al. 2000). The samples were then homogenized using a mechanical grinder (BIOREBA, Switzerland, Homex 6 [115V]) and crude extracts were transferred into 1.5-ml microcentrifuge tubes. Samples were stored at -80°C for up to two years prior to this experiment.

These samples were tested for multiple viruses using the primers and methods outlined in Table 5.1. From this tested sample collection from Chapter 2, nine GLRaV-1, twenty-five GLRaV-2, twenty-five GLRaV-3, six GLRaV-4, twenty-five GRSPaV, twenty-five GVA, fifteen GVB, six GFkV, eight ToRSV, and twenty-five GRBaV-positive grapevine samples were selected for use in this experiment.

Each virus positive sample was thawed on ice and 10µl of nucleic acid extract sample was spotted onto both NPN membranes and FTA cards using a pipette. The membrane was allowed to air dry for 24 hours on the lab bench (Osman and Rowhani 2006). Once dry, 3 mm discs of membrane or card containing blotted, dried sample were punched out using a 3mm micro-punch (Harris Uni-core Sampling Tool 3.0, Electron Microscopy Sciences, Hatfield, PA).

Two different procedures were used for nucleic acid preparation based on membrane type. Each discs of the NPN membranes was placed in a 50µl microcentrifuge tube with 50µl of a grapevine extraction serum (GES) (0.1 M Glycine, 0.05 M NaCl, 1 mM EDTA, containing 0.5% Triton X-100). These tubes were incubated at 95°C for 10 minutes, vortexted vigorously, and placed on ice. FTA card discs were prepared by two washes with FTA® purification reagent using the manufacturer's protocol (Whatman) followed by two washes with TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) (Osman and Rowhani 2006). The discs were then allowed to dry a minimum of 24 hours.

Then, 2 µl aliquots of suspended nucleic acid from the NPN membrane discs, or the dry FTA card disc itself was added to a 25 µl total volume of 13.4 µl nuclease-free H2O, 2.5 µl 10X PCR buffer containing (New England Biolabs, Ipswitch, MA), 2.5 µl sucrose/cresol red (20% w/v sucrose, 1 mM cresol red) (Sigma-Aldrich Co. LLC, St. Louis, MO), 1.25 µl virus specific forward primer (20 µM), 1.25 µl virus specific reverse primer (20 µM), 1.25 µl 100mM dithiothreitol (Sigma-Aldrich Co. LLC, St. Louis, MO), 0.5 µl dNTPs (10 mM) (Invitrogen, Grand Island, NY), 0.1 µl RnaseOUT (40 U/µl) (Invitrogen), 0.035 µl Superscript III RTase (200 U/µl)(Invitrogen), and 0.25 µl Taq DNA polymerase (5 U/µl) (New England Biolabs) (Naidu et al. 2006; Osman et al. 2007; Rowhani et al. 2000). For conventional PCR reactions, an additional 0.135 µl of H20 was used to substitute the RTase or RNaseOUT. This 25 µl reaction mix was

used for RT-PCR and PCR for the viruses listed in Table 5.1 using the primers and methods listed. Negative (water) and positive (virus positive sample traditionally extracted and not deposited on membrane) controls were included to validate test results. PCR products were observed through gel electrophoresis on a 1.5% agarose gel in TBE (89mM Tris Borate, 2mM EDTA, pH 8.2) stained with GelRed (Biotium, Hayward, CA) through a UV trans-illuminator and imager.

NPN membrane nucleic acid extraction methods: macerating buffer, disc treatments for DNA/RNA recovery, and template. In order to determine the proper methodology for nucleic acid recovery from blotted NPN membranes, sample maceration buffer, membrane disc treatments, and template types were to be examined.

Three buffers were examined as potential candidates for use in maceration of fresh petiole tissues: 1) in-house Grapevine Extraction Buffer (GEB) (listed above), 2) a commercially available grapevine sample buffer (GSB) (#M004-K1, AC Diagnostics, Fayetteville, AR), and 3) Water. Forty-eight fresh grapevine petiole samples from GLRaV-3-positive vines, and 48 fresh grapevine petiole samples from GRBaV-positive were collected from the field in July 2015. Each sample contained five grapevine petioles arbitrarily taken from the infected vine's canopy. Petiole samples were cut into small pieces using household scissors. In between samples, scissors were disinfected in 10% household bleach and the rinsed with clean water. One gram for each sample was placed into the bottom of a small, sterile disposable medicine cup (cat#EF5639, Daigger Scientific, Vernon Hills, IL). One gram contained enough tissue to fill the entire bottom of the disposable medicine cup with one full layer of tissue. Then, 1 ml of GEB, GSB, or water was added to each sample cup. Using the blunt ends of sterile 6" long wood applicators

(cat#72303, Electron Microscopy Sciences, Hatfield, PA), petiole tissue was macerated in the cup until a liquid mixture was formed. The applicator was then dipped into the mixture and gently touched to blot the solution onto a NPN membrane. The membrane was allowed to dry for a minimum of 12 hours.

Five methods (referred to as Method A, B, C, D, and E below and in Table 5.2) of membrane preparation (= washing of the membrane in buffers) for nucleic acids recovery were examined. For all methods, a 3mm disc of a NPN membrane, which contained a blot of either GLRaV-3- or GRBaV-positive grape sap, was placed in a 50ml microcentrifuge tube using a sterile micro-punch. The micro-punch was cleaned in 10% household bleach with gentle agitation for 60s, rinsed with deionized water for 60s, and blotted dry in between samples.

Method A of preparation involved no washes, vortexing, or incubation. The 3mm membrane discs were used directly in the RT-PCR and PCR reactions to be mentioned later. The methods B and C followed NPN membrane protocols for other plant viruses (Chang et al. 2011; Chang and Tolin 2008, 2010). Discs for method B were placed in a 500µl tube with 200µl of FTA reagent and discs for methods C were placed in 5% Triton X-100 in deionized water added to the disc-containing tubes. Triton X-100 is a non-ionic detergent used for solubilizing membrane proteins. These tubes were then vortexted for 5s, allowed to stand at room temperature for 5min, the solution was removed, and the wash was repeated twice. Then the discs were rinsed twice in 200µl of TE buffer, and allowed to air dry for at least 24 hours prior to RT-PCR/PCR (GLRaV-3/GRBaV) reactions. Methods D and E followed the aforementioned protocol for FTA cards and Nylon membranes (Osman and Rowhani 2006). Here, 50µl of GES for method D and 50µl of GES containing 1% beta-mercaptoethanol for method E was added to

each disc-containing tube. These tubes were incubated at 95°C for 10 minutes, removed, vortexted vigorously, and placed on ice for immediate use in RT-PCR and PCR reactions.

Two template types were examined for use in PCR reactions either the 3mm NPN membrane disc itself or 2µl solution from a tube holding the membrane disc. For method A, B, and C, the 3mm membrane disc was placed directly in the PCR reaction tube as the template. For methods D and E, the 3mm membrane disc, which was placed directly in the PCR reaction tube as the template, was tested against using 2µl of the vortexed solution from the PCR tube containing the membrane. The 48 samples for GLRaV-3-positive vine samples were tested using a one-tube one-step RT-PCR protocol (Rowhani et al. 2000) with the GLRaV-3 primer set listed in Table 5.1. The 48 samples of GRBaV-positive vine samples were tested using a previously reported conventional PCR (Al Rwahnih et al. 2013) with the primers listed in Table 5.1. Negative (water) and positive (previously confirmed positive samples from Chapter 2 using methods from Chapter 2) controls were included to validate test results. PCR products were observed through gel electrophoresis as describe above.

Additional testing: Twenty-five fresh petiole samples (ten petioles per sample) of grapevines with known infections of GLRaV-2, GLRaV-3, GRSPaV-1, GVA, GVB, and GRBaV were sampled from the field in July 2015, and testing was conducted using the NPN membrane (Table 5.3). Tissue was ground in GSB, nucleic acid recovery was performed using GES with 1% beta-Mercaptoethanol, and then 2µl of the solution containing the released nucleic acid from the membrane was used as a template for RT-PCR or PCR reactions. Select membrane-deposited PCR positive samples from each virus species following PCR were subjected to a purification using the QIAquick PCR purification kit (QIAgen Inc USA, CA) with the manufacturers

protocol. Cleaned products were sequenced at the Virginia Bioinformatics Institute, Virginia Tech (Blacksburg, VA, U.S.) using an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA).

Membranes contained several subsamples for these samples, and they were stored on the laboratory bench top in a Rubbermaid container stacked on top of each other with paper dividers. After 18 months, subsamples were tested for the appropriate virus to confirm long term membrane storage of recoverable nucleic acid.

Validation of the NPN membrane based sampling with GRBaV qPCR. The recently developed qPCR technique for GRBaV detection described in Chapter 3 was also examined in its ability to detect this virus from the membrane storage medium. Nucleic acid recovery from sample-deposited NPN membranes was performed using GES with 1% beta-Mercaptoethanol, and 2µl of the solution containing the released nucleic acids from the membrane was used as a template for the GRBaV qPCR reactions. A total of 50 known GRBaV positive vines were tested.

These 50 samples from fresh petioles were subjected to the membrane deposition method listed above and also were ground and extracted using the Isolate II Plant DNA Kit (Bioline, Taunton, MA) following the manufacturers standard protocol. Samples were run in triplicate in 25µl total volume reactions using primer-probe set RB3 (900nM Forward: 5'-CGACAGCTGATTAGGCGTG-3'; 900nM Reverse: 5'-TCTTCTCTGCTTCCGTTG-GC-3'; 250nM Probe: 5'-6FAM-ACAACCCTCAAACCACTCCTCGCA-TAMRA-3'), 2µl of template, and a run method of 2 min @ 50°C, 10 min @ 95°C, with 40 cycles of 15 sec @ 95°C followed by 1 min @ 59°C. All qPCR data was recorded using the ABI Step-One Plus real-time PCR System (Applied Biosystems, Foster City, CA). The *Vitis vinifera* resveratrol synthase gene was

selected for use as an endogenous control due to its reliable copy number within the genome (Velasco et al. 2007). For the resveratrol synthase gene, a primer/probe set of ResF, ResR, and ResP was used (Saito et al. 2013; Valsesia et al. 2005). As a result of a lack of known copy number standards, relative comparisons with the comparative C_t method of $2^{-\Delta Ct}$ was used to compare gene expression levels among samples (Livak and Schmittgen 2001; Schmittgen and Livak 2008).

ΔCt and $2^{-\Delta Ct}$ values were compared between membrane and conventional nucleic acid extraction methods. A standard curve was generated using one reference sample of unknown DNA copy number. An arbitrary copy number of 10 was assigned to its highest concentration, and then the sample was diluted at a 1:5 ratio for 7 times, and estimated copy numbers were assigned to each diluted sample (Mehle et al. 2013).

The slope and R^2 value of the log-linear section of the ΔCt value curve was used to determine amplification efficiency for the membrane based sampling method, and compared to the previously reported curve in Chapter 3. In order to examine pairwise association of the significance between the two methods of extraction a matched pairs analysis was estimated using JMP Pro 11 (SAS institute, Cary, NC) for both the GRBaV V2 gene and the resveratrol synthase gene. The average difference between Ct values of two methods for each gene was also calculated for comparisons of overall difference between two methods.

5.3 Results

Initial testing of NPN membranes, confirmation of FTA cards, and sample viability. Initial testing of previously sampled and frozen grapevine petiole tissue on FTA cards and NPN membranes using 10µl spot application was successful for all of nine GLRaV-1, twenty-five GLRaV-2, twenty-five GLRaV-3, six GLRaV-4, twenty-five GRSPaV, twenty-five GVA, fifteen GVB, six GFkV, eight ToRSV, and twenty-five GRBaV positive grapevine samples. Thus, the FTA card methods worked with GLRaV-4, ToRSV, and GRBaV in addition to the previously reported GLRaV-1, -2, -3, -5, -9, GVA, GVB, GRSPaV, and GFkV (Osman and Rowhani 2006). In addition, the methods previously set forth for Nylon membranes (Osman and Rowhani 2006) successfully worked to store nucleic acid on NPN membranes using conventionally extracted samples.

NPN membrane nucleic acid extraction methods: macerating buffer, disc treatments for DNA/RNA recovery, and template. Table 5.2 shows combination of macerating buffer, NPN membrane disc treatments, and template used for the recovery of GLRaV-3 RNA and GRBaV DNA, and a representative display of typical gel analysis is shown in Figure 5.1. The use of water as a buffer to store and blot the nucleic acid had a 0% success rate. Both GEB and GSB resulted in range of 0% to 100% success rate for both GLRaV-3 and GRBaV, and the success rate depended on the disc treatment and template (Table 5.2).

Untreated and FTA reagent treated NPN membrane discs were not successful (= 0% success rate) in nucleic acids recovery for both GLRaV-3 and GRBaV, regardless of maceration buffer or template used (Table 5.2, Fig. 5.1). Triton X-100 resulted in up to 50% success rate depending on the combination of maceration buffer and virus to be tested (Table 5.2). The most successful membrane disc nucleic acid preparation method was achieved by using GES with 1% beta-Mercaptoethanol as a disc treatment buffer. For both GEB and GSB maceration treatments,

up to 100% success rate was observed. Removing the beta-Mercaptoethanol from the GES solution resulted in a considerable loss of success rate (Table 5.2).

When the selection of PCR template was compared with the samples macerated with GEB or GSB, followed by the resulting disc treatment with GES + 1% beta-Mercaptoethanol, the use of 2µl of the solution containing the released nucleic acid from the membrane resulted in higher success rates than the use of the blotted 3mm NPN membrane discs (Table 5.2, Fig. 5.1). For GLRaV-3, the used of the NPN membrane discs used as templates detected between 83% and 88% with GEB and GSB, respectively. For GRBaV, the disc template resulted in 90% for GEB and 94% for GSB (Table 5.2) On the other hand, when 2µl of the solution containing the released nucleic acid from the membrane was used, it detected 100% of GLRaV-3 and GRBaV samples for both GEB and GSB (Table 5.2). Both GEB and GSB resulted in similar success rates; however, the GSB was selected as a choice of maceration buffer for our study because GSB is stable at room temperature.

Based on the results above, the final procedure was created based on the most reliable method of storage and detection. Fresh grapevine petiole samples from known positive grapevines were taken randomly from the infected vine's canopy. Petiole samples were lined up and cut into small pieces using household scissors. In between samples, scissors were disinfected in 10% household bleach and rinsed with clean water after. Petiole cuttings were placed into the bottom of the disposable medicine cup until one layer of petiole tissue covered the bottom. To each sample cup, 1ml of GSB was added. The blunt ends of sterile 12" long wood applicators are then used to macerate petiole tissue in the cup. The wood applicator was then dipped into the mixture and gently touched/blotted onto a NPN membrane, then repeated two additional times. The membrane was allowed to dry for a minimum of 24 hours.

Once dry, a 3mm disc of the blotted membrane was removed and placed in a 50ml microcentrifuge tube using a sterile micro-punch. To the tube containing the disc, 50µl of GES containing 1% beta-Mercaptoethanol was added. Tubes were incubated at 95°C for 10 minutes, removed, vortexted vigorously, and placed on ice. Then, 2µl of the solution from tubes containing discs was used in a 25µl total volume PCR reaction specific for each virus and primer set (Table 5.1).

Membrane sampling kit final product: Using the successful method developed in the lab above, a "kit" was created for sampling and was distributed to grapevine growers to test. A large piece of paper with instructions on how to blot the membrane and a membrane attached, along with 15 wooden applicator sticks, 15 1mL bottles of GSB, 15 medicine cups were placed in a Ziploc bag. Instructions were also provided via a YouTube video (http://youtu.be/xc_0jQAx8WQ). Growers were instructed on how to sample and blot the membranes and then membranes could be sent via mail in an envelope to the research station for virus testing. Three membranes were sent back in envelopes, each containing a small number of samples (11 total samples) from vineyards with suspected leafroll infections. Nine samples were positive for GLRaV-3 with two samples positive for GRBaV.

Additional testing: Using the established method, fresh petiole samples were sampled from vines infected from GLRaV-2, GLRaV-3, GRSPaV, GVA, GVB, or GRBaV. For each virus, 25 samples were tested, and all samples were correctly identified through this appropriate PCR methodology for each virus (Table 5.3). All samples taken were preserved on NPN membranes by placing in a Rubbermaid plastic container on the lab bench at room temperature, each membrane separated by a piece of paper. After 18 months of storage, samples were tested again using appropriate PCR methods (Table 5.3). The results showed that all samples were

successfully amplified, showing that the NPN membranes contained recoverable amounts of nucleic acid after 18 months at room temperature (Table 5.3).

Validation of the NPN membrane based sampling with GRBaV qPCR. The GRBaV qPCR successfully amplified all 50 samples, 50 with the traditional extraction method using the Bioline kit and the same 50 with the NPN membrane based sampling method. However, ΔCt values of the NPN membrane method was slightly higher than the traditional extraction method samples (Fig. 5.2). This indicated that the samples from the NPN membrane method amplified later during cycling, thus, the NPN membrane method might be slightly less sensitive than testing using a more conventional extraction method. On average, the Ct values of GRBaV differed within samples between the two extraction methods by 1.4 ($2^{-\Delta Ct}$= 0.38), and the difference between two methods for the grape resveratrol synthase gene was 2.1 ($2^{-\Delta Ct}$ = 0.23) (Fig. 5.2). When pairwise comparisons were conducted, the Ct values from the NPN membrane method on both genes were significantly higher than that from the traditional extraction method ($P < 0.05$) (Fig. 5.2). Standard curve analysis confirmed the NPN membrane method was less efficient at amplification. With the traditional method, the amplification efficiency was 93% (Chapter 3); on the other hand, the NPN membrane method resulted in 66% amplification efficiency (Fig 5.3).

5.4 Discussion

The objective of this study was to develop an easy and robust method of grapevine virus sampling that could be readily used by vineyard managers while efficiently storing recoverable nucleic acid for routine molecular diagnostic assays. To our knowledge, this study is the first to report the NPN membrane as a storage medium of nucleic acids of several grapevine viruses (members of *Betaflexiviridae, Comoviridae, Closteroviridae, Flexiviridae, Geminiviridae,* and

Tymoviridae) in plant sap. This study also showed these viral nucleic acids can be recovered from the membrane up to 18 months from the time of sampling, and amplified through conventional PCR or RT-PCR, or in the case of GRBaV, through qPCR.

It was previously found that FTA cards and Nylon membranes could effectively store 13 different grapevine viruses (including GLRaV-1, -2, -3, -5, -9, GVA, GVB, GRSPaV, GFkV, *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), *Prunus nectrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV)) and *Xylella fastidiosa* and multiple phytoplasma nucleic acids, but only for up to four days to 2+months (F. Osman and Rowhani 2006). With this study, we confirmed the FTA card method also successfully detects GLRaV-4, ToRSV, and GRBaV.

The use of NPN membranes has a distinct advantage over those previously reported methods. We were able to store, recover, and amplify GLRaV-2, GLRaV-3, GRSPaV, GVA, GVB, and GRBaV after 18 months of storage at room temperature. For GLRaV-3 and GRBaV, we were able to confirm the presence of high enough quality RNA and DNA for sequencing after this time period as well.

On the other hand, there was a critical difference in the preparation of the membranes prior to the PCR reactions. Other reports (Chang et al. 2011; Chang and Tolin 2008, 2010; Osman and Rowhani 2006) have shown that a piece of membrane blotted with sap can be directly placed in PCR reaction mixtures and tubes;however, our results indicated that even with the best combination of maceration (GSB) and washing (GES, β-mercaptoethanol) buffers, the efficacy of amplification was lower (88%) when the NPN membrane was directly used while the use of the solution from the mix yielded an efficacy of 100% (Osman and Rowhani 2006). Our results suggest that vortexing the tubes with buffer allows the virus nucleic acid particles to be

suspended off of the membrane, and therefore, the suspension became a better template for replication as opposed to remaining nucleic acids attached to the membrane.

Sample preparation of grapevines for molecular testing can be difficult due to phenolic compounds and polysaccharides that inhibit these reactions (Osman and Rowhani 2006). These inhibitors found in woody plants (grapevines in this case) are most likely the reason membrane preparation with water, FTA reagent, and Triton X-100 did not provide good results. On the other hand, the grapevine specific buffers that were made specifically to aid in inhibitor suppression resulted in a higher rate of both GLRaV-3 RNA and GRBaV DNA recovery.

Our results also showed that the NPN membrane storage and extraction method can be applicable to the GRBaV qPCR procedure from Chapter 3. However, when traditional nucleic acid extraction methods were compared with the extraction using the NPN membrane, higher Ct values with the NPN membrane from the GRBaV qPCR procedure showed consistently lower amplification efficiency. This result was expected since the extraction method developed for the NPN membrane in this study most likely resulted in more inhibitory materials for the qPCR procedures than commercially available extraction kits. The NPN membrane sampling method is, therefore, better used solely for detection purposes, but not for detailed quantitative work with qPCR.

Our research resulted in the development of a kit for grapevine virus sampling. This kit can have up to 15 samples blotted per membrane, allowing growers to sample on their own time throughout the season due to the stability of nucleic acids on the NPN membrane. Although the sample size was small, the success of kits returned to the lab and comments from participants suggests that the kits can work as designed, especially if managers take time to read the instructions and follow the YouTube video.

There are several immediate short-term benefits of this kit. First, the method of transportation of membranes from/to the research lab became much simpler as coolers or expensive express services are no longer required. Second, because nucleic acid on the NPN membrane is stable for up to 18 months at room temperature, the need for cold storage is eliminated. For long-term benefits, we envision that this kit can be used to store and test for virus, bacteria, or phytoplasma infected samples from grapevines or other crops located at remote locations where neither a cold storage nor quick shipment are available.

In summary, we developed a simple and robust NPN membrane-based grapevine virus sampling and storage method that allows growers to blot grapevine sap to the membrane that will store recoverable nucleic acid for at least 18 months at room temperature. The results were utilized to create a sampling kit for grapevine growers. We also developed a very efficient extraction method for the NPN membrane which yielded 100% amplification of GLRaV-2, GLRaV-3, GRSPaV, GVA, GVB, or GRBaV, as well as successful amplification using the GRBaV qPCR from Chapter 3.

Table 5.1: Primers used for conventional and RT-PCR detection of grapevine viruses.

Virus	Primer name and Reference	PCR parameters (temp in °C)
GLRaV-1	dCP1-1/dCP1-2 (Esteves et al. 2013)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GLRaV-2	P19qtF4 and p24qtR (Beuve et al. 2007)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GLRaV-3	GEN-11112F/GEN-11233R (Chooi et al. 2013)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GLRaV-4 (strains 4, 5, 9)	LRAmp-F/LRAmp-R (Abou Ghanem-Sabanadzovic et al. 2012)	52°, 1h; 94, 2min; 40x (94, 30s; 50, 35s; 72,45s); 72, 7min
RSPaV-1	RSP13/RSP14 (B. Meng et al. 1999)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GFkV	GFkV-585 F/GFkV-1117 R (R. A. Naidu and Mekuria 2010)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GVA	H587/C995 (Minafra et al. 1997)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GVB	C410/H28 (Minafra and Hadidi 1994)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GVCV	GVCV-F1/GVCV-R1 (Y. Zhang et al. 2011)	94°, 1min; 34x (94, 30s; 52, 40s; 72, 1min); 72, 7min
ToRSV	ToRSV5/ToRSV6 (Li et al. 2011)	50°, 30min; 94, 2min; 30x (94, 45s; 60, 45s; 68, 2min); 68, 5min
GRBaV	GVGF1/GVGR1 (Al Rwahnih et al. 2013)	94°, 2min; 35x (94, 30s; 60, 30s; 72, 1min); 72, 5min

Table 5.2: GLRaV-3 and Red Blotch nucleic acid recovery from NPN Membranes.

Maceration Buffer	Method	Washing Buffer	Template for PCR[a]	GLRaV-3[b]	GRBaV[b]
GEB	A	No trt	Disc	0/48	0/48
	B	Triton X-100	Disc	16/48	23/48
	C	FTA reagent	Disc	0/48	0/48
	D	GES	Disc	33/48	41/48
			2ul solution	37/48	44/48
	E	GES+beta-M	Disc	40/48	43/48
			2ul solution	48/48	48/48
GSB	A	No trt	Disc	0/48	0/48
	B	Triton X-100	Disc	13/48	24/48
	C	FTA reagent	Disc	0/48	0/48
	D	GES	Disc	31/48	43/48
			2ul solution	34/48	47/48
	E	GES+beta-M	Disc	42/48	45/48
			2ul solution	48/48	48/48
Water	A	No trt	Disc	0/48	0/48
	B	Triton X-100	Disc	0/48	0/48
	C	FTA reagent	Disc	0/48	0/48
	D	GES	Disc	0/48	0/48
			2ul solution	0/48	0/48
	E	GES+beta-M	Disc	0/48	0/48
			2ul solution	0/48	0/48

[a] Disc = Membrane were used directly in PCR, 2ul = 2ul of solution in tube containing membrane was used in PCR. Negative controls were used for all membrane reactions for false positives.
[b] Number of samples correctly identified as positive / total known positive samples tested

Table 5.3: Grapevine virus testing results at the time of sampling and after storage for 18 months using the NPN membrane sampling method

Virus	Number Sampled	Number Positive	Number positive after 18 months storage
GLRaV-2	25	25	25
GLRaV-3	25	25	25
GRSPaV-1	25	25	25
GVA	25	25	25
GVB	25	25	25
GRBaV	25	25	25

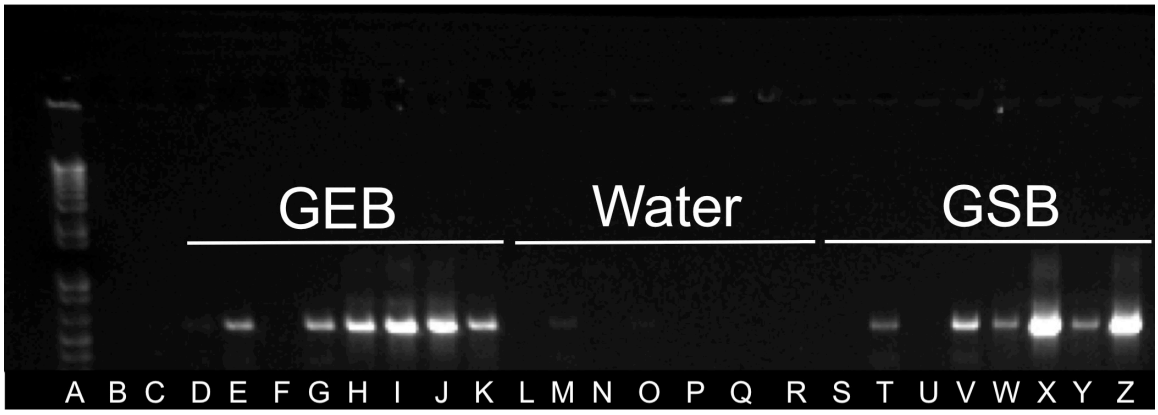


Figure 5.1: PCR amplification of the V2 gene of GRBaV (~446bp) isolates using varying extraction buffers and template combinations. Lanes D-K were membrane discs blotted with GRBaV petioles macerated in GEB, Lanes L-R were membrane discs blotted with GRBaV petioles macerated in Water, Lanes S-Z were membrane discs blotted with GRBaV petioles macerated in GSB. Lane specific washing buffer and template treatments are as follows: A: 1kb Ladder, B: H20 control, C: Untreated disc, D: Untreated disc, E: Triton X-100 disc, F: FTA reagent disc, G: GES+BM disc, H: GES+BM 2µl, I: GES disc, J: GES 2µl, K: positive GRBaV control, L: Untreated disc, M: Triton X-100 disc, N: FTA reagent disc, O: GES+BM disc, P: GES+BM 2 µl, Q: GES disc, R: GES 2 µl, S: Untreated disc, T: Triton X-100 disc, U: FTA reagent disc, V: GES+BM 2µl, W: GES+BM disc, X: GES 2µl, Y: GES disc, Z: positive GRBaV control.

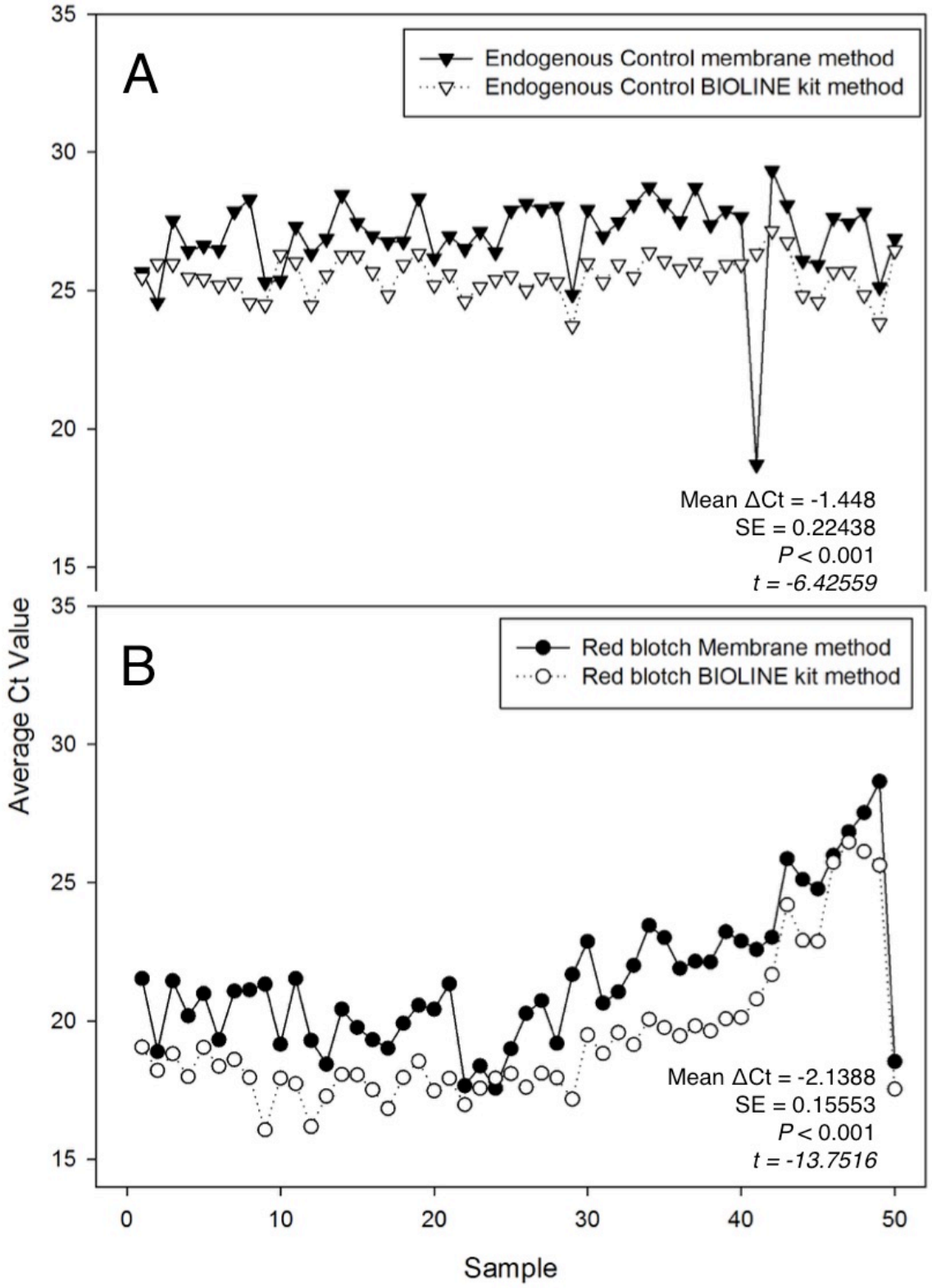


Figure 5.2: Mean Ct values per sample based on three independent amplifications per sample for the resveratrol synthase gene (endogenous control) (A) and V2 gene of GRBaV (B). White

circles represent the mean Ct values of BIOLINE kit and black circles are the NPN membrane method. Mean ΔCt values, and results from pairwise t-test (JMP Pro 11) were also shown.

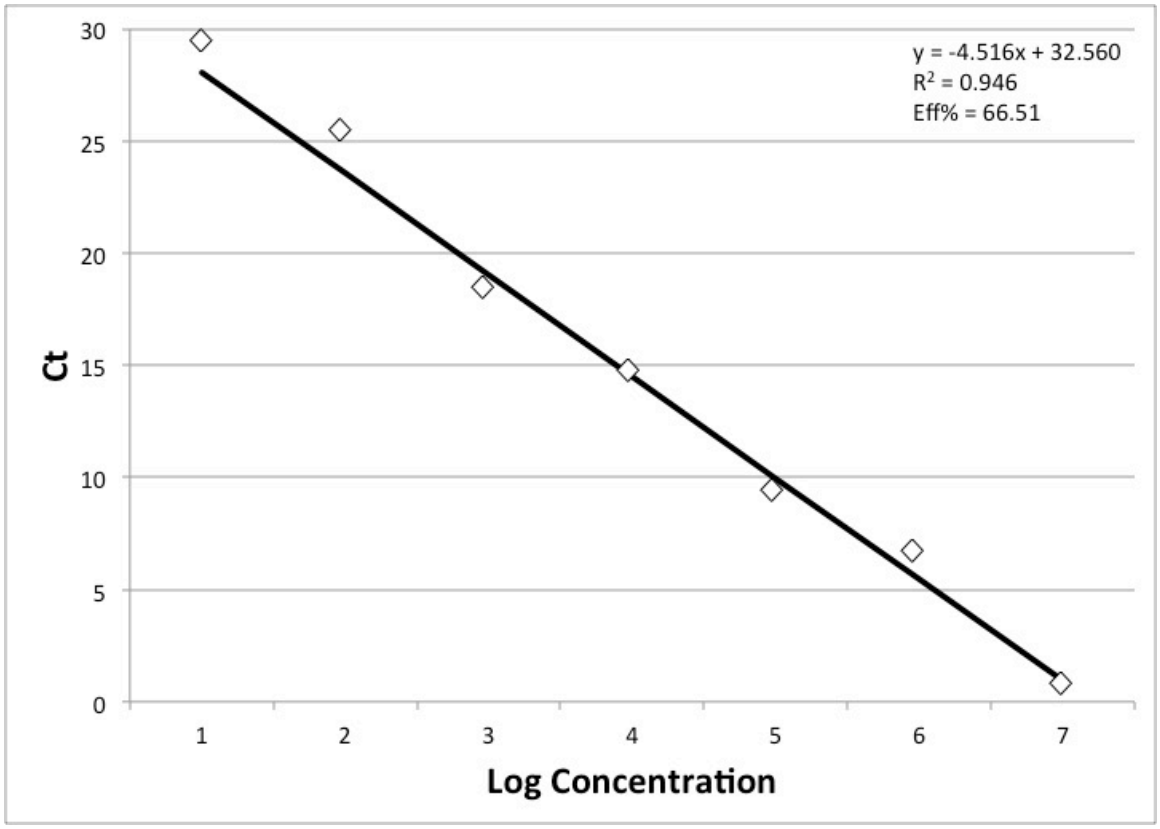


Figure 5.3: Determination of efficiency of qPCR assays using standard curve analysis and C_t slope method with seven concentrations covering a 6-log dilution range for the NPN membrane extraction method for GRBaV. Equation, R^2 value, and efficiency percentage are shown.

5.5 References:

Al Rwahnih, M., Dave, A., Anderson, M. M., Rowhani, A., Uyemoto, J. K., & Sudarshana, M. R. (2013). Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology, 103*(10), 1069-1076, doi:10.1094/PHYTO-10-12-0253-R.

Alley, C., Goheen, A., Olmo, H., & Koyama, A. (1963). The effect of virus infections on vines, fruit and wines of Ruby Cabernet. *American Journal of Enology and Viticulture, 14*(3), 164-170.

Atallah, S. S., Gomez, M. I., Fuchs, M. F., & Martinson, T. E. (2012). Economic Impact of Grapevine Leafroll Disease on Vitis vinifera cv. Cabernet franc in Finger Lakes Vineyards of New York. *American Journal of Enology and Viticulture, 63*(1), 73-79, doi:10.5344/ajev.2011.11055.

Beuve, M., Sempe, L., & Lemaire, O. (2007). A sensitive one-step real-time RT-PCR method for detecting *Grapevine leafroll-associated virus 2* variants in grapevine. *Journal of Virological Methods, 141*(2), 117-124, doi:10.1016/j.jviromet.2006.11.042.

Cabaleiro, C., Segura, A., & Garcia-Berrios, J. J. (1999). Effects of *grapevine leafroll-associated virus 3* on the physiology and must of Vitis vinifera L. cv. Albarino following contamination in the field. *American Journal of Enology and Viticulture, 50*(1), 40-44.

Chang, P. G. S., McLaughlin, W. A., & Tolin, S. A. (2011). Tissue blot immunoassay and direct RT-PCR of cucumoviruses and potyviruses from the same NitroPure nitrocellulose membrane. *Journal of Virological Methods, 171*(2), 345-351, doi:10.1016/j.jviromet.2010.11.018.

Chang, P. S., & Tolin, S. A. (2008). Detection and partial characterization of three RNA viruses using improved membrane-based technologies. *Phytopathology, 98*(6), S214-S214.

Chang, P. S., & Tolin, S. A. (2010). Nitrocellulose membranes as a solid matrix for Cucumber mosaic virus immuno-detection and subgroup identification by RT-PCR. *Phytopathology, 100*(6), S173-S173.

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). *59*, 330-337.

Chooi, K. M., Cohen, D., & Pearson, M. N. (2013). Generic and sequence-variant specific molecular assays for the detection of the highly variable *Grapevine leafroll-associated virus 3*. *Journal of Virological Methods, 189*(1), 20-29, doi:10.1016/j.jviromet.2012.12.018.

Credi, R., & Babini, A. R. (1997). Effects of virus and virus-like infections on growth, yield, and fruit quality of Albana and Trebbiano Romagnolo grapevines. *American Journal of Enology and Viticulture, 48*(1), 7-12.

Daane, K. M., Cooper, M. L., Triapitsyn, S. V., Walton, V. M., Yokota, G. Y., Haviland, D. R., et al. (2008). Vineyard managers and researchers seek sustainable solutions for mealybugs, a changing pest complex. *California Agriculture, 62*(4), 167-176, doi:10.3733/ca.v062n04p167.

Daane, K. M., Middleton, M. C., Sforza, R., Cooper, M. L., Walton, V. M., Walsh, D. B., et al. (2011). Development of a multiplex PCR for identification of vineyard mealybugs. *Environmental Entomology, 40*(6), 1595-1603, doi:10.1603/en11075.

Esteves, F., Teixeira Santos, M., Eiras-Dias, J. E., & Fonseca, F. (2013). Molecular data mining to improve antibody-based detection of *Grapevine leafroll-associated virus 1* (GLRaV-1). *Journal of Virological Methods, 194*(1-2), 258-270, doi:10.1016/j.jviromet.2013.09.004.

Fuchs, M. (2007). Grape leafroll disease factsheet. http://www.nysipm.cornell.edu/factsheets/grapes/diseases/grape_leafroll.pdf. Accessed June 2012.

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, doi:10.1094/pdis-93-4-0395.

Ghanem-Sabanadzovic, N., Sabanadzovic, S., Gugerli, P., & Rowhani, A. (2012). Genome organization, serology and phylogeny of *Grapevine leafroll-associated viruses 4* and *6*: Taxonomic implications. *Virus Research, 163*(1), 120-128, doi:10.1016/j.virusres.2011.09.001.

Goheen, A. C., & Cook, J. A. (1959). Leafroll (red-leaf or rougeau) and its effects on vine growth, fruit quality, and yields. *Amer Jour Enol and Viticulture, 10*((4)), 173-181.

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.

Guidoni, S., Mannini, F., Ferrandino, A., Argamante, N., & Di Stefano, R. (1997). The effect of grapevine leafroll and rugose wood sanitation on agronomic performance and berry and leaf phenolic content of a Nebbiolo clone (Vitis vinifera L.). *American Journal of Enology and Viticulture, 48*(4), 438-442.

Habili, N. (2013). Highlights of a newly-emerging grapevine virus: *Grapevine red blotch-associated virus*.

Jones, T. J., Rayapati, N., & Nita, M. (2015). Occurrence of *Grapevine leafroll associated virus-2, −3* and *Grapevine fleck virus* in Virginia, U.S.A., and factors affecting virus infected vines. *European Journal of Plant Pathology*, 1-14, doi:10.1007/s10658-015-0605-z.

Kliewer, W. M., & Lider, L. (1976). Influence of leafroll virus on composition of Burger fruits. *American Journal of Enology and Viticulture, 27*(3), 118-124.

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.

Krenz, B., Thompson, J. R., Fuchs, M., & Perry, K. L. (2012). Complete genome sequence of a new circular DNA virus from grapevine. *Journal of Virology, 86*(14), 7715-7715.

Krenz, B., Thompson, J. R., McLane, H., Fuchs, M., & Perry, K. L. (2014). *Grapevine red blotch-associated virus* is widespread in the United States. *Phytopathology*, doi:10.1094/phyto-02-14-0053-r.

La Notte, P., 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, doi:10.1016/s0166-0934(97)00038-4.

Li, R., Mock, R., Fuchs, M., Halbrendt, J., Howell, B., & Liu, Z. (2011). Characterization of the partial RNA1 and RNA2 3′ untranslated region of *Tomato ringspot virus* isolates from North America. *Canadian Journal of Plant Pathology, 33*(1), 94-99.

Lider, L., Goheen, A., & Ferrari, N. (1975). A comparison between healthy and leafroll-affected grapevine planting stocks. *American Journal of Enology and Viticulture, 26*(3), 144-147.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *methods, 25*(4), 402-408.
Mannini, F., Argamante, N., & Credi, R. (1996). Improvements in the quality of grapevine 'Nebbiolo' clones obtained by sanitation. In S. Poni, E. Peterlunger, F. Iacono, & C. Intrieri (Eds.), *First Ishs Workshop on Strategies to Optimize Wine Grape Quality* (pp. 319-324, Acta Horticulturae, Vol. 427). Leuven 1: International Society Horticultural Science.
Martelli, G. P. (2014). Directory of virus and virus-like diseases of the grapevine and their agents. *Journal of Plant Pathology, 96*(1), 1-136.
Martelli, G. P., Abou Ghanem-Sabanadzovic, N., Agranovsky, A. A., Al Rwahnih, M., Dolja, V. V., Dovas, C. I., et al. (2012). Taxonomic revision of the family Closteroviridae with special reference to the grapevine leafroll-associated members of the genus Ampelovirus and the putative species unassigned to the family. *Journal of Plant Pathology, 94*(1), 7-19.
Martelli, G. P., & Boudon-Padieu, E. (2006). *Directory of infectious diseases of grapevines*. (Vol. 55): International Centre for Advanced Mediterranean Agronomic Studies. Options Méditerranéennes, Ser. B, Studies and Research. 55: 59-75.
Martelli, G. P., Viruses, I. C. f. t. S. o., Grapevine, V. D. o. t., Food, & Nations, A. O. o. t. U. (1993). *Graft-transmissible Diseases of Grapevines: Handbook for Detection and Diagnosis*: Food and Agriculture Organization of the United Nations.
Martin, R. R., Eastwell, K. C., Wagner, A., Lamprecht, S., & Tzanetakis, I. E. (2005). Survey for viruses of grapevine in Oregon and Washington. *Plant Disease, 89*(7), 763-766, doi:10.1094/pd-89-0763.
Mehle, N., Prezelj, N., Hren, M., Boben, J., Gruden, K., Ravnikar, M., et al. (2013). A Real-Time PCR Detection System for the Bois Noir and Flavescence Dorée Phytoplasmas and Quantification of the Target DNA. In M. Dickinson, & J. Hodgetts (Eds.), *Phytoplasma* (Vol. 938, pp. 253-268, Methods in Molecular Biology): Humana Press.
Mekuria, Karasev, A. V., Martin, R. R., & Naidu, R. A. (2009a). First report of *Grapevine leafroll-associated virus-3* in six wine grape cultivars in Idaho. *Plant Disease, 93*(11), 1218, doi:10.1094/pdis-93-11-1218c.
Mekuria, Soule, M. J., Jarugula, S., & Naidu, R. A. (2009b). Current status of grapevine viruses in Washington State vineyards. *Phytopathology, 99*, S83.
Meng, B., Zhu, H. Y., & Gonsalves, D. (1999). *Rupestris stem pitting associated virus-1* consists of a family of sequence variants. *Archives of Virology, 144*(11), 2071-2085, doi:10.1007/s007050050623.
Milkus, B. N., & Goodman, R. N. (1999). A survey of Missouri vineyards for the presence of five grape viruses. *American Journal of Enology 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, doi:10.1016/0166-0934(94)90076-0.
Minafra, A., Saldarelli, P., & Martelli, G. P. (1997). *Grapevine virus A*: nucleotide sequence, genome organization, and relationship in the Trichovirus genus. *Archives of Virology, 142*(2), 417-423, doi:10.1007/s007050050088.
Naidu, R. A., Maree, H. J., & Burger, J. T. (2015). Grapevine Leafroll Disease and Associated Viruses: A Unique Pathosystem. *Annual Review of Phytopathology, 53*, 613-634.

Naidu, R. A., & Mekuria, T. A. (2010). First report of *Grapevine fleck virus* from Washington vineyards. *Plant Disease, 94*(6), 784, doi:10.1094/pdis-94-6-0784a.

Naidu, R. A., Soule, M. J., & Jarugula, S. (2006). Single and mixed infections of *Grapevine leafroll-associated viruses* in Washington State vineyards. *Phytopathology, 96*, S83.

Osman, F., Leutenegger, C., Golino, D., & Rowhani, A. (2007). Real-time RT-PCR (TaqMan®) assays for the detection of *Grapevine Leafroll associated viruses 1-5* and *9*. *Journal of Virological Methods, 141*(1), 22-29, doi:10.1016/j.jviromet.2006.11.035.

Osman, F., & Rowhani, A. (2006). Application of a spotting sample preparation technique for the detection of pathogens in woody plants by RT-PCR and real-time PCR (TaqMan). *Journal of Virological Methods, 133*(2), 130-136, doi:10.1016/j.jviromet.2005.11.005.

Pearson, R. C., & Goheen, A. C. (1988). *Compendium of grape diseases*: APS Press.

Poojari, S., Alabi, O. J., Fofanov, V. Y., & Naidu, R. A. (2013). A leafhopper-transmissible DNA virus with novel evolutionary lineage in the family Geminiviridae implicated in grapevine redleaf disease by next-generation sequencing. *PLoS ONE, 8*(6), e64194.

Rayapati, N. (2012). Virus Diseases. http://wine.wsu.edu/research-extension/plant-health/virology/virus-diseases/. Accessed November 2012.

Rayapati, N., O'Neal, S., & Walsh, D. (2008). Grapevine leafroll disease. http://pubs.wsu.edu. Accessed 2012 April.

Rosa, C., Jimenez, J. F., Margaria, P., & Rowhani, A. (2011). Symptomatology and Effects of Viruses Associated with Rugose Wood Complex on the Growth of Four Different Rootstocks. *American Journal of Enology and Viticulture, 62*(2), 207-213, doi:10.5344/ajev.2011.10104.

Rowhani, A., Biardi, L., Johnson, R., Saldarelli, P., Zhang, Y. P., Chin, J., et al. Simplified sample preparation method and one-tube RT-PCR for grapevine viruses. In *Proceedings of XIII International Council for the Study of Viruses and Virus-Like Diseases of the Grapevine, Adelaide, 2000* (pp. 82)

Saito, S., Dunne, K. J., Evans, K. J., Barry, K., Cadle-Davidson, L., & Wilcox, W. F. (2013). Optimisation of techniques for quantification of Botrytis cinerea in grape berries and receptacles by quantitative polymerase chain reaction. *Australian Journal of Grape and Wine Research, 19*(1), 68-73, doi:10.1111/ajgw.12011.

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nature protocols, 3*(6), 1101-1108.

Thompson, J. R., Fuchs, M., & Perry, K. L. (2012). Genomic analysis of *Grapevine leafroll associated virus-5* and related viruses. *Virus Research, 163*(1), 19-27, doi:10.1016/j.virusres.2011.08.006.

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, doi:10.1094/phyto-100-8-0830.

Valsesia, G., Gobbin, D., Patocchi, A., Vecchione, A., Pertot, I., & Gessler, C. (2005). Development of a high-throughput method for quantification of *Plasmopara viticola* DNA in grapevine leaves by means of quantitative real-time polymerase chain reaction. *Phytopathology, 95*(6), 672-678, doi:10.1094/PHYTO-95-0672.

Velasco, R., Zharkikh, A., Troggio, M., Cartwright, D. A., Cestaro, A., Pruss, D., et al. (2007). A high quality draft consensus sequence of the genome of a heterozygous grapevine variety. *PLoS ONE, 2*(12), e1326.

Wallingford, A. K., Fuchs, M. F., Martinson, T., Hesler, S., & Loeb, G. M. (2015). Slowing the Spread of Grapevine Leafroll-Associated Viruses in Commercial Vineyards With Insecticide Control of the Vector, *Pseudococcus maritimus* (Hemiptera: Pseudococcidae). *Journal of insect science, 15*(1).

Wolf, T. K. (2008). *Wine grape production guide for eastern North America*. (NRAES-145). Ithaca, NY.: PALS Publishing (formerly NRAES).

Wolpert, J. A., & Vilas, E. P. (1992). Effect of mild leafroll disease on growth, yield, and fruit maturity indices of Riesling and Zinfandel. *American Journal of Enology and Viticulture, 43*(4), 367-369.

Woodham, R., Antcliff, A., Krake, L., & Taylor, R. (1984). Yield differences between Sultana clones related to virus status and genetic factors. *Vitis, 23*(2), 73-83.

Zhang, Y., Singh, K., Kaur, R., & Qiu, W. P. (2011). Association of a novel DNA virus with the grapevine vein-clearing and vine decline syndrome. *Phytopathology, 101*(9), 1081-1090, doi:10.1094/PHYTO-02-11-0034.

Chapter 6

Changes in basic grapevine berry chemistry due to effects of single and co-infections of Grapevine leafroll associated virus-3, Grapevine rupestris stem pitting associated virus, and Grapevine red blotch associated virus.

6.1 Introduction

Many grapevine viruses are known to cause changes in infected grapevines. The effects of viral infection can appear on growth, amount of leaf gas exchange, reduction in yield, and change in fruit chemistry such as berry weight, soluble solids, pH, titratable acidity (TA), polyamines and phenols (Alley et al. 1963; Cabaleiro et al. 1999; Credi and Babini 1997; Endeshaw et al. 2014; Goheen and Cook 1959; Guidoni et al. 1997; Kliewer and Lider 1976; Kovacs et al. 2001; Lee et al. 2009; Lee and Martin 2009; Naidu et al. 2014; Naidu et al. 2015b; Reynolds et al. 1997; Rosa et al. 2011; Sampol et al. 2003; Wolpert and Vilas 1992; Woodham et al. 1984; Woodham et al. 1983). These changes are poorly understood because virus biotic stress studies on plants are complicated and results from virus infections are highly variable due to influences from multiple factors such as environmental conditions, vine age, cultivar selection, rootstocks selected, and vine field management practices (Balachandran et al. 1997; Credi and

Babini 1997; Guidoni et al. 1997; Mannini et al. 1996; Sampol et al. 2003; Woodham et al. 1983). In addition, many viruses cause physical leaf symptoms that could lead to other issues. In other plant systems such as tomato and tobacco, leaf symptoms from viruses are correlated with reduced CO2 assimilation, chloroplast numbers, chlorophyll metabolism, and stomatal conductance (Almasi et al. 1996; Hunter and Peat 1973), and these changes in leaf function can result in reduced fruit quality parameters (Guidoni et al. 1997; Martelli 1993).

Some of the critical parameters growers and winemakers examine to determine berry maturity levels for harvest and winemaking include °Brix, pH, TA, yeast-assimilable nitrogen (YAN), and anthocyanins. °Brix is a degree measurement of the soluble solids (or sugars) present within the grape berries, an important indicator of grape berry maturity. Generally, sugar accumulates in grape berries as the season progresses while acidity falls as berries mature (Keller 2010). Thus, pH is also used as a variable for ripening. Additionally, pH values can be an important measurement for post-fermentation to evaluate risk of oxidation because wines with high in pH will undergo oxidation more easily (Boulton 1980; Boulton 1998; Crowe 2007). Two measurements of acidity, pH and TA are closely related; however TA can better describe acidity in terms of the sensory perception of acidity in wines (Boulton 1980; Boulton 1998; Crowe 2007). Therefore, for an approximation of total acidity, measurements of TA in the berries is used (Boulton 1980; Boulton 1998; Crowe 2007). Therefore, one of the main goals at harvest is to have berries with well-balanced levels of °Brix, pH, and TA.

YAN is the measurement of free amino acid, ammonia, and ammonium content that is available for the wine yeast (*Saccharomyces cerevisiae*) in harvested grapes (R. B. Boulton 1998). Sugar (measured in °Brix) and nitrogen (measured in YAN) are the two most important micronutrients for the wine yeast. Low YAN levels can results in slow or lagging fermentation

can take place; on the other hand, high YAN levels can result in undesirable aroma compounds such as ethyl acetate (Boulton 1998; Crowe 2007). Therefore, the target ranges of YAN (dependent on °Brix) is between 200 and 350mg/L on average (Boulton 1998; Crowe 2007). Finally, anthocyanins are compounds found in skin tissues of grape berries and are important compounds for red-fruited cultivars as they are mainly responsible for color and phenolic compounds in the resulting wine (Markakis 2012).

Two recent surveys of Virginia (Jones et al. 2015 and Chapter 2) revealed that *Grapevine leafroll-associated virus-3* (GLRaV-3, family *Closteroviridae*, genus *Ampelovirus*), *Grapevine rupestris stem-pitting-associated virus-1* (GRSPaV-1, family *Flexiviridae*, genus *Foveavirus*), and *Grapevine red blotch-associated virus* (GRBaV, family *Geminiviridae*) are the three most common viruses found infecting grapevines throughout vineyards. GLRaV-3, and other GLRaVs, lower vine vigor, decrease yield, cause uneven ripening, delay ripening due to a reduction in sugar accumulation, and reduce anthocyanin production (Cabaleiro et al. 1999; Endeshaw et al. 2014; Golino 1993; Kliewer and Lider 1976; Kovacs et al. 2001; Lee et al. 2009; Lee and Martin 2009; Wolpert and Vilas 1992). With GLRaV-3 infection, average berry weight (ABW) can be reduced and TA can be increased (Kovacs et al. 2001). Furthermore, of vines infected with both GLRaV-3 and *Grapevine fleck virus* (GFkV, family *Tymoviridae*, genus *Maculavirus*) were reduced by 7% and increased by 14%, respectively, which were inferior to those of healthy vines and vines infected only with GLRaV-3 (ABW 5% reduction, TA 5-9% increase) (Kovacs et al. 2001). However, other critical components of fruit chemistry, such as YAN, seem to not be influenced by GLRaV-2 or GLRaV-3, based on a single study done with cultivar Pinot noir (Lee et al. 2009).

There are few reports of actual effects of GRBaV on berry chemistry. So far, up to 4-5 degree decrease in °Brix occurs sometimes accompanied with an increase in TA (Al Rwahnih et al. 2013; Poojari et al. 2013; Oberholster 2015). Also, GRBaV can delay fruit ripening and inhibit proper color development (Al Rwahnih et al. 2013; Poojari et al. 2013; Oberholster 2015). Rupestris stem pitting virus is known to reduce PH and TA in Riesling (Reynolds et al. 1997).

With GRSPaV, information on its effect on berry fruit chemistry is poorly understood. GRSPaV is widespread and causes a slow decline of vines and graft incompatibility issues (Rosa et al. 2011). Canes of severely infected vines will ripen irregularly or not at all (Martelli et al. 1993). Symptoms caused by GRSPaV and other viruses in the Rugose wood disease complex develop slowly over the course of multiple years; however, when multiple viruses are found within a vine, symptoms may become more severe (Guidoni et al. 1997; Mannini et al. 1996).

In Virginia, U.S.A., the wine and grape industry is quickly becoming a major contributor to the state economy. The recent estimate of the Virginia wine industry in 2010 showed an annual economic impact of about $740 million to the state (VWB 2012). This growth represents a 106% increase from the previous economic study conducted five years earlier (VWB 2012). Currently, Virginia ranks fifth in the United States with 1,100 growing hectares of wine grape cultivars and close to 250 wineries (Caldwell 2012; VWB 2012).

With this growing industry, it is critical to maintain high quality grape production so that resulting wines are appealing to customers; however, our previous studies showed high levels of infection of GLRaV-3, GRBaV, and GRSPaV-1 among our samples (Chapter 2). The aim of this study is to analyze basic fruit composition (°Brix, pH, TA), anthocyanin levels, and YAN amounts from grapevines infected with the three most common viruses in Virginia: GLRaV-3,

GRBaV, and GRSPaV-1 in order to understand potential effects of these virus infections to berry chemistries.

6.2 Materials and Methods

Vine Selection: Data from Chapter 2 were utilized to identify potential blocks within a vineyard containing the same cultivar where grapevines existed with combinations of single and co-infections of GLRaV-3, GRBaV, and GRSPaV-1. We identified three cultivars in a commercial vineyard in Orange County, VA to meet the criteria. The vineyard manager had noticed significant differences in fruit composition over the last ten years with these cultivars. Identified cultivars and viruses were: 1) 18-year-old 'Mourvedre' with infections of GLRaV-3 and GRSPaV-1; 2) 12-year-old 'Pinotage' with infections of GRBaV and GRSPaV-1; and 3) 18-year-old 'Syrah' with infections of GLRaV-3 and GRBaV. Locations of these cultivars with in the commercial vineyard were geographically separated. All three cultivars were trained using a Lyre system (i.e., divided canopy with vertical shoot growth) with vine spacing between rows and between vines within a row at 3.6 m and 2.1 m, respectively. Within each cultivar block, three rows of 40 individual vines were sampled and tested for GLRaV-1, -2, -3, -4, GRSPaV-1, GVA, GVB, and GRBaV. Petiole sampling of vines in each cultivar block occurred on September 8th, 2015, about one week before predicted harvest dates. Each sample consisted of 14 petioles taken arbitrarily across the canopy, seven from each side of the Lyre-trained vines. Samples were placed in Ziploc bags, placed on ice, and transported back to the lab where they were temporarily stored at -20C until they were processed into crude nucleic acid extracts.

Samples were processed in the same manner as in our previous studies where we used one tube one step RT-PCR (Jones et al. 2015; Rowhani et al. 2000). Petiole samples were removed from their Ziploc bags and 0.25 g of petiole sections, cut using sterile razor blades, were placed into grinding bags (BIOREBA, Switzerland) containing 5 ml of a filter-sterilized grapevine extraction buffer (1.59 g/liter Na_2CO_3, 2.93 g/liter $NaHCO_3$, 2% Polyvinylpyrrolidone-40, 0.2% Bovine Serum Albumin, and 0.05% Tween 20) (Sigma-Aldrich Co. LLC, St. Louis, MO) (Rowhani et al. 2000). The samples were then ground using a mechanical grinder (BIOREBA, Switzerland, Homex 6 [115V]) and crude extracts were transferred into 1.5-ml microcentrifuge tubes and stored at -80°C indefinitely until used in future molecular diagnostic assays.

Thawed crude extract was added to 50 μl GES denaturing buffer (0.1 M glycine, pH 9.0; 50 mM NaCl; 1 mM EDTA; 0.5% Triton X-100) in a 0.5ml microcentrifuge tube. Following incubation at 95°C for 10 minutes, samples were placed on ice for a minimum of 5 minutes before adding 2 μl of the GES denatured homogenate to PCR tubes containing: 13.4 µl nuclease-free H2O, 2.5 µl 10X PCR buffer containing (New England Biolabs, Ipswitch, MA), 2.5 µl sucrose/cresol red (20% w/v sucrose, 1 mM cresol red) (Sigma-Aldrich Co. LLC, St. Louis, MO), 1.25 µl virus specific forward primer (20 µM) 1.25 µl virus specific reverse primer (20 µM), 1.25 µl 100mM dithiothreitol (Sigma-Aldrich Co. LLC, St. Louis, MO), 0.5 µl dNTPs (10 mM) (Invitrogen, Grand Island, NY), 0.1 µl RnaseOUT (40 U/µl) (Invitrogen), 0.035 µl Superscript III RTase (200 U/µl)(Invitrogen), and 0.25 µl Taq DNA polymerase (5 U/µl) (New England Biolabs) (Naidu et al. 2006; Osman et al. 2007; Rowhani et al. 2000). For conventional PCR reactions, the RTase and RNaseOUT was substituted with 0.135 µl of H20.

The PCR tubes were placed in a thermal cycler and subjected to the cycles outlined Table 6.1 for each primer pair set. Following amplification, gel electrophoresis using a 1.2% agarose gel resolved PCR products and DNA bands were visualized using a Fotodyne digital imaging system (Fotodyne, Hartland, WI, U.S.). Positive (known positives from the Chapter 2 survey) and negative (water) controls were used to validate test results.

Fruit Sampling and Testing: Berries were sampled on September 15th, one day prior to expected harvest for the Pinotage and Syrah blocks and two days prior to harvest for the Mourvedre block based on recommendations given by the vineyard manager. For each cultivar block, five single vines were sampled for virus-free, single-infected, and mix-infected vine samples. Samples were taken from closely located vines so that field effects on fruit chemistry parameters will be minimal. Sample per vine consisted of 100 berries, which were collected from the clusters along both sides of the Lyre system's divided canopies, and picked from both outside and inside of clusters for a realistic representation of the berry chemistry. Each 100-berry sample was placed in 3.79 L Ziploc bags, placed in an ice box, and transported back to the lab for immediate processing. Each 100-berry sample was divided into two batches of 50, one batch was used to test for soluble solids (°Brix), pH, titratable acidity (TA), and yeast assimilable nitrogen (YAN) while the other batch was used to test for total anthocyanins.

Sample preparation and testing for °Brix, pH, TA, and YAN: Each 50-berry sample was placed in a separate 3.79 L Ziploc bag, and evenly crushed using a heavy book until all juice was pressed out. Then juice was poured into a sterile, 50mL falcon tube. Soluble solids (°Brix) were measured using a digital refractometer (Pocket PAL-1, ATAGO USA, Inc., Bellevue, WA) while

Juice pH and TA were simultaneously measured using an 848 Titrino Plus auto-titrator (Metrohm, USA, Riverview, FL) titrating to an endpoint of pH 8.2 with 0.1 N NaOH base. Leftover juice was immediately placed at -20C until being shipped overnight one day later to the Enology Services Lab in the Food Science Department at Virginia Polytechnic Institute and State University (Blacksburg, VA, USA) for YAN analysis.

Sample preparation and testing for total anthocyanin analysis: For anthocyanin analysis, homogenization and color expression methods of Iland et al. (1996, 2000) was used. Each 50-berry sample was homogenized in a Magic Bullet blender (Homeland Housewares LLC., Pacoima, CA) for 1 minute. Homogenate was scraped out of blender, mixed well, and 3g of homogenate was transferred into a pre-tared 50ml falcon tube. Then, 30mL of a 50% v/v ethanol solution was added to the homogenate, and the solution was agitated by inverting the mixture every 10 minutes over the course of 1 hour. Following the agitation process, the homogenate solutions were centrifuged at 1,800g for 10 minutes allowing a pellet to form. Supernatant was pipetted into a new sterile falcon tube and stored at -20C for one day. These frozen samples were then sent to the Enology Services Lab in the Food Science Department at Virginia Polytechnic Institute and State University for total anthocyanin analysis.

Statistical Analysis: A generalized linear mixed model (PROC GLIMMIX, SAS ver. 9.4) was used for one-way ANOVA for comparison of measurements among three virus status. The virus status was considered as a fixed factor, and the normal distribution was assumed for each measurement. When there was a significant effect of factor(s), Fisher's least significant difference (LSD) was used for examination of differnce among virus status.

6.3 Results

Data from RT-PCR for GLRaV-3 and GRPSaV-1 and PCR for GRBaV were used to isolate single vines within each cultivar block that had specific single and co-infections to be used in fruit analysis. The Mourvedre block had a mix of virus-free vines, GLRaV-3 infected vines, and GLRaV-3 and GRSPaV-1 co-infected vines. The Pinotage block had a mix of virus-free vines, vines infected with GRBaV, and vines co-infected with both GRBaV and GRSPaV-1. The Syrah block had a mix of virus-free vines, GLRaV-3 positive vines, and GLRaV-3 and GRBaV co-infected vines.

Mourvedre: virus-free, GLRaV-3, and GLRaV-3 + GRSPaV-1: °Brix, pH, and anthocyanin were significantly affected by the virus status (Table 6.2). °Brix ranged from 17 to 20 °Brix, and berries from virus-free vines resulted in significantly higher °Brix than that from GLRaV-3 and GLRaV-3 + GRSPaV-1 co infected vine (Table 6.3). The pH ranged from 3.4 to 3.5, and even though the differences were small, berries from virus-free vines resulted in significantly higher °Brix than that from GLRaV-3 and GLRaV-3 + GRSPaV-1 co-infected vine. The total anthocyanin content varied from 313 to 370 mg/L, and as with °Brix and pH, berries from virus-free vines resulted in significantly higher °Brix than that from GLRaV-3 and GLRaV-3 + GRSPaV-1 co-infected vine. Although values for both TA and YAN was numerically higher with berries from virus-free vines, the effect of the virus status was not significant (Tables 6.2 and 6.3).

Pinotage: virus-free, GRBaV, and GRBaV + GRSPaV-1: °Brix, pH, and TA were significantly affected by the virus status (Table 6.2). °Brix measurements ranged from 20-22.2 with a

significant higher °Brix in virus-free vines compared to both GRBaV and GRBaV + GRSPaV infected vines (Table 6.3). The pH was significantly higher in the virus-free vine compared to the co-infected and single infected vines, ranging from 3.53 to 3.74 (Table 6.3). This was the only cultivar, virus-status combination where a significant effect on TA was found (Table 6.2). TA was significantly lower in fruit from virus-free vines compared to the co-infected and singly infected vines, ranging from 4.9 to 6.0g/L (Table 6.3). YAN (ranged from 333 to 345 mg/L N) and anthocyanin (ranged between 333-416mg/L) were not significant to the virus-status of the vines. However, between samples, anthocyanin levels were numerically higher in virus-free vines compared to the other two virus-statuses.

Healthy Syrah vs. Syrah singly infected with GLRaV-3 and mix-infected with GLRaV-3 + GRBaV: °Brix, pH, YAN, and anthocyanin levels showed significant virus-status effect (Table 6.2). °Brix measurements ranged from 16.3 to 20.3. The mean °Brixwas significantly higher with the virus-free berries compared to the GLRaV-3 infected berries, which was significantly higher the GLRaV-3 + GRBaV co-infected vines (Table 6.3). The pH was significantly higher in virus-free vines fruit than in the other two virus-statuses, ranging from 3.47 to 3.58 (Table 6.3). Anthocyanin levels, which ranged from 250 to 350mg/L, were significantly higher with the virus-free berries than the GLRaV-3-infected berries, and as with °Brix, the mean pH of the GLRaV-3-infected berries was higher than that of the GLRaV-3 + GRBaV co-infected berries (Table 6.3). This was the only cultivar-virus status combination where a significant effect on YAN was found (Table 6.2). YAN values ranged from 287 to 321mg/L (Table 6.3). The virus-free fruit yielded significantly higher YAN measurements compared to both the GLRaV-3 and GLRaV-3 + GRBaV co-infected vines fruit (Table 6.3). TA of virus-free vines were not

significantly different from the single or co-infected vines. However, the virus-free fruit was numerically higher than the co-infected fruit (Table 6.3).

6.4 Discussion

There are very few studies that examine the effect of grapevine virus infection on basic fruit chemistry, and far fewer that have looked into cases of co-infection, especially with GRBaV-infected vines. The Virginia wine industry is rapidly becoming a major contributor to the state economy; however, 90.3% of vineyards present in the state have at least one virus-infected grapevines (Chapter 2). The most common viruses found were GLRaV-3, GRBaV, and GRSPaV-1; therefore, the knowledge of how these viruses effect fruit chemistry is vitally important for wine grape production in Virginia.

Between two cultivars which contained vines with single infection of GLRaV-3, a consistent reduction in °Brix (-14.9% and -10.4% with Mourvedre and Syrah, respectively), increase in pH (4.5% with Mourvedre, 5% with Syrah), and reduction in anthocyanin content (-14.5% with Mourvedre and -17.3% with Syrah) were observed. Thus, our results agree with previous studies showing significant decreases in °Brix (Endeshaw et al. 2014) (Lee et al. 2009; Lee and Martin 2009; Montero et al. 2016), and anthocyanin contents (Lee et al. 2009; Lee and Martin 2009; Alabi et al. 2016). Our results also agree with studies that show non-significant effects on TA (Endeshaw et al. 2014) and YAN (Lee et al. 2009; Lee and Martin 2009; Montero et al. 2016) by GLRaV-3. On the other hand, some studies have shown GLRaV-3-infections have no effects on pH and anthocyanin contents (Endeshaw et al. 2014).

Moreover, although it was not statistically significant, the mean TA of the virus-free vines were numerically lower than that of GLRaV-3-infected Mourvedre and Syrah. Thus, although the mean pH values were decreased, the mean TA, which is the measurement of the sensible acidity increased. A recent study has shown the clear effect of these berry chemical changes by GLRaV-3 on wine, where they found the resulting wine to have significantly lower alcohol, polymeric pigments, and anthocyanin contents along with differences in color, aroma profiles, and astringency (Alabi et al. 2016).

GRBaV has become a hot topic recently with its discovery and unknown vector while it is spreading and generally causing a decrease of 4-5° °Brix (Al Rwahnih et al. 2013; Al Rwahnih et al. 2015; Krenz et al. 2014b; Naidu et al. 2014; Poojari et al. 2013; Sudarshana et al. 2015). GRBaV has also been shown to negatively impact photosynthesis, stomatal conductance, berry weight, anthocyanins, berry metabolism, and solute translocation in Cabernet Sauvignon in California (Calvi 2011). But little else is known about what this virus is capable of in terms of berry chemistry. Based on our study, with a single infection of GRBaV, reduction in both °Brix (-8.7%) and pH (-5.0%) were observed; on the other hand, TA was significantly increased by 21.9% with GRBaV-infected vine.

Co-infection of GRBaV and GLRaV-3 on Syrah vines seemed to have greater impact on °Brix, pH, and anthocyanin contents than a single infection of GLRaV-3. This indicates additional effects of co-infected vines. In our previous survey, we revealed that both GLRaV-3 and GRBaV were present in more than 22% of our samples (Chapter 2). Given the negative impacts of both viruses shown in this and other studies, growers should be encouraged to test their vines for these viruses.

Single infections of GLRaV-3 and co-infections of GLRaV-3 and GRSPaV were found in Mouvedre, and a significant effect of the virus status was found within °Brix, pH and anthocyanin content. In this instance, both the single and mix-infected berry chemistries showed similar significant changes to °Brix, pH, and anthocyanin content. In the Pinotage plot, a significant difference was found between the healthy vines and both the GRBaV and GRBaV + GRSPaV co-infected vines; however, no difference was found between the singly- and co-infected vines. Therefore, our results suggest that the effect of GRSPaV most likely did not relate to the measured chemical compositions of grape berries. Generally, GRSPaV has been shown to decrease yield, winter pruning weights, berry weights as well as causing a lower TA and higher pH in fruit while not effecting °Brix (Reynolds et al. 1997).

One issue on determining the effect of virus infection on berry chemistry is that viruses, such as the GLRaVs and GRBaV will impact grapevines differently based on a wide range of parameters such as cultivar, scion/rootstock combination, vine age, and location (Alabi et al. 2016; Cabaleiro et al. 1999; Golino 1993; Guidoni et al. 1997; Kliewer and Lider 1976; Kovacs et al. 2001; Krake 1993; Lee and Martin 2009; Singh Brar et al. 2008). For example, in one study, GRBaV affected fruit yield for cultivars Chardonnay and Zinfandel, but did not affected Cabernet Sauvignon (Sudarshana et al. 2015). This type of fluctuations creates many reports that seem to differ in results, as we observed in comparison of our results to previous studies. All of the vines tested in this study were relatively older vines (Pinotage-12 years, Mourvedre and Syrah-18 years). If we were to assume these vines have been infected for the majority of their planted years, the results seen may be more severe due to the long-term infections these vines have endured.

In summary, significant impacts of GLRaV-3, GRBaV, and GLRaV-3 + GRBaV co-infection on °Brix, pH, TA, YAN, and anthocyanin levels were found, and previous studies indicated that these changes will negatively impact wine quality. Since there is no cure for virus-infected vines, only removal of infected vines could increase harvest quality. It has been shown with cases of Grapevine Bois noir, Grapevine virus A, GLRaV-1, and GLRaV-3 that removal of infected vines helps yield, berry chemistry, and sensory characteristics of the final wine product (Endeshaw et al. 2012; Komar et al. 2007).

Table 6.1: Primers used for conventional and RT-PCR detection of grapevine viruses.

Virus	Primer name and Reference	PCR parameters (temp in °C)
GLRaV-1	dCP1-1/dCP1-2 (Esteves et al. 2013)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GLRaV-2	P19qtF4 and p24qtR (Beuve et al. 2007)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GLRaV-3	GEN-11112F/GEN-11233R (Chooi et al. 2013)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GLRaV-4 (strains 4, 5, 9)	LRAmp-F/LRAmp-R (Abou Ghanem-Sabanadzovic et al. 2012)	52°, 1h; 94, 2min; 40x (94, 30s; 50, 35s; 72,45s); 72, 7min
GRSPaV-1	RSP13/RSP14 (B. Meng et al. 1999)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GVA	H587/C995 (Minafra et al. 1997)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GVB	C410/H28 (Minafra and Hadidi 1994)	52°, 1h; 35x (94, 30s; 54,45s; 72, 1min); 72,2min
GRBaV	GVGF1/GVGR1 (Al Rwahnih et al. 2013)	94°, 2min; 35x (94, 30s; 60, 30s; 72, 1min); 72, 5min

Table 6.2: ANOVA table for the effect of virus status on harvest parameter measurements on °Brix, pH, TA, YAN, and Anthocyanin, Orange County, VA 2015.

	Cultivars					
	Mouvedre[a]		**Pinotage**[b]		**Syrah**[c]	
Measurement	F^d	*P*-value[d]	F^d	*P*-value[d]	F^d	*P*-value[d]
°Brix	351.61	<0.001**	46.87	<0.001**	283.47	<0.001**
pH	17.55	<0.001**	50.64	<0.001**	9.31	<0.001**
TA	1.19	0.34	8.63	<0.001**	2.63	0.11
YAN	1.41	0.28	2.00	0.18	3.88	0.05*
Anthocyanin	16.24	<0.001**	1.41	0.28	126.55	<0.001**

[a] Mouvedre co-infection virus status was GLRaV-3 and GLRaV-3+GRSPaV
[b] Pinotage co-infection virus status was GRBaV and GRBaV+GRSPaV
[c] Syrah co-infection virus status was GLRaV-3 and GLRaV-3+GRBaV
[d] *F* statistic from a generalized linear mixed model (PROC GLIMMIX, SAS 9.4), *P*-value is to indicate significantly high treatment effect. One and Two asterisks indicate 95% and 99% confidence, respectively.

Table 6.3: Significant differences in °Brix, pH, TA, YAN, and anthocyanin levels within each cultivar based on virus status.

Cultivar	Virus status	°Brix[a]	pH[a]	TA[a] (g/L)	YAN[a] (mg/L N)	Anthocyanin[a] (mg/L)
Mourvedre	Virus-free	20.1 A (0.092)	3.51 A (0.022)	8.792 (0.227)	323.6 (10.941)	370.98 A (8.031)
	GLRaV-3	17.1 B (0.092)	3.36 B (0.022)	9.182 (0.227)	300.2 (10.941)	317.06 B (8.031)
	GLRaV-3 + GRSPaV	17.0 B (0.092)	3.36 B (0.022)	9.252 (0.227)	302.20 (10.941)	313.00 B (8.031)
Pinotage	Virus-free	22.2 A (0.175)	3.74 A (0.016)	4.998 B (0.210)	345.0 (4.445)	416.26 (3.203)
	GRBaV	20.2 B (0.175)	3.55 B (0.016)	6.092 A (0.210)	333.4 (4.445)	333.20 (3.203)
	GRBaV + GRSPaV	20.0 B (0.175)	3.54 B (0.016)	6.034 A (0.210)	335.0 (4.445)	336.80 (3.203)
Syrah	Virus-free	20.3 A (0.119)	3.58 A (0.018)	8.126 (0.300)	321.6 A (9.638)	350.54 A (4.463)
	GLRaV-3	18.2 B (0.119)	3.52 B (0.018)	8.412 (0.300)	287.4 B (4.445)	289.90 B (4.463)
	GLRaV-3 + GRBaV	16.3 C (0.119)	3.47 B (0.018)	9.074 (0.300)	290.2 B (4.445)	250.90 C (4.463)

[a] Significant effect of factor(s) examined if ANOVA determined significance using Fisher's least significant difference (LSD). Different letters within dates show significant differences ($P \leq 0.05$) between "treatments". Standard error shown in parentheses below values.

6.5 References:

Agrios, G. N. (2005). *Plant Pathology*: Elsevier Academic Press.

Aigrain, P. (2001). Conjuncture vitiviniculture mondiale. *Office Int Vigne Vin Bull, 74*, 209-225.

Al Rwahnih, M., Dave, A., Anderson, M. M., Rowhani, A., Uyemoto, J. K., & Sudarshana, M. R. (2013). Association of a DNA virus with grapevines affected by red blotch disease in California. *Phytopathology, 103*(10), 1069-1076, doi:10.1094/PHYTO-10-12-0253-R.

Al Rwahnih, M., Dolja, V. V., Daubert, S., Koonin, E. V., & Rowhani, A. (2012a). Genomic and biological analysis of *Grapevine leafroll-associated virus 7* reveals a possible new genus within the family Closteroviridae. *Virus Research, 163*(1), 302-309, doi:10.1016/j.virusres.2011.10.018.

Al Rwahnih, M., Osman, F., Sudarshana, M., Uyemoto, J., Minafra, A., Saldarelli, P., et al. (2012b). Detection of *Grapevine leafroll-associated virus 7* using real time qRT-PCR and conventional RT-PCR. *Journal of Virological Methods, 179*(2), 383-389, doi:10.1016/j.jviromet.2011.11.026.

Al Rwahnih, M., Rowhani, A., & Golino, D. A. (2015). First report of *Grapevine red blotch associated virus* in archival grapevine material from Sonoma County, California. *Plant Disease*, 150127132800003, doi:10.1094/PDIS-12-14-1252-PDN.

Al Rwahnih, M., Rowhani, A., Golino, D. A., Islas, C. M., Preece, J. E., & Sudarshana, M. R. (2015). Detection and genetic diversity of *grapevine red blotch-associated virus* isolates in table grape accessions in the National Clonal Germplasm Repository in California. *Canadian Journal of Plant Pathology, 37*(1), 130-135, doi:10.1080/07060661.2014.999705.

Alabi, O. J., Al Rwahnih, M., Karthikeyan, G., Poojari, S., Fuchs, M., Rowhani, A., Naidu, R.A. (2011). *Grapevine leafroll-associated virus 1* Occurs as Genetically Diverse Populations. *Phytopathology, 101*(12), 1446-1456, doi:10.1094/phyto-04-11-0114.

Alabi, O. J., Casassa, L. F., Gutha, L. R., Larsen, R. C., Henick-Kling, T., Harbertson, J. F., Naidu, R.A. (2016). Impacts of Grapevine Leafroll Disease on Fruit Yield and Grape and Wine Chemistry in a Wine Grape (Vitis vinifera L.) Cultivar: e0149666. *PLoS ONE, 11*(2), doi:10.1371/journal.pone.0149666.

Alkowni, R., Rowhani, A., Daubert, S., & Golino, D. (2004). Partial characterization of a new ampelovirus associated with grapevine leafroll disease. *Journal of Plant Pathology*, 123-133.

Alleweldt, G., & Dettweiler, E. (1994). *The Genetic Resources of Vitis: World List of Grapevine Collections. (2nd edition)*: Geilweilerhof.

Alley, C., Goheen, A., Olmo, H., & Koyama, A. (1963). The effect of virus infections on vines, fruit and wines of Ruby Cabernet. *American Journal of Enology and Viticulture, 14*(3), 164-170.

Alley, L., & Golino, D. The origins of the grape program at foundation plant materials service. In *Proceedings of the ASEV 50th Anniversary Meeting, Seattle, WA, 2000* (pp. 222-230): American Society for Enology and Viticulture

Almasi, A., Ekes, M., & Gaborjanyi, R. (1996). Comparison of ultrastructural changes of Nicotiana benthamiana infected with three different viruses. *Acta Phytopathologica et Entomologica Hungarica, 31*, 181-190.

Almeida, R. P. P., Daane, K. M., Bell, V. A., Blaisdell, G. K., Cooper, M. L., Herrbach, E., et al. (2013). Ecology and management of grapevine leafroll disease. *Frontiers in Microbiology, 4*, 13, doi:10.3389/fmicb.2013.00094.

Atallah, S. S., Gomez, M. I., Fuchs, M. F., & Martinson, T. E. (2012). Economic Impact of Grapevine Leafroll Disease on Vitis vinifera cv. Cabernet franc in Finger Lakes Vineyards of New York. *American Journal of Enology and Viticulture, 63*(1), 73-79, doi:10.5344/ajev.2011.11055.

Avgelis, A., & Boscia, D. (2001). *Grapevine leafroll-associated closterovirus 7* in Greece. *Phytopathologia Mediterranea, 40*(3), 289-292.

Azzam, A. I., Gonsalves, D., & Golino, D. A. (1991). Detection of dsRNA in grapevines showing symptoms of rupestris stem pitting disease and the variabilities encountered. *Plant Disease, 75*(9), 960-964, doi:10.1094/PD-75-0960.

Bahder, B. W., Zalom, F., Jayanth, M., & Sudarshana, M. R. (2016). Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying Spissistilus festinus (Say) as a vector of *Grapevine red blotch-associated virus*. *Phytopathology*, doi:10.1094/phyto-03-16-0125-fi.

Balachandran, S., Hurry, V., Kelley, S., Osmond, C., Robinson, S., Rohozinski, J., et al. (1997). Concepts of plant biotic stress. Some insights into the stress physiology of virus - infected plants, from the perspective of photosynthesis. *Physiologia Plantarum, 100*(2), 203-213.

Bangels, E., Peusens, G., Belien, T., & Gobin, B. (2011). Compatibility of spirotetramat (Movento) in integrated pome fruit production. *Acta Hort., 917*, 77-83.

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, doi:10.1071/ea9941205.

Bell, J., Krueger, S., & Steffens, R. (2008). Development of MoventoTM for sucking pest control on annual and perennial crops in the United States. *Bayer CropScience Journal, 61*(2), 307-328.

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. Natural spread of grapevine leafroll disease in a vineyard of Northern Italy. In *Extended Abstracts 11th meeting of ICVG, 1993, Montreaux, 1993* (pp. 110)

Bertazzon, N., & Angelini, E. (2004). Advances in the detection of *Grapevine leafroll-associated virus 2* variants. *Journal of Plant Pathology, 86*(4, Special issue), 283-290.

Beuve, M., Candresse, T., Tannieres, M., & Lemaire, O. (2015). First Report of *Grapevine Pinot gris virus* (GPGV) in grapevine in France. *Plant Disease, 99*(2), 293-294, doi:10.1094/PDIS-10-14-1008-PDN.

Beuve, M., Sempe, L., & Lemaire, O. (2007). A sensitive one-step real-time RT-PCR method for detecting *Grapevine leafroll-associated virus 2* variants in grapevine. *Journal of Virological Methods, 141*(2), 117-124, doi:10.1016/j.jviromet.2006.11.042.

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.

Bonavia, M., Digiaro, M., Boscia, D., Boari, A., Bottalico, G., Savino, V., et al. (1996). Studies on "corky rugose wood" of grapevine and on the diagnosis of grapevine virus B. *Vitis, 35*(1), 53-58.

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., Martelli, G. P., Savino, V., & Castellano, M. A. (1991). Identification of the agent of grapevine fleck disease. *Vitis, 30*(2), 97-105.

Boscia, D., Minafra, A., & Martelli, G. P. (1997). Filamentous viruses of grapevine: Putative trichoviruses and capilloviruses. In P. L. Monette (Ed.), *Filamentous viruses of woody plants*. (pp. 19-28): Trivandrum: Research Signpost.

Boscia, D., Savino, V., Minafra, A., Namba, S., Elicio, V., Castellano, M. A., et al. (1993). Properties of a filamentous virus isolated from grapevines affected by corky bark. *Archives of Virology, 130*(1-2), 109-120, doi:10.1007/BF01319000.

Boulila, M., Boscia, D., Terlizzi, B. d., Castellano, M. A., Minafra, A., Savino, V., et al. (1990). Some properties of a phloem-limited non mechanically-transmissible grapevine virus. *Journal of Phytopathology, 129*(2), 151-158, doi:10.1111/j.1439-0434.1990.tb04298.x.

Boulton, R. (1980). The Relationships between Total Acidity, Titratable Acidity and pH in Wine. *American Journal of Enology and Viticulture, 31*(1), 76.

Boulton, R. B. (1998). *Principles and practices of winemaking* (Vol. Book, Whole). Gaithersburg, Md: Aspen.

Brady, S. G., Gadau, J., & Ward, P. S. (2000). Systematics of the ant genus *Camponotus* (Hymenoptera: Formicidae): a preliminary analysis using data from the mitochondrial gene cytochrome oxidase I. In A. D. Austin, & M. Dowton (Eds.), *Hymenoptera: evolution, biodiversity and biological control. Fourth International Hymenoptera Conference, held in Canberra, Australia, in January 1999*. (pp. 131-139). Collingwood: CSIRO Publishing.

Braun, A., Palmiter, D., & Keplinger, J. (1966). Nematodes found in apple orchards in the Hudson Valley, 1956-1965. *Plant Dis. Reptr, 50*(10), 783-786.

Brück, E., Elbert, A., Fischer, R., Krueger, S., Kühnhold, J., Klueken, A. M., et al. (2009). Movento< sup>®</sup>, an innovative ambimobile insecticide for sucking insect pest control in agriculture: Biological profile and field performance. *Crop Protection, 28*(10), 838-844.

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. http://biology.anu.edu.au/Groups/MES/vide/August 1996.

Burrows, M. E., Caillaud, M. C., Smith, D. M., & Gray, S. M. (2007). Biometrical genetic analysis of luteovirus transmission in the aphid *Schizaphis graminum*. *Heredity, 98*(2), 106-113, doi:10.1038/sj.hdy.6800909.

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, doi:10.1007/s10658-007-9254-1.

Cabaleiro, C., Segura, A., & Garcia-Berrios, J. J. (1999). Effects of grapevine leafroll-associated virus 3 on the physiology and must of Vitis vinifera L. cv. Albarino 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, doi:10.1094/pdis.1997.81.3.283.

Caldwell, J. (2012). Virginia wine industry jobs grow by 50%; Economic impact doubles, new study finds. http://www.governor.virginia.gov/News/viewRelease.cfm?id=1114.

Calvi, B. L. (2011). *Effects Of Red-leaf Disease On Cabernet Sauvignon At The Oakville Experimental Vineyard And Mitigation By Harvest Delay And Crop Adjustment*. ProQuest Dissertations Publishing,

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.

Chang, P. G. S., McLaughlin, W. A., & Tolin, S. A. (2011). Tissue blot immunoassay and direct RT-PCR of cucumoviruses and potyviruses from the same NitroPure nitrocellulose membrane. *Journal of Virological Methods, 171*(2), 345-351, doi:10.1016/j.jviromet.2010.11.018.

Chang, P. S., & Tolin, S. A. (2008). Detection and partial characterization of three RNA viruses using improved membrane-based technologies. *Phytopathology, 98*(6), S214-S214.

Chang, P. S., & Tolin, S. A. (2010). Nitrocellulose membranes as a solid matrix for Cucumber mosaic virus immuno-detection and subgroup identification by RT-PCR. [*Phytopathology, 100*(6), S173-S173.

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). *59*, 330-337.

Chooi, K. M., Cohen, D., & Pearson, M. N. (2013). Generic and sequence-variant specific molecular assays for the detection of the highly variable *Grapevine leafroll-associated virus 3*. *Journal of Virological Methods, 189*(1), 20-29, doi:10.1016/j.jviromet.2012.12.018.

Conti, M. (1980). A Closterovirus from a Stem-Pitting-Diseased Grapevine. *Phytopathology, 70*(5), 394, doi:10.1094/Phyto-70-394.

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, doi:10.1017/s0007485300053888.

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, doi:10.1017/s0007485300055048.

Credi, R., & Babini, A. R. (1997). Effects of virus and virus-like infections on growth, yield, and fruit quality of Albana and Trebbiano Romagnolo grapevines. *American Journal of Enology and Viticulture, 48*(1), 7-12.

Crowe, A. (2007). *The Winemaker's Answer Book: Solutions to Every Problem; Answers to Every Question*: Storey Publishing, LLC.

Daane, K. M., Almeida, R. P., Bell, V. A., Walker, J. T., Botton, M., Fallahzadeh, M., et al. (2012). Biology and management of mealybugs in vineyards. In *Arthropod Management in Vineyards:* (pp. 271-307): Springer.

Daane, K. M., Bentley, W. J., Walton, V. M., Malakar-Kuenen, R., Millar, J. G., Ingels, C. A., et al. (2006). New controls investigated for vine mealybug. *California Agriculture, 60*(1), 31-38, doi:10.3733/ca.v060n01p31.

Daane, K. M., Cooper, M. L., Triapitsyn, S. V., Walton, V. M., Yokota, G. Y., Haviland, D. R., et al. (2008). Vineyard managers and researchers seek sustainable solutions for mealybugs, a changing pest complex. *California Agriculture, 62*(4), 167-176, doi:10.3733/ca.v062n04p167.

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, doi:10.1016/j.biocontrol.2004.04.010.

Daane, K. M., Middleton, M. C., Sforza, R., Cooper, M. L., Walton, V. M., Walsh, D. B., et al. (2011). Development of a multiplex PCR for identification of vineyard mealybugs. *Environmental Entomology, 40*(6), 1595-1603, doi:10.1603/en11075.

de Saporta, G. (1879). *Le monde des plantes avant l'apparition de l'homme*.: G. Masson.

Demeke, T., & Adams, R. P. (1992). The effects of plant polysaccharides and buffer additives on PCR. *BioTechniques, 12*(3), 332-332.

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, doi:10.1146/annurev.py.32.090194.001401.

Dolja, V. V., Kreuze, J. F., & Valkonen, J. P. T. (2006). Comparative and functional genomics of closteroviruses. *Virus Research, 117*(1), 38-51, doi:10.1016/j.virusres.2006.02.002.

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, doi:10.1007/s10658-008-9269-2.

du Preez, J., Stephan, D., Mawassi, M., & Burger, J. T. (2011). The grapevine-infecting vitiviruses, with particular reference to grapevine virus A. *Archives of Virology, 156*(9), 1495-1503, doi:10.1007/s00705-011-1071-3.

Elbeaino, T., Numic, F., Digiaro, M., Sabanadzovic, S., & Martelli, G. P. (2009). Partial characterization of a grapevine leafroll-associated virus isolated from an infected cypriot vine of cv. Mavro. *Journal of Plant Pathology, 91*(2), 479-484.

Endeshaw, S. T., Murolo, S., Romanazzi, G., & Neri, D. (2012). Effects of Bois noir on carbon assimilation, transpiration, stomatal conductance of leaves and yield of grapevine (Vitis vinifera) cv. Chardonnay. *Physiologia Plantarum, 145*(2), 286-295, doi:10.1111/j.1399-3054.2012.01576.x.

Endeshaw, S. T., Sabbatini, P., Romanazzi, G., Schilder, A. C., & Neri, D. (2014). Effects of *grapevine leafroll associated virus 3* infection on growth, leaf gas exchange, yield and basic fruit chemistry of Vitis vinifera L. cv. Cabernet Franc. *Scientia Horticulturae, 170*, 228-236, doi:10.1016/j.scienta.2014.03.021.

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, doi:10.1016/j.jviromet.2009.11.009.

Engelbrecht, D., & Kasdorf, G. (1987). Occurrence and transmission of grapevine virus A in South African grapevines. *South African Journal for Enology and Viticulture, 8*(1), 23-29.

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.

Esteves, F., Teixeira Santos, M., Eiras-Dias, J. E., & Fonseca, F. (2013). Molecular data mining to improve antibody-based detection of *Grapevine leafroll-associated virus 1* (GLRaV-1). *Journal of Virological Methods, 194*(1-2), 258-270, doi:10.1016/j.jviromet.2013.09.004.

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.

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.

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. http://www.nysipm.cornell.edu/factsheets/grapes/diseases/grape_leafroll.pdf. Accessed June 2012.

Fuchs, M., Marsella-Herrick, P., Loeb, G. M., Martinson, T. E., & Hoch, H. C. (2009a). Diversity of ampeloviruses in mealybug and soft scale vectors and in grapevine hosts from leafroll-affected vineyards. *Phytopathology, 99*(10), 1177-1184, doi:10.1094/phyto-99-10-1177.

Fuchs, M., Martinson, T. E., Loeb, G. M., & Hoch, H. C. (2009b). Survey for the three major leafroll disease-associated viruses in Finger Lakes vineyards in New York. *Plant Disease, 93*(4), 395-401, doi:10.1094/pdis-93-4-0395.

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, doi:10.1071/zo9760237.

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, doi:10.1094/phyto-96-1223.

Garau, R., Prota, V. A., Boscia, D., Fiori, M., & Prota, U. (1995). Pseudococcus-affinis mask, new vector of grapevine trichovirus-A and trichovirus-B. *Vitis, 34*(1), 67-68.

Garau, R., Prota, V. A., Piredda, R., Boscia, D., & Prota, U. (1994). On the possible relationship between Kober stem grooving and *grapevine virus A*. *Vitis, 33*(3), 161-163.

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, doi:10.1603/0022-0493-94.1.291.

Ghanem-Sabanadzovic, N., Sabanadzovic, S., Gugerli, P., & Rowhani, A. (2012). Genome organization, serology and phylogeny of *Grapevine leafroll-associated viruses 4* and *6*: Taxonomic implications. *Virus Research, 163*(1), 120-128, doi:10.1016/j.virusres.2011.09.001.

Giampetruzzi, A., Roumi, V., Roberto, R., Malossini, U., Yoshikawa, N., La Notte, P., et al. (2012). A new grapevine virus discovered by deep sequencing of virus- and viroid-derived small RNAs in Cv Pinot gris. *Virus Research, 163*(1), 262-268, doi:10.1016/j.virusres.2011.10.010.

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., Predajňa, L., & Komínek, P. (2011). Grapevine fleck virus isolates split into two distinct molecular groups. *Journal of Phytopathology, 159*(11/12), 805-807, doi:10.1111/j.1439-0434.2011.01845.x.

Glasa, M., Predajňa, L., Komínek, P., Nagyová, A., Candresse, T., & Olmos, A. (2014). Molecular characterization of divergent *grapevine Pinot gris virus* isolates and their detection in Slovak and Czech grapevines. *Archives of Virology, 159*(8), 2103-2107, doi:10.1007/s00705-014-2031-5.

Goheen, A. C., & Cook, J. A. (1959). Leafroll (red-leaf or rougeau) and its effects on vine growth, fruit quality, and yields. *Amer Jour Enol and Viticulture, 10*((4)), 173-181.

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. (1995). Transmission studies of grapevine leafroll associated virus and grapevine corky bark associated virus by the obscure mealybug. *American Journal of Enology and Viticulture, 46*, 408.

Golino, D., Sim, S., & Rowhani, A. Identification of the latent viruses associated with young vine decline in California. In *In: Extended Abstracts of the 13th meeting of ICVG, Adelaide, 2000, 85-86, 2000* (pp. 85-86)

Golino, D. A. (1993). Potential Interactions Between Rootstocks and Grapevine Latent Viruses. *American Journal of Enology and Viticulture, 44*(2), 148-152.

Golino, D. A., Sim, S. T., Gill, R., & Rowhani, A. (2002). California mealybugs can spread grapevine leafroll disease. *California Agriculture, 56*(6), 196-201, doi:10.3733/ca.v056n06p196.

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, doi:10.3733/ca.v062n04p156.

Goszczynski, D. E., & Jooste, A. E. C. (2003). Identification of divergent variants *of Grapevine virus A. European Journal of Plant Pathology, 109*(4), 397-403, doi:10.1023/A:1023555018700.

Goszczynski, D. E., Kasdorf, G. G. F., Pietersen, G., Tonder, H. v., Goszczynski, D. E., Kasdorf, G. G. F., et al. (1996). Detection of two strains of *grapevine leafroll-associated virus 2*. *Vitis, 35*(3), 133-135.

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, doi:10.1111/j.1570-7458.2008.00786.x.

Gray, S. M., Caillaud, M. C., Burrows, M., & Smith, D. M. (2007). Transmission of Two Viruses that Cause Barley Yellow Dwarf is Controlled by Different Loci in the Aphid, *Schizaphis graminum*. *Journal of insect science, 7*(25), 1-15, doi:10.1673/031.007.2501.

Greif, C., Garau, R., Boscia, D., Prota, V. A., Fiori, M., Bass, P., et al. (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.

Gugerli, P. 25 years of serological identification of grapevine leafroll-associated viruses: antiserum and monoclonal antibodies to GLRaV-1 to GLRaV-9. In *16th Meetig of ICVG, Dijon, France, 2009* (16 ed., pp. 24-28, Extended Abstracts 16th Meetig of ICVG, Dijon, France)

Gugerli, P., Brugger, J. J., & Bovey, R. (1984). L'enroulement de la vigne: mise en evidence de particules virales et drsveloppement dune methode immuno-enzymatique pour le diagnostic rapide. *Revue Suisse de Viticulture Arboriculture et Horticulture, 16*, 299-304.

Gugerli, P., & Ramel, M. E. Grapevine leafroll associated virus II analyzed by monoclonal antibodies. In *Extended Abstracts 11th Meeting ICVG, Montreux, 1993* (pp. 23-24, Vol. 1993)

Guidoni, S., Mannini, F., Ferrandino, A., Argamante, N., & Di Stefano, R. (1997). The effect of grapevine leafroll and rugose wood sanitation on agronomic performance and berry and leaf phenolic content of a Nebbiolo clone (Vitis vinifera L.). *American Journal of Enology and Viticulture, 48*(4), 438-442.

Gullan, P. J., Downie, D. A., & Steffan, S. A. (2003). A new pest species of the mealybug genus *Ferrisia fullaway* (Hemiptera : Pseudococcidae) from the United States. *Annals of the Entomological Society of America, 96*(6), 723-737, doi:10.1603/0013-8746(2003)096[0723:anpsot]2.0.co;2.

Guo, Q., Honesty, S., Xu, M. L., Zhang, Y., Schoelz, J., & Qiu, W. P. (2014). Genetic diversity and tissue and host specificity of *Grapevine vein clearing virus*. *Phytopathology, 104*(5), 539-547, doi:10.1094/PHYTO-03-13-0075-R.

Habili, N. (2013). Highlights of a newly-emerging grapevine virus: Grapevine red blotch-associated virus.

Habili, N., Kominek, P., & Little, A. (2007). *Grapevine leafroll-associated virus 1* as a common grapevine pathogen. *PLant Viruses, 1*, 63-68.

Habili, N., & Nutter, F. W. (1997). Temporal and spatial analysis of grapevine leafroll-associated virus 3 in Pinot Noir grapevines in Australia. *Plant Disease, 81*(6), 625-628, doi:10.1094/pdis.1997.81.6.625.

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.

Haviland, D. R., Beede, R., Godfrey, K. E., & Daane, K. M. (2006). *Ferrisia gilli*: A new mealybug pest of pistachios and other deciduous crops. http://anrcatalog.ucdavis.edu/pdf/8207.pdf. Accessed November 2012.

Haviland, D. R., Buckey, J. H., & Rill, S. M. (2010a). *In-season control of vine mealybug in 'Autumn Royal' table grapes in Kern County, 2009* (Vol. 35, Vol. 1).

Haviland, D. R., Buckey, J. H., & Rill, S. M. (2010b). *In-season control of vine mealybug in 'Flame Seedless' table grapes in Kern County, 2008* (Vol. 35, Vol. 1).

Haviland, D. R., Hashim-Buckey, J., & Rill, S. M. (2011). *In-season control of vine mealybug in 'Red Globe' table grapes in Kern County, 2010* (Vol. 36, Vol. 1).

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.

Hogenhout, S. A., Ammar, E. D., Whitfield, A. E., & Redinbaugh, M. G. (2008). Insect vector interactions with persistently transmitted viruses. *Annual Review of Phytopathology, 46*(1), 327-359, doi:10.1146/annurev.phyto.022508.092135.

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.

Hunter, C., & Peat, W. (1973). The effect of tomato aspermy virus on photosynthesis in the young tomato plant. *Physiological Plant Pathology, 3*(4), 517-524.

Iland, P., Cynkar, W., Francis, I., Williams, P., & Coombe, B. (1996). Optimisation of methods for the determination of total and red ‐ free glycosyl glucose in black grape berries of Vitis vinifera. *Australian Journal of Grape and Wine Research, 2*(3), 171-178.

Iland, P., Ewart, A., Sitters, J., Markides, A., & Bruer, N. (2000). *Techniques for accurate chemical analysis and quality monitoring during winemaking*. Campbell Town, Australia: Wine Promotions.

Jelkmann, W., Mikona, C., Turturo, C., Navarro, B., Rott, M. E., Menzel, W., et al. (2012). Molecular characterization and taxonomy of grapevine leafroll-associated virus 7. *Archives of Virology, 157*(2), 359-362.

Jones, T. J., & Nita, M. (2016). Spatio-temporal association of GLRaV-3-infected grapevines, and effect of insecticidal treatments on mealybug populations in Virginia vineyards. *European Journal of Plant Pathology*, 1-16, doi:10.1007/s10658-016-0878-x.

Jones, T. J., Rayapati, N., & Nita, M. (2015). Occurrence of *Grapevine leafroll associated virus-2, −3* and *Grapevine fleck virus* in Virginia, U.S.A., and factors affecting virus infected vines. *European Journal of Plant Pathology*, 1-14, doi:10.1007/s10658-015-0605-z.

Karasev, A. V. (2000). Genetic diversity and evolution of closteroviruses. *Annual Review of Phytopathology, 38*, 293-324, doi:10.1146/annurev.phyto.38.1.293.

Keller, M. (2010). *The science of grapevines: anatomy and physiology* (Vol. Book, Whole). Amsterdam;Boston;: Academic Press/Elsevier.

King, A., Leftkowitz, E., Adams, M. J., & Carstens, E. B. (2011). *Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses*: Elsevier.

Kliewer, W. M., & Lider, L. (1976). Influence of leafroll virus on composition of Burger fruits. *American Journal of Enology and Viticulture, 27*(3), 118-124.

Komar, V., Vigne, E., Demangeat, G., & Fuchs, M. (2007). Beneficial Effect of Selective Virus Elimination on the Performance of *Vitis vinifera* cv. Chardonnay. *American Journal of Enology and Viticulture, 58*(2), 202-210.

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.

Krake, L. (1993). Characterization of grapevine leafroll disease by symptomatology. *Australian & New Zealand Wine Industry Journal, 8*(1), 40-44.

Krenz, B., Thompson, J. R., Fuchs, M., & Perry, K. L. (2012). Complete genome sequence of a new circular DNA virus from grapevine. *Journal of Virology, 86*(14), 7715-7715.

Krenz, B., Thompson, J. R., McLane, H., Fuchs, M., & Perry, K. L. (2014). *Grapevine red blotch-associated virus* is widespread in the United States. *Phytopathology*, doi:10.1094/phyto-02-14-0053-r.

La Notte, P., Buzkan, N., Choueiri, E., Minafra, A., & Martelli, G. P. (1997a). Acquisition and transmission of grapevine virus A by the mealybug *Pseudococcus longispinus*. *Journal of Plant Pathology, 78*(1), 79-85.

La Notte, P., Minafra, A., & Saldarelli, P. (1997b). A spot-PCR technique for the detection of phloem-limited grapevine viruses. *Journal of Virological Methods, 66*(1), 103-108, doi:10.1016/s0166-0934(97)00038-4.

Lee, J., Keller, K. E., Rennaker, C., & Martin, R. R. (2009). Influence of grapevine leafroll associated viruses (GLRaV-2 and -3) on the fruit composition of Oregon *Vitis vinifera* L. cv. Pinot noir: Free amino acids, sugars, and organic acids. *Food Chemistry, 117*(1), 99-105, doi:10.1016/j.foodchem.2009.03.082.

Lee, J., & Martin, R. R. (2009). Influence of grapevine leafroll associated viruses (GLRaV-2 and -3) on the fruit composition of Oregon *Vitis vinifera L.* cv. Pinot noir: Phenolics. *Food Chemistry, 112*(4), 889-896, doi:10.1016/j.foodchem.2008.06.065.

Levadoux, L. (1956). Les populations sauvages et cultivees de *Vitis vinifera* L.. *Ann. Amelior. Plantes, 6*, 59-117.

Li, R., Mock, R., Fuchs, M., Halbrendt, J., Howell, B., & Liu, Z. (2011). Characterization of the partial RNA1 and RNA2 3' untranslated region of *Tomato ringspot virus* isolates from North America. *Canadian Journal of Plant Pathology, 33*(1), 94-99.

Lider, L., Goheen, A., & Ferrari, N. (1975). A comparison between healthy and leafroll-affected grapevine planting stocks. *American Journal of Enology and Viticulture, 26*(3), 144-147.

Lim, S., Igori, D., Zhao, F., Moon, J. S., Cho, I. S., & Choi, G. S. (2016). First report of *Grapevine red blotch-associated virus* on grapevine in Korea. *Plant Disease*, doi:10.1094/PDIS-03-16-0283-PDN.

Littell, R. C., Milliken, G. A., Stroup, W. W., Wolffinger, R. D., & Schabenberger, O. (2006). *SAS for Mixed Models, Second Edition*. Cary, NC: SAS Institute, Inc.

Little, A., Fazeli, C. F., & Rezaian, M. A. (2001). Hypervariable genes in Grapevine leafroll associated virus 1. *Virus Research, 80*(1), 109-116, doi:10.1016/S0168-1702(01)00343-4.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *methods, 25*(4), 402-408.

Loeb, G. M. (2012). Grape Insect and mite pests-2012 field season. http://www.fruit.cornell.edu/grape/pdfs/Loeb-Grape%20Insect%20Mite%20Pests%202012.pdf. Accessed November 2012.

Lunden, S., Meng, B., Avery, J., & Qiu, W. (2010). Association of *Grapevine fanleaf virus, Tomato ringspot virus and Grapevine rupestris stem pitting-associated virus* with a grapevine vein-clearing complex on var. Chardonnay. *European Journal of Plant Pathology, 126*(2), 135-144.

Madden, L. V., Hughes, G., & Bosch, F. v. d. (2007). *The study of plant disease epidemics*. St. Paul: American Phytopathological Society (APS Press).

Mahfoudhi, N., Digiaro, 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, doi:10.1094/pdis-93-10-0999.

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, doi:10.1016/j.virusres.2008.02.015.

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, doi:10.1007/s00705-008-0290-8.

Malossini, U., Moscon, R., Ferrazza, M., Bianchedi, P., Varner, M., & Credi, R. Caratteristiche vegeto-produttive di viti Pinot grigio e Traminer aromatico affette da una nuova virosi segnalata in Trentino. In *IV Convegno Nazionale di Viticoltura (Conavi), 2012* (pp. 37 (032))

Mannini, F., Argamante, N., & Credi, R. (1996). Improvements in the quality of grapevine 'Nebbiolo' clones obtained by sanitation. In S. Poni, E. Peterlunger, F. Iacono, & C. Intrieri (Eds.), *First Ishs Workshop on Strategies to Optimize Wine Grape Quality* (pp. 319-324, Acta Horticulturae, Vol. 427). Leuven 1: International Society Horticultural Science.

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. near pseudococci , with implications for integrated pest management in vineyards. *Phytoparasitica, 39*(4), 369-376, doi:10.1007/s12600-011-0170-8.

Maree, H. J., Almeida, R. P. P., Bester, R., Chooi, K. M., Cohen, D., Dolja, V. V., et al. (2013). *Grapevine leafroll-associated virus 3*. *Frontiers in Microbiology, 4*, 21, doi:10.3389/fmicb.2013.00082.

Markakis, P. (2012). *Anthocyanins as Food Colors*: Elsevier Science.

Martelli, G. P. (1993). *Graft-transmissible diseases of grapevines: handbook for detection and diagnosis*. Rome: Food and Agriculture Organization (FAO).

Martelli, G. P. (2014). Directory of virus and virus-like diseases of the grapevine and their agents. *Journal of Plant Pathology, 96*(1), 1-136.

Martelli, G. P., Abou Ghanem-Sabanadzovic, N., Agranovsky, A. A., Al Rwahnih, M., Dolja, V. V., Dovas, C. I., et al. (2012). Taxonomic revision of the family Closteroviridae with special reference to the grapevine leafroll-associated members of the genus Ampelovirus and the putative species unassigned to the family. *Journal of Plant Pathology, 94*(1), 7-19.

Martelli, G. P., Adams, M. J., Kreuze, J. F., & Dolja, V. V. (2007). Family Flexiviridae: a case study in virion and genome plasticity. *Annual Review of Phytopathology, 45*, 73-100, doi:10.1146/annurev.phyto.45.062806.094401.

Martelli, G. P., Agranovsky, A. A., Bar-Joseph, M., Boscia, D., Candresse, T., Coutts, R. H. A., et al. (2002a). The family Closteroviridae revised. *Archives of Virology, 147*(10), 2039-2044, doi:10.1007/s007050200048.

Martelli, G. P., & Boudon-Padieu, E. (2006). *Directory of infectious diseases of grapevines*. (Vol. 55): International Centre for Advanced Mediterranean Agronomic Studies. Options Méditerranéennes, Ser. B, Studies and Research. 55: 59-75.

Martelli, G. P., & Jelkmann, W. (1998). *Foveavirus*, a new plant virus genus. *Archives of Virology, 143*(6), 1245-1249, doi:10.1007/s007050050372.

Martelli, G. P., Sabanadzovic, S., Abou Ghanem-Sabanadzovic, N., & Saldarelli, P. (2002b). Maculavirus, a new genus of plant viruses. *Archives of Virology, 147*(9), 1847-1853, doi:10.1007/s007050200046.

Martelli, G. P., Sabanadzovic, S., Abou-Ghanem Sabanadzovic, N., Edwards, M. C., & Dreher, T. (2002c). The family Tymoviridae. *Archives of Virology, 147*(9), 1837-1846, doi:10.1007/s007050200045.

Martelli, G. P., Viruses, I. C. f. t. S. o., Grapevine, V. D. o. t., Food, & Nations, A. O. o. t. U. (1993). *Graft-transmissible Diseases of Grapevines: Handbook for Detection and Diagnosis*: Food and Agriculture Organization of the United Nations.

Martin, R. R., Eastwell, K. C., Wagner, A., Lamprecht, S., & Tzanetakis, I. E. (2005). Survey for viruses of grapevine in Oregon and Washington. *Plant Disease, 89*(7), 763-766, doi:10.1094/pd-89-0763.

Martin, R. R., James, D., & Lévesque, C. A. (2000). Impacts of molecular diagnostic technologies on plant disease management. *Annual Review of Phytopathology, 38*(1), 207-239.

McGovern, P. E. (2003). *Ancient wine The search for the origins of viniculture*. Princeton: Princeton University Press.

Mehle, N., Prezelj, N., Hren, M., Boben, J., Gruden, K., Ravnikar, M., et al. (2013). A Real-Time PCR Detection System for the Bois Noir and Flavescence Dorée Phytoplasmas and Quantification of the Target DNA. In M. Dickinson, & J. Hodgetts (Eds.), *Phytoplasma* (Vol. 938, pp. 253-268, Methods in Molecular Biology): Humana Press.

Mekuria, Karasev, A. V., Martin, R. R., & Naidu, R. A. (2009a). First report of *Grapevine leafroll-associated virus-3* in six wine grape cultivars in Idaho. *Plant Disease, 93*(11), 1218, doi:10.1094/pdis-93-11-1218c.

Mekuria, Soule, M. J., Jarugula, S., & Naidu, R. A. (2009b). Current status of grapevine viruses in Washington State vineyards. *Phytopathology, 99*, S83.

Melzer, M. J., Sether, D. M., Borth, W. B., Mersino, E. F., & Hu, J. S. (2011). An assemblage of closteroviruses infects Hawaiian ti (Cordyline fruticosa L.). *Virus Genes, 42*(2), 254-260, doi:10.1007/s11262-010-0537-9.

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., 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, doi:10.1007/s11262-004-2197-0.

Meng, B., Zhu, H. Y., & Gonsalves, D. (1999). Rupestris stem pitting associated virus-1 consists of a family of sequence variants. *Archives of Virology, 144*(11), 2071-2085, doi:10.1007/s007050050623.

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.

Mikona, C., & Jelkmann, W. (2010). Replication of *Grapevine leafroll-associated virus-7* (GLRaV-7) by Cuscuta species and its transmission to herbaceous plants. *Plant Disease, 94*(4), 471-476.

Milkus, B. N., & Goodman, R. N. (1999). A survey of Missouri vineyards for the presence of five grape viruses. *American Journal of Enology and Viticulture, 50*(1), 133-134.

Millar, J. G., Daane, K. M., McElfresh, J. S., Moreira, J. A., Malakar-Kuenen, R., Guillen, M., et al. (2002). Development and optimization of methods for using sex pheromone for monitoring the mealybug *Planococcus ficus* (Homoptera : Pseudococcidae) in California vineyards. *Journal of Economic Entomology, 95*(4), 706-714, doi:10.1603/0022-0493-95.4.706.

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, doi:10.1016/0166-0934(94)90076-0.

Minafra, A., Saldarelli, P., & Martelli, G. P. (1997). *Grapevine virus A*: nucleotide sequence, genome organization, and relationship in the Trichovirus genus. *Archives of Virology, 142*(2), 417-423, doi:10.1007/s007050050088.

Monis, J., & Bestwick, R. K. (1996). Detection and localization of grapevine leafroll associated closteroviruses in greenhouse and tissue culture grown plants. *American Journal of Enology and Viticulture, 47*(2), 199-205.

Montero, R., Mundy, D., Albright, A., Grose, C., Trought, M. C. T., Cohen, D., et al. (2016). Effects of *Grapevine Leafroll associated Virus 3* (GLRaV-3) and duration of infection on fruit composition and wine chemical profile of *Vitis vinifera L.* cv. Sauvignon blanc. *Food Chemistry, 197*, 1177-1183, doi:10.1016/j.foodchem.2015.11.086.

Morales, R. Z., & Monis, J. (2007). First detection of *Grapevine leafroll associated virus-7* in California vineyards. *Plant Disease, 91*(4), 465-465, doi:10.1094/pdis-91-4-0465b.

Morelli, M., Catarino, A. D. M., Susca, L., Saldarelli, P., Gualandri, V., & Martelli, G. P. (2014). First report of *Grapevine Pinot gris Virus* from table grapes in Southern Italy. *Journal of Plant Pathology, 96*(2), 439-439.

Myles, S., Boyko, A. R., Owens, C. L., Brown, P. J., Grassi, F., Aradhya, M. K., et al. (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, doi:10.1073/pnas.1009363108.

Naidu, R., Rowhani, A., Fuchs, M., Golino, D., & Martelli, G. P. (2014). Grapevine Leafroll: A Complex Viral Disease Affecting a High-Value Fruit Crop. *Plant Disease, 98*(9), 1172-1185, doi:10.1094/PDIS-08-13-0880-FE.

Naidu, R. A., Maree, H. J., & Burger, J. T. (2015b). Grapevine Leafroll Disease and Associated Viruses: A Unique Pathosystem. *Annual Review of Phytopathology, 53*, 613-634, doi:10.1146/annurev-phyto-102313-045946.

Naidu, R. A., & Mekuria, T. A. (2010). First report of *Grapevine fleck virus* from Washington vineyards. *Plant Disease, 94*(6), 784, doi:10.1094/pdis-94-6-0784a.

Naidu, R. A., Soule, M. J., & Jarugula, S. (2006). Single and mixed infections of *Grapevine leafroll-associated viruses* in Washington State vineyards. *Phytopathology, 96*, S83.

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, doi:10.3186/jjphytopath.45.497.

Nassuth, A., Pollari, E., Helmeczy, 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, doi:10.1016/s0166-0934(00)00211-1.

Nauen, R., Reckmann, U., Thomzik, J., & Thielert, W. (2008). Biological profile of spirotetramat (Movento®) - a new two-way systemic (ambimobile) insecticide against sucking pest species. *Bayer CropScience Journal, 61*(2), 245-278.

Newbury, H. J., & Possingham, J. V. (1977). Factors Affecting the Extraction of Intact Ribonucleic Acid from Plant Tissues Containing Interfering Phenolic Compounds. *Plant Physiology, 60*(4), 543-547, doi:10.1104/pp.60.4.543.

Ng, J. C. K., & Falk, B. W. (2006). Virus-vector interactions mediating nonpersistent and semipersistent transmission of plant viruses. *Annual Review of Phytopathology, 44*(1), 183-212, doi:10.1146/annurev.phyto.44.070505.143325.

Nita, M., Ellis, M. A., & Madden, L. V. (2008). Variation in disease incidence of Phomopsis cane and leaf spot of grape in commercial vineyards in Ohio. *Plant Disease, 92*(7), 1053-1061, doi:10.1094/pdis-92-7-1053.

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, doi:10.1111/j.1439-0434.2011.01857.x.

Oberhansli, T., Altenbach, D., & Bitterlin, W. (2011). Development of a duplex TaqMan real-time PCR for the general detection of phytoplasmas and 18S rRNA host genes in fruit trees and other plants. *Bulletin of Insectology, 64*, S37-S38.

Oberholster, A. Impact of red blotch disease on grape and wine composition and quality. . In *American Society of Enology and Viticulture National Conference Technical Abstracts. p. 75, 2015* (pp. 75)

Osman, F., Leutenegger, C., Golino, D., & Rowhani, A. (2007). Real-time RT-PCR (TaqMan®) assays for the detection of *Grapevine Leafroll associated viruses 1-5* and *9*. *Journal of Virological Methods, 141*(1), 22-29, doi:10.1016/j.jviromet.2006.11.035.

Osman, F., Leutenegger, C., Golino, D., & Rowhani, A. (2008). Comparison of low-density arrays, RT-PCR and real-time TaqMan ® RT-PCR in detection of grapevine viruses. *Journal of Virological Methods, 149*(2), 292-299, doi:10.1016/j.jviromet.2008.01.012.

Osman, F., & Rowhani, A. (2006). Application of a spotting sample preparation technique for the detection of pathogens in woody plants by RT-PCR and real-time PCR (TaqMan). *Journal of Virological Methods, 133*(2), 130-136, doi:10.1016/j.jviromet.2005.11.005.

Pearson, R. C., & Goheen, A. C. (1988). *Compendium of grape diseases*: APS Press.

Perry, J. N. (1998). Measures of spatial pattern for counts. *Ecology, 79*(3), 1008-1017, doi:10.1890/0012-9658(1998)079[1008:mospfc]2.0.co;2.

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.

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, doi:10.1046/j.1365-3059.1997.d01-44.x.

Pfeiffer, D. G., Baudoin, A. B., Bergh, J. C., & Nita, M. (2016). Grapes: Diseases and Insects in Vineyards. In *Horticultural and Forest Crops* (Vol. Publication 456-017): Virginia Cooperative Extension.

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. 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. Accessed May 2012.

Pietersen, G. Spatio temporal distribution dynamics of grapevine leafroll disease in western cape vineyards. In *Extended Abstracts 15th Meeting of ICVG, Stellenbosch, South Africa, April 2006, pp 126-127, 2006* (pp. 126-127)

Pietersen, G. (2010). Grapevine leafroll disease control in South Africa. https://41.203.19.179/index.php?option=com_zine&view=article&id=548%3Agrapevine-leafroll-disease-control-in-south-africa&Itemid=10. Accessed May 2012.

Plesko, I. M., Marn, M. V., & Zezlina, I. (2014). First Report of *Grapevine Pinot gris virus* Infecting Grapevine in Slovenia. *Plant Disease, 98*(7), 1014-1015, doi:10.1094/PDIS-11-13-1137-PDN.

Poojari, S., Alabi, O. J., Fofanov, V. Y., & Naidu, R. A. (2013). A leafhopper-transmissible DNA virus with novel evolutionary lineage in the family Geminiviridae implicated in grapevine redleaf disease by next-generation sequencing. *PLoS ONE, 8*(6), e64194.

Powell, C., Forer, L., & Stouffer, R. (1982). Reservoirs of *tomato ringspot virus* in fruit orchards. *Plant Disease, 66*(7), 583-584.

Powell, C., Forer, L., Stouffer, R., Cummins, J., Gonsalves, D., Rosenberger, D., et al. (1984). Orchard weeds as hosts of *tomato ringspot* and *tobacco ringspot viruses*. *Plant Disease, 68*(3), 242-244.

Powell, C. A., Longenecker, J. L., & Forer, L. B. (1990). Incidence of *Tomato Ringspot Virus* and *Tobacco Ringspot Virus* in grapevines in Pennsylvania. *Plant Disease, 74*(9), 702-704, doi:10.1094/pd-74-0702.

Qiu, W., Avery, J., & Lunden, S. (2007). Characterization of a severe virus-like disease in Chardonnay grapevines in Missouri. *Plant Health Progress*.

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. http://wine.wsu.edu/research-extension/plant-health/virology/virus-diseases/. Accessed November 2012.

Rayapati, N., O'Neal, S., & Walsh, D. (2008). Grapevine leafroll disease. http://pubs.wsu.edu. Accessed 2012 April.

Reynolds, A. G., Lanterman, W. S., & Wardle, D. A. (1997). Yield and berry composition of five *Vitis* cultivars as affected by Rupestris stem pitting virus. *American Journal of Enology and Viticulture, 48*(4), 449-458.

Rezaian, M. A., & Krake, L. R. (1987). Nucleic acid extraction and virus detection in grapevine. *Journal of Virological Methods, 17*(3), 277-285, doi:10.1016/0166-0934(87)90137-6.

Rosa, C., Jimenez, J. F., Margaria, P., & Rowhani, A. (2011). Symptomatology and Effects of Viruses Associated with Rugose Wood Complex on the Growth of Four Different Rootstocks. *American Journal of Enology and Viticulture, 62*(2), 207-213, doi:10.5344/ajev.2011.10104.

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. Transmission of grapevine leafroll disease and an associated closterovirus to healthy grapevine by the mealybug *Planococcus ficus* Signoret. In *Proceedings 9th Meeting of ICVG, Kiryat Anavim, 1989* (pp. 1987, 1967-1969)

Rosenberger, D., Cummins, J., & Gonsalves, D. Development of apple union necrosis and decline in apple-trees inoculated with *Tomato Ringspot Virus*. In *Phytopathology, 1985* (Vol. 75, pp. 1312-1312, Vol. 11): Amer Phytopathological Soc 3340 Pilot Knob Road, St Paul, Mn 55121

Rosenberger, D., Harrison, M., & Gonsalves, D. (1983). Incidence of apple union necrosis and decline, *tomato ringspot virus*, and *Xiphinema* vector species in Hudson Valley orchards. *Plant Disease, 67*(4), 356-360.

Rosenberger, D., & Meyer, F. (1988). Control of dagger and lesion nematodes in apple and plum orchards with fenamiphos, carbofuran, and carbosulfan. *Plant Disease, 72*(6), 519-522.

Rott, M. E., Gilchrist, A., Lee, L., & Rochon, D. A. (1995). Nucleotide sequence of *tomato ringspot virus* RNA1. *Journal of General Virology, 76*(2), 465-473.

Rott, M. E., Tremaine, J. H., & Rochon, D. M. (1991). Nucleotide sequence of *tomato ringspot virus* RNA-2. *Journal of General Virology, 72*(7), 1505-1514, doi:10.1099/0022-1317-72-7-1505.

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, doi:10.1094/phyto.1998.88.11.1238.

Rowhani, A., Biardi, L., Johnson, R., Saldarelli, P., Zhang, Y. P., Chin, J., et al. Simplified sample preparation method and one-tube RT-PCR for grapevine viruses. In *Proceedings of XIII International Council for the Study of Viruses and Virus-Like Diseases of the Grapevine, Adelaide, 2000* (pp. 82)

Sabanadzovic, S., Abou Ghanem-Sabanadzovic, N., Saldarelli, P., & Martelli, G. P. (2001). Complete nucleotide sequence and genome organization of *Grapevine fleck virus*. *Journal of General Virology, 82*(8), 2009-2015.

Saito, S., Dunne, K. J., Evans, K. J., Barry, K., Cadle‐Davidson, L., & Wilcox, W. F. (2013). Optimisation of techniques for quantification of Botrytis cinerea in grape berries and receptacles by quantitative polymerase chain reaction. *Australian Journal of Grape and Wine Research, 19*(1), 68-73, doi:10.1111/ajgw.12011.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4*(4), 406-425.

Saldarelli, P., Giampetruzzi, A., Morelli, M., Malossini, U., Pirolo, C., Bianchedi, P., et al. (2015). Genetic Variability of *Grapevine Pinot gris virus* and Its Association with Grapevine Leaf Mottling and Deformation. *Phytopathology, 105*(4), 555-563, doi:10.1094/PHYTO-09-14-0241-R.

Saldarelli, P., Minafra, A., & Martelli, G. P. (1996). The nucleotide sequence and genomic organization of *grapevine virus B*. *Journal of General Virology, 77*(10), 2645-2652, doi:10.1099/0022-1317-77-10-2645.

Saldarelli, P., Rowhani, A., Routh, G., Minafra, A., & Digiaro, 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, doi:10.1023/a:1008608506699.

Sampol, B., Bota, J., Riera, D., Medrano, H., & Flexas, J. (2003). Analysis of the virus‐induced inhibition of photosynthesis in malmsey grapevines. *New Phytologist, 160*(2), 403-412.

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, doi:10.2503/jjshs.50.169.

Scheu, G. (1935). Die Rollkrankheit des Rebstockes. *Der Deutsche Weinbau., 14*, 222-223, 345-346, 356-358.

Scheu, G. (1936). *Mein Winzerburch*. Berlin: Reichnährstand Verlag.

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nature protocols, 3*(6), 1101-1108.

Schooley, N., Baudoin, A. B., Derr, J. F., Pfeiffer, D. G., Weaver, M. J., & Wolf, T. K. (2010). Crop profile for grapes in Virginia. http://www.ipmcenters.org/cropprofiles/docs/vagrapes.pdf.

Seddas, Haidar, Greif, Jacquet, Cloquemin, & Walter (2000). Establishment of a relationship between grapevine leafroll closteroviruses 1 and 3 by use of monoclonal antibodies. *Plant Pathology, 49*(1), 80-85, doi:10.1046/j.1365-3059.2000.00415.x.

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, doi:10.1023/b:ejpp.0000003750.34458.71.

Shi, B.-j., Habili, N., Gafny, R., & Symons, R. H. (2004). Extensive Variation of Sequence within Isolates of *Grapevine Virus B*. *Virus Genes, 29*(2), 279-285, doi:10.1023/B:VIRU.0000036388.41242.c1.

Sim, S. T., Rowhani, A., Alkowni, R., & Golino, D. A. Experimental transmission of *Grapevine leafroll associated virus 5* and *9* by longtailed mealybugs. In *Extended Abstract 14th Meeting of ICVG, Locorotondo, Italy. 211-212, 2003* (pp. 211-212)

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., & Flook, P. (1994). Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America, 87*(6), 651-701.

Singh Brar, H., Singh, Z., Swinny, E., & Cameron, I. (2008). Girdling and grapevine leafroll associated viruses affect berry weight, colour development and accumulation of anthocyanins in 'Crimson Seedless' grapes during maturation and ripening. *Plant Science, 175*(6), 885-897, doi:10.1016/j.plantsci.2008.09.005.

Smegal, D. C. Human Health Risk Assessment Chlorpyrifos. In O. o. P. U.S. Environmental Protection Agency, Pesticides and Toxic Substances, Office of Pesticide Programs, Health Effects Division, U.S. Government Printing Office: Washington, DC, 2000; pp 1-131 (Ed.).

Smith, H., & Cowles, R. (2009). Scale insect pests of Connecticut trees and ornamentals. http://www.hort.uconn.edu/ipm/nursery/caes_fs/caes_fs_scalepests.htm. Accessed November 2012.

Stouffer, R., & Uyemoto, J. Association of *tomato ringspot virus* with apple union necrosis and decline. In *X International Symposium on Fruit Tree Virus Diseases 67, 1976* (pp. 203-208)

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.

Sudarshana, M., Perry, K. L., & Fuchs, M. (2015). *Grapevine red blotch-associated virus*, an emerging threat to the grapevine industry. *Phytopathology*, doi:10.1094/phyto-12-14-0369-fi.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution, 28*(10), 2731-2739, doi:10.1093/molbev/msr121.

Tanne, E., Ben-Dov, Y., & Raccah, 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, doi:10.1016/j.tig.2006.07.008.
Thompson, J. R., Fuchs, M., & Perry, K. L. (2012). Genomic analysis of *Grapevine leafroll associated virus-5* and related viruses. *Virus Research, 163*(1), 19-27, doi:10.1016/j.virusres.2011.08.006.
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, doi:10.1094/phyto-100-8-0830.
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, doi:10.1094/phyto-98-10-1093.
Tuskan, R. G., & Tolin, S. A. (1984). Properties of grape and soybean isolates of Tomato Ringspot Virus. *Phytopathology, 74*(7), 808-808.
Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., et al. (2012). Primer3—new capabilities and interfaces. *Nucleic Acids Research, 40*(15), e115-e115, doi:10.1093/nar/gks596.
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, doi:10.3733/ca.v055n04p28.
Valsesia, G., Gobbin, D., Patocchi, A., Vecchione, A., Pertot, I., & Gessler, C. (2005). Development of a high-throughput method for quantification of Plasmopara viticola DNA in grapevine leaves by means of quantitative real-time polymerase chain reaction. *Phytopathology, 95*(6), 672-678, doi:10.1094/PHYTO-95-0672.
Varela, L. G. (2005). Grape and Vine Mealybug Life Cycles in the North Coast. http://cesonoma.ucdavis.edu/files/27229.pdf.
Varela, L. G., Elkins, R. B., Van Steenwyk, R. A., & Ingels, C. A. (2012). Pear Mealybugs. http://www.ipm.ucdavis.edu/PMG/r603301211.html. Accessed 2012 November.
Varela, L. G., & Smith, R. J. (2009). Mealybugs. http://ucanr.org/sites/SoCo/files/27649.pdf. Accessed November 2012.
Velasco, R., Zharkikh, A., Troggio, M., Cartwright, D. A., Cestaro, A., Pruss, D., et al. (2007). A high quality draft consensus sequence of the genome of a heterozygous grapevine variety. *PLoS ONE, 2*(12), e1326.
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.
VWB (2012). The Economic Impact of Wine and Wine Grapes on the State of Virginia--2010. St. Helena, CA: A Frank, Rimerman + Co. LLP.
VWB (2016). Virginia 2015 Commercial Grape Report. Virginia Wine Marketing Office.
Wakita, T., Kinoshita, K., Yamada, E., Yasui, N., Kawahara, N., Naoi, A., et al. (2003). The discovery of dinotefuran: a novel neonicotinoid. *Pest Management Science, 59*(9), 1016-1022, doi:10.1002/ps.727.

Walker, L., Bagewadi, B., Schultz, A., & Naidu, R. A. (2015). First Report of *Tobacco ringspot virus* Associated With Fanleaf Disease in a Washington State Vineyard. *Plant Disease, 99*(9), 1286-1286, doi:10.1094/PDIS-02-15-0140-PDN.

Wallingford, A. K., Fuchs, M. F., Martinson, T., Hesler, S., & Loeb, G. M. (2015). Slowing the Spread of Grapevine Leafroll-Associated Viruses in Commercial Vineyards With Insecticide Control of the Vector, Pseudococcus maritimus (Hemiptera: Pseudococcidae). *Journal of insect science, 15*(1).

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. 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, doi:10.1603/0022-0493-99.4.1280.

Walton, V. M., & Pringle, K. L. (2001). Effects of pesticides and fungicides used on grapevines on the mealybug predatory beetle Nephus 'boschianus' (Coccinellidae, Scymnini). *South African Journal for Enology and Viticulture, 22*(2), 107-110.

Wieczorek, A., & Sanfacon, H. (1993). Characterization and subcellular localization of tomato ringspot nepovirus putative movement protein. *Virology, 194*(2), 734-742, doi:10.1006/viro.1993.1314.

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.

Wolf, T. K. (2008). *Wine grape production guide for eastern North America*. (NRAES-145). Ithaca, NY.: PALS Publishing (formerly NRAES).

Wolpert, J. A., & Vilas, E. P. (1992). Effect of mild leafroll disease on growth, yield, and fruit maturity indices of Riesling and Zinfandel. *American Journal of Enology and Viticulture, 43*(4), 367-369.

Woodham, R., Antcliff, A., Krake, L., & Taylor, R. (1984). Yield differences between Sultana clones related to virus status and genetic factors. *Vitis, 23*(2), 73-83.

Woodham, R., Krake, L., & Cellier, K. (1983). The effect of grapevine leafroll plus yellow speckle disease on annual growth, yield and quality of grapes from Cabernet Franc under two pruning systems. *Vitis, 22*(4), 324-330.

Wunderlich, L. R., Cooper, M. L., & Daane, K. M. (2013). Seasonal Biology of *Ferrisia gilli* (Hemiptera: Pseudococcidae) in California Sierra Foothill Vineyards. *Journal of Economic Entomology, 106*(4), 1716-1725, doi:10.1603/EC13046.

Xiao, H., Shabanian, M., McFadden-Smith, W., & Meng, B. (2015). First Report of *Grapevine Pinot gris virus* in Commercial Grapes in Canada. *Plant Disease, 100*(5), 1030-1030, doi:10.1094/PDIS-12-15-1405-PDN.

Yokomi, R. K., & DeBorde, R. L. (2005). Incidence, transmissibility, and genotype analysis of Citrus tristeza virus (CTV) isolates from CTV eradicative and noneradicative districts in central California. *Plant Disease, 89*(8), 859-866, doi:10.1094/PD-89-0859.

Zhang, Y., Singh, K., Kaur, R., & Qiu, W. P. (2011). Association of a novel DNA virus with the grapevine vein-clearing and vine decline syndrome. *Phytopathology, 101*(9), 1081-1090, doi:10.1094/PHYTO-02-11-0034.

Zhang, Y.-P., Uyemoto, J. K., Golino, D. A., & Rowhani, A. (1998). Nucleotide sequence and RT-PCR detection of a virus associated with grapevine rupestris stem-pitting disease. *Phytopathology, 88*(11), 1231-1237.

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.