Progress report to the Virginia Wine Board – 2016 FY

PROJECT TITLE: Understanding Grapevine Virus Complex, and Development of Grapevine Leafroll Disease management

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OBJECTIVES:

1. Survey economically significant and newly discovered grapevine viruses among commercial vineyards in VA
2. Evaluate foliarly-applied insecticides for mealybug management
3. Development of membrane-based sampling method, and suitable qRT-PCR method for testing Red Blotch
4. Determine transmission of GLRaV-3 by the Gill's mealybug

Reports for each objective

1) Survey economically significant and newly discovered grapevine viruses among commercial vineyards in VA

During 2009-2013, we sampled around 1,300 (about 600 of which are used in individual virus testing, the other 700 used in intensive field sampling for virus spread/pattern analysis) cultivated grapevine samples comprising 39 different wine grape varieties. In our previously used molecular assay, we have detected RNAs of Grapevine leafroll-associated virus (GLRaV-2 and -3) and grapevine fleck virus (GFkV), because both GLRaV-2 and -3 are very common among wine grape production worldwide, and GFkV is known to cause detrimental damage when combined with GLRaV-3. Thus far, 8%, 25%, and 1% of vines were positive for GLRaV-2,

GLRaV-3, and GFkV, respectively. With just those three viruses, 64% of the total vineyards surveyed were positive for at least one infected grapevine.

In recent few years, we expanded our detection into more varieties of viruses. During 2013-14 seasons, we have tested over 722 samples that were collected during between 2009 and 2014 for several viruses that are known to cause serious threat to wine grape production (Table 1). We have tested for GLRaV-1, -4, -5 and -9, and Respestris stem pitting associated virus (RSPaV-1), grapevine virus A and B (GVA and GVB). RSPaV, GVA and GVB are among the Rugose Wood Complex viruses that cause slow decline of grapevines. Also, GVA can be transmitted by mealybugs, the same vector as GLRaV-3.

Table 1 shows the total numbers of positive grapevines found so far in VA as well as the number of those vines that are involved in cases of mixed infection. Our current results support that GLRaV-3 was the most common virus form the leafroll-complex (23% positive, the number decreased because we added more to the sample size) and RSPaV-1 (52% positive) was the most commonly found virus in the state and is involved in slightly more mixed infection cases than GLRaV-3.

Moreover, Table 2 shows that results of our testing on some of newly found grapevine viruses. The most notable one is GRBaV (grapevine red blotch-associated virus) where 22% our sample turned out to be positive. Since the vector insect of GRBaV is not known yet (Virginia creeper leafhopper is speculated as a potential vector), and it seemed that movements within infected vineyards are limited, it is highly likely that these are introduced through contaminated nursery materials. Nonetheless, this study demonstrated that Virginia has a large number of infected vineyards and better management strategies need to be implemented across the state.

Table 1. Current results of virus survey out of 722 total grapevine samples tested.

Virus	Number of Positive Vines	% Positive	Number of those that are involved in mixed infections
GLRaV-1	15	2.07%*	5
GLRaV-2	64	8.86%*	36
GLRaV-3	**166**	**22.99%***	**79**
GLRaV-4	6	0.83%*	6
GLRaV-4s5	3	0.41%*	3
GLRaV-4s9	3	0.41%*	3
RSPaV-1	**372**	**51.52%***	**91**
GVA	29	4.01%*	25
GVB	13	1.80%*	11
GFkV	6	0.83%*	4

Table 2. Current results of virus survey out of 572 total grapevine samples tested.

Virus	Number of Positive Vines	% Positive	Number of those that are involved in mixed infections
ToRSV	9	1.57	7
GpgV	0	--	--
GVCV	0	--	--
GRBaV	**125**	**21.78**	**78**

In addition to wine grapes, a total of 100 wild grapevines were sampled. Some of these are taken from a field adjacent to vineyards, and others are taken from mountains. None of wild grape samples was positive for any viruses. This is a promising result since recently, a wild grapevine in California (*Vitis californica*) has tested positive for GLRaV-2, GLRaV-3, GVA, and

GVB. This also indicates that we need to maintain our leafroll management in order to avoid escape of viruses to wild grapes.

When we compared samples based on their environment, vines planted prior to 1990 had a significantly higher chance of being infected with either GLRaV-2 or -3 than vines planted after 1990 (Table 3). It can be suggested that older vines were not subjected to the new molecular testing methods of the current era; therefore, the virus screening was not as good as the current standard. Similarly, vines that were infested with mealybugs had a significantly higher chance of being infected with GLRaV-3, but not with GLRaV-2 (Table 4). This is expected since mealybugs are efficient vectors of GLRaV-3, but not a vector for GLRaV-2. It was also found that visual symptoms are not a good indicator of virus infection (Table 4). There were vines with 100% foliar symptoms that contained no viruses and there were also vines that were symptomless that were, in fact, infected with a virus.

Table 3. Probability of finding vines infested with either GLRaV-2 or -3 based on age of vine.

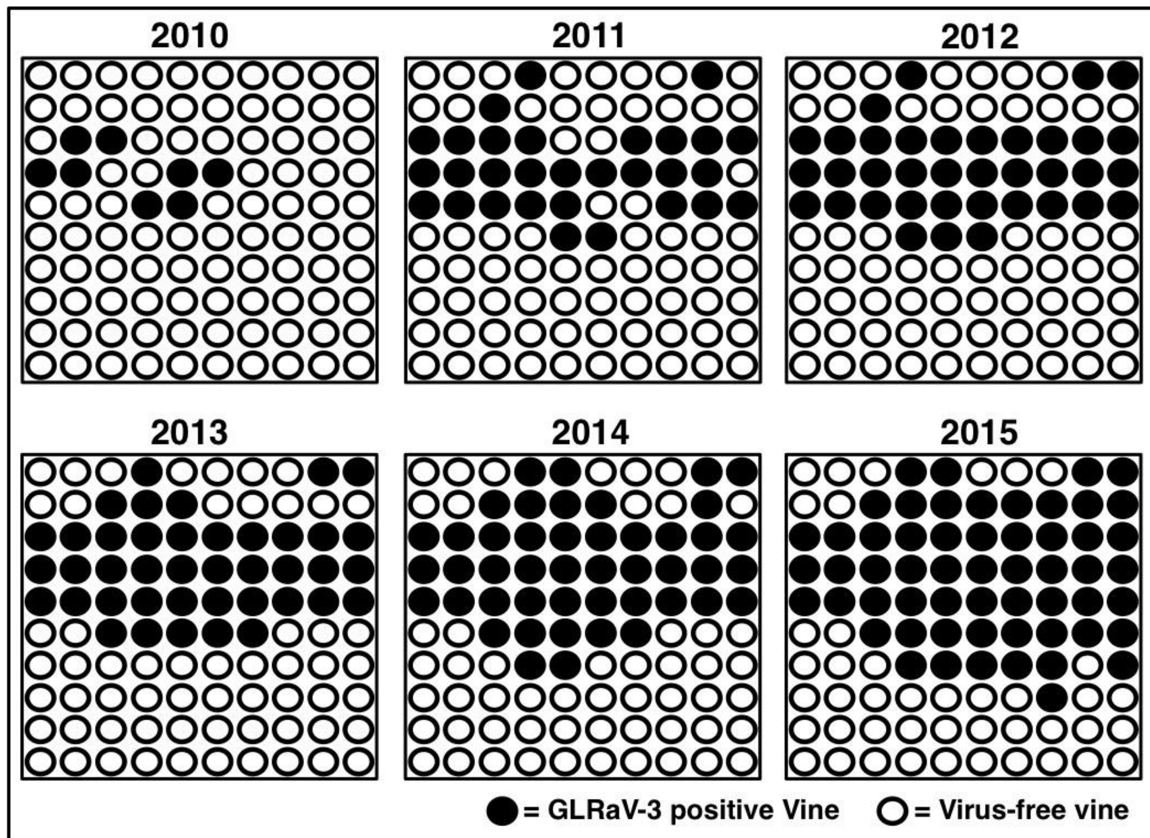
	GLRaV-2		GLRaV-3	
Age group	**LSMean^z**		**LSMean^z**	
Pre-1990	18.4%	A	71.4%	A
1990’s	9.1%	B	38.6%	B
2000’s	5.0%	B	12.2%	C

Table 4. Results from χ^2 tests on probability of GLRaV-2 or -3 infected vine and presence of visual symptoms or mealybugs

Condition	**Virus**	χ^2	***P*-value**
Symptoms	GLRaV-2	0.99	0.32
	GLRaV-3	0.03	0.85
Mealybugs	GLRaV-2	0.23	0.63
	GLRaV-3	16.2	< 0.0001

In addition to simple detection of samples, we have conducted several intensive sampling studies to monitor the movement of GLRaV-3 in a vineyard. One intensive sampling block that was tested (at the Winchester AREC) in three consecutive years showed spread of GLRaV-3 in over three years (Fig. 1). This block, which was two years old at the time of the first sampling, was planted directly next to a leafroll-infected block. At the end of the 2010 season, only 8 vines were infected with GLRaV-3; however, by the end of the 2011 season, the disease had spread to a total of 30 infected vines, a 275% increase. In the 2012 season, only 6 more cases of leafroll were found. It is important to note here that mealybug populations were very high in the 2011 season, which most likely was the cause for the quick spread of the disease. In all three years, there were significant levels of aggregation, meaning that GLRaV-3 tended to spread to nearby, adjacent vines from year to year.

Figure 1. Yearly observations of GLRaV-3 in a vineyard are showing rapid spread of virus among vines.



We have also conducted survey for mealybug species. In 2012, mealybugs were collected from 7 different vineyards in the state and species identification of these insects is currently underway. We have now shown that the Gill's mealybug, grape mealybug, and striped mealybug are present in vineyards in Virginia. Of those, only the grape mealybug is known to transmit grapevine leafroll disease. More preliminary data shows that we can detect GLRaV-3 in Gill's mealybug, which suggested it probably be able to transmit the disease. Transmission studies will be conducted during the field season of 2015 to obtain preliminary data.

In order to compare wine quality of vines with or without GLRaV-3, we have started a preliminary wine making process using our Chardonnay vines in 2012. At the time of harvest, there were no differences in Brix or pH, thus we are not expecting to see major differences in wine. Vineyard sites have been identified in 2015 that contain mixed and single infections of GLRaV-3, Red Blotch, RSPaV-1, and healthy vines. Berries from these vines will be analyzed at the end of the season for Brix, pH, TA, and YAN. We expect to find significant differences based on the combination of virus-infected vines being used.

To examine potential positive effects of a new bio-based liquid product, ecoAgra Plant Protect, the concentrate was applied foliarly three times to leafroll-infected vines at the end of the 2013 season prior to harvest. This product has been shown to treat Goss' wilt on popcorn and yellow corn, as well as sanitize virus infected lemon trees dying of yellow disease in Mexico and has benefitted other crops such as blackberries, sugar cane, and papaya. In 2014, we were not able to duplicate the experiment due to lack of fruits.

Grapes were harvested and juice samples were sent for analysis. Our results form this study show no significant difference between treated and untreated vines in terms of pH, Brix, and other acids.

In addition, juice was analyzed from three different cultivars in 2015 immediately prior to harvest. Within each cultivar, vines were selected that had different virus infections between GLRaV-3, Red Blotch, and RSPaV-1 (the three most common viruses in VA) and berry chemistry was performed. Results can be seen in Table 5. In general, Red blotch and GLRaV-3 both significantly impacted Brix, pH, TA and Anthocyanin. Only GLRaV-3 and Red Blotch in Syrah significantly effected YAN.

Table 5. Difference of harvest parameters between virus-free and infected vines 2015. Difference from the virus-free control are shown. Significant difference between vines were denoted with non-overlapping letters.

Variety	Vine	°Brix	pH	TA (g/L)	YAN (mg/L N)	Anthocyanin (mg/L)
Pinotage	virus-free	22.16 a	3.7346 a	4.998 a	345 a	416.26 a
	Red Blotch	-8.66% b	-4.97% b	21.89% b	-3.36% a	-19.95% b
	Red Blotch and RSPaV-1	-9.93% b	-5.35% b	20.73% b	-2.90% a	-19.08% b
Syrah	virus-free	20.3 a	3.5768 a	8.126 a	321.6 a	350.54 a
	GLRaV-3	-10.44% b	-1.63% b	3.52% a	-10.63% b	-17.30% b
	GLRaV-3 and Red Blotch	-19.80% c	-3.04% c	11.67% b	-9.76% b	-28.42% c
Mourvedre	virus-free	20.06 a	3.5138 a	8.79 a	323.6 a	370.98 a
	GLRaV-3	-14.86% b	-4.45% b	4.40% a	-7.23% a	-14.54% b
	GLRaV-3 and RSPaV-1	-15.05% b	-4.44% b	5.20% a	-6.61% a	-15.63% b

Objective 2) Evaluate foliarly-applied insecticides for mealybug management

Cabernet Sauvignon AHS AREC trial: A three-year study was implemented in 2012 at a field of vines at the AHS-AREC in Winchester VA contained 3 rows of 13 panels of vines with each panel containing three vines in an attempt to prevent and watch the spread of GLRaV-3 from old infected vines to newly planted vines under insecticidal treatment. Vines were trained using a Lyre system. In each panel, one vine was an old (originally planted in 1990) Cabernet Sauvignon, infected with GLRaV-3 and harboring overwintering female grape and Gill's mealybugs, and the other two vines next to it within the panel were virus-free Cabernet franc nursery vines newly planted in 2012 at 5 feet and 10 feet from the old vine.

There were three foliarly applied treatments applied individually to specific panels and assigned at random to each block: 1) two applications of a tetramic-acid derivative (spirotetramat,

Movento, 0.439 L/ha, Bayer CropScience LP); 2) two applications of a pyrethroid (beta-cyfluthrin, Baythroid XL, 0.219 L/ha, Bayer CropScience LP), and 3) no insecticide spray as a control. The same treatment was applied to the same vines in each of the three years. Fungal diseases were controlled by a standard fungicide application program that does not affect mealybug activities. Applications of insecticides according to year were performed twice each year at 1-2inch shoot growth (just post bud-break) and then again at bloom. Throughout the seasons, mealybugs were counted every one to two weeks by a rater spending a total of 2.5 minutes per side of a Lyre trained vine visually inspecting, counting, and recording mealybugs.

In all three seasons, Baythroid treated vines maintained significantly higher populations of mealybugs ($P<0.05$) than the Movento treated vines. No evidence of mealybug movement to healthy, young vines was witnessed in 2012, but in 2013 there was one case of a mealybug found on a young, Baythroid treated vine and in 2014, mealybugs were found on young vines despite treatments, albeit at very low numbers. 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 Baythroid treatment block. In 2014, mealybug populations were generally higher than in 2012 and 2013 and were more abundantly spread throughout all treatment blocks. This caused an increased spread of GLRaV-3 in many blocks regardless of treatment. Eight, six, and one vines were newly infected with GLRaV-3 following the 2014 season for the control, Baythroid, and Movento treated vines respectively.

Merlot AHS AREC trial: A separate three-year field trial was implemented in 2012 at the same vineyard at the AHS AREC in Winchester, VA but in a different plot in order to prevent the entry of GLRaV-3 from a neighboring plot of Chardonnay in which GLRaV-3 was confirmed to be actively spreading. Mealybugs and GLRaV-3 have not been found in this plot previously. VSP trained vines in 4 rows containing 5 panels of 5 vines per panel were used in a completely randomized design with four replicates of five treatments using each panel in each row as a treatment. The five treatments were applied twice per season, once at budbreak and once at bloom, with the high rate of Lorsban being applied initially at delayed dormant a few weeks before other application stated. Foliarly applied treatments were: 1) the tetramic acid derivative spirotetramat, (Movento, 0.439 L/ha, Bayer CropScience LP); 2) the pyrethroid (beta-cyfluthrin, Baythroid XL, 0.219 L/ha, Bayer CropScience LP); 3) low rate of chloropyifos (Lorsban, 1.4 L/ha, Dow AgroSciences LLC, Indianapolis, IN); 4) high rate of chloropyifos (Lorsban, 1.6 L/ha, Dow AgroSciences LLC, Indianapolis, IN); and 5) water as a control. Fungal diseases were controlled by a standard fungicide application program that does not affect mealybug activities. Applications of insecticides according to year were performed on the following dates: 2012: April 12 and May 31; 2013: April 12 (for Lorsban high rate) then May 17 and June 12; 2014: April 22 (for Lorsban high rate) then May 12 and June 18. Throughout the seasons, mealybugs were counted every one to two weeks by a rater spending a total of 3 minutes per panel of 5 vines (which is also per treatment) visually inspecting, counting, and recording mealybugs.

In all three years, no significant differences were found between treatments, as mealybug numbers were consistently low each year. However, by the end of the third year, panels of all treatments were infected with at least one positive vine for GLRaV-3, suggesting that none of these materials were suitable to prevent the entry of GLRaV-3.

Orange County, VA Chardonnay trial: A field trial during the 2013 and 2014 seasons was implemented at a commercial vineyard on a single row of Chardonnay (planted in 1989) that had a previous infestation of mealybugs in order to determine the effectiveness of two foliarly applied treatments at decreasing the number of mealybugs over time in this plot. A completely randomized design with four replications where treatments were randomly assigned to a vine randomly with buffer/untreated vines separating all treated vines was used. The two treatments examined were applied twice per season (delayed dormant and bloom) and were: acetamiprid

(Assail, 0.182 L/ha, United Phosphorus, Inc. [UPI], King of Prussia, PA) and insecticidal soap (M-Pede, 18.7 L/ha Dow AgroSciences LLC, Indianapolis, IN). Throughout the seasons, mealybugs were counted every one to two weeks by a rater spending a total of 5 minutes per treated vine (Lyre trained) visually inspecting, counting, and recording mealybugs. 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; however, other pesticides were regularly used in this vineyard, but not on rows directly adjacent to the trial row.

During the 2013 and 2014 seasons, there were few numbers of mealybugs present in both years. In 2013 and 2014 no significant differences (P<0.05) were found between the treatments or the control.

Orange County, VA Rkatsiteli trial: A field trial in a separate commercial vineyard in Orange County, VA was implemented between 2013 and 2014 in a plot of Lyre trained Rkatsiteli. This trial was another attempt to eliminate or at least knock back the populations of mealybugs in this area using six treatments: 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 chlorpyrifos (Lorsban), 5) a high rate of chlorpyrifos (Lorsban,1.6L/ha Dow AgroSciences LLC, Indianapolis, IN), and 6) no spray as a control. As before, mealybugs were counted every one to two weeks throughout the season by a rater spending a total of 5 minutes per treated vine visually inspecting, counting, and recording mealybugs. A final, early season count was made in May 2015 to examine potential effects from previous year treatments.

No significant differences found in 2012; however, in 2013 and 2014, Scorpion, Lorsban, and Movento treated vines all performed significantly better than the control or Baythroid treatments. In all, Movento and high rate of Lorsban were the most effective treatments at eliminating the mealybug population.

Summary of Objective 2

Our results indicated how quickly mealybugs and GLRaV- 3 could be transmitted to nearby vines, and this rapid movement can happen with some insecticide treatments. The discovery of GLRaV-3 in a newly planted vine six months after planting showed that mealybugs were efficiently transmitting GLRaV-3 to new vines, even though their mobility is somewhat limited.

Our experiments demonstrated that the use of a contact insecticide may not be effective, and could actually increase mealybug populations. At both AREC and Orange locations, we have used Baythroid as one of treatment. In both cases, the mealybug population was not significantly different from untreated check. Moreover, in 2009-2011 studies, we have showed that Baythroid application actually can increase the mealybug population.

Both spirotetramat (Movento) and dinotefuran (Scorpion) treatments worked well in controlling the mealybug populations. With significant population declines in both treatments compared to the untreated check, these two treatments seem to effectively control the population. Spirotetramat may have residual effects on the following years population levels as well. When the same treatments were applied on the same vines two years in a row, the number of mealybugs treated with dinotefuran was numerically lower (difference not statistically significant) than spirotetramat in 2011. The overall counts of mealybugs in 2012 were statistically lower ($P < 0.05$) in spirotetramat-treated vines than dinotefuran-treated vines. Furthermore, the initial count of mealybugs in 2013 showed that vines sprayed with spirotetramat resulted in significantly lower counts of mealybugs than that of dinotefuran.

Use of Assail: Although other neonicotinoid insecticide (Scorpion) resulted in significant decrease in mealybug population, use of Assail did not result in low number of mealybugs in

2013. Also, in the AREC plot, the delayed-dormant application of acetamiprid was tested in 2009-2011, but it did not provide a significant reduction in mealybug numbers.

One commercial vineyard site in Orange VA that consisted of a single row of Chardonnay and examined the effects of Acetamiprid (Assail 2 oz/A) and M-Pede (insecticidal soap) in elimination of the mealybug vector during the 2013 and 2014 seasons showed no significant differences ($P<0.05$) between the treatments or the control. The second field trial at a separate vineyard in the same location from 2012-2014 attempting to eliminate the mealybug vectors examined the effects of Dinotefuran (Scorpion, 0.292 L/ha), Dinotefuran (Movento 6 oz/A), ß-cyfluthrin (Baythroid 3 oz/A), and Low and High rates of Chlorpyrifos (Lorsban 1.6L/ha) found no significant differences in 2012; however, in 2013 and 2014, Scorpion, Lorsban, and Movento treated vines all performed significantly better than the control or Baythroid treatments. Movento and the high rate of Lorsban were the most effective treatments at eliminating the mealybug population in this trial.

The research vineyard at the AHS Jr. AREC in Winchester, VA, containing healthy young vines interplanted with old, GLRaV-3 positive vines, examining the control of mealybugs using Dinotefuran (Movento 6 oz/A) and ß-cyfluthrin (Baythroid 3 oz/A) and the resulting spread of GLRaV-3 to the healthy vines during the 2012, 2013, and 2014 seasons resulted in all three seasons, control and Baythroid treated vines maintained significantly higher populations of mealybugs ($P<0.05$) than the Movento treated vines. No evidence of mealybug movement to healthy, young vines was witnessed but GLRaV-3 did spread to healthy vines regardless of treatment. Mealybugs were first found moving to new, healthy vines in 2013 and by the end of 2014, all vines were positive for GLRaV-3, regardless to treatment. The plot of Merlot also located at the Winchester AREC, evaluating the efficacy of Dinotefuran (Movento 6 oz/A), ß-cyfluthrin (Baythroid 3 oz/A), and Low and High rates of Chlorpyrifos (Lorsban 1.6L/ha) in trying to prevent the entry of GLRaV-3 into this vineyard resulted in all three years having no significant differences between treatments as mealybug numbers were consistently low each year. However, by the end of the third year, panels of all treatments were infected with GLRaV-3, suggesting that none of these materials were suitable to prevent the entry of GLRaV-3.

Objective 3) Development of membrane-based sampling method, and suitable qRT-PCR method for testing Red Blotch

An efficient, Real-Time PCR method for grapevine red blotch was also tested as conventional PCR is the only available routine-use test while methods such as ELISA and Real Time PCR have yet to be developed and could help aid in mass testing of potentially infected materials, such as those located in nursery stocks. 48 red blotch positive grapevine isolates from the survey were obtained via sequencing at the above-mentioned VBI. Multiple sequence alignment was achieved with these 48 partial sequences along with 17 corresponding sequences from GenBank using Clustal X (Conway Institute, UK) for the V2 portion of the GRBaV genome under default parameters. Using this alignment, a consensus sequence was generated and used to create three primer/probe sets using Primer3 software for potential detection under Real-Time PCR methodology. A set of primers, and a respective probe, was found to work and maintain appropriate slope levels under standard curve analysis (the red blotch primer set had an efficiency of 93.84% and the endogenous control efficiency was at 89.76%) for qPCR analysis. 574 samples were tested in triplicate with the new red blotch primer/probe set and the endogenous control. All 140 positive samples previously detected via conventional PCR were also detected through this new real-time PCR method. $2^{-\Delta Ct}$ calculations were made for each positive sample using the average C_t value for red blotch and endogenous control. When sampling date was examined as a factor, no significant differences were found in $2^{-\Delta Ct}$ values. However, a general trend was seen with $2^{-\Delta Ct}$ values generally increasing slightly each month between June and August, and leveling out from August to October, suggesting GRBaV best time of sampling would occur at

some point in August, but it is still detectable between June and October. When cultivar selection was examined as a factor, no significant differences existed between cultivars and $2^{-\Delta Ct}$ values, suggesting GRBaV replicates at the same rate in all varieties. The old method was found to successfully detect all 140 positive GRBaV samples; however C_t values were always different and generally higher than the same samples tested from the Bioline kit. Therefore, if the sole purpose of is for detection, the old extract/test method works fine as it will detects all GRBaV positive samples; however, if comparative methods are needed, further purification/uninhibited samples must be used.

In addition, we have been investigating the possibilities of using a piece of membrane (= paper) that can trap viral DNA and RNA from the sap of grapevines. This method will help us collecting samples in the future. For example, it will allow us to send a sheet of paper to growers if they have suspicious vines. All they need to do is rub sap from petiole to the paper, and send it back to us. Since DNA or RNA can be very unstable, we are currently relying on the freshness of the sample; however, the structure of the membrane will hold both DNA and RNA in tact for a period of time.

The preliminary results of possible buffers and solutions to achieve this objective are shown in Table 6. We were able to trap viral RNA in a nitrocellulose membrane, and recover viable nucleic acid using either an ELISA or PCR buffer solution. Our results also showed that we could increase the probability of recovering RNA by washing the paper with buffer. In 2015, the membrane testing was completed. Results showed that GLRaV-1, -2, -3, -4, -5, -9, RSPaV-1, GVA, GVB, GFkV, and Red Blotch were all reliably detected using this membrane technology with a 100% success rate in a controlled, laboratory environment. The qPCR method created/described above also worked with 100% efficiency in detecting 100 red blotch samples using the membrane and qPCR method. With this knowledge, a kit was developed with the hopes of allowing growers to sample grapevines at their own time and place for viruses using an easy to use kit containing common items. This kit will save time for growers and researchers in terms of sampling for viruses. Once a grower uses the kit, the membrane can easily be shipped back through the mail to the Winchester AREC for PCR analysis for any viruses that need to be tested for.

Table 6. GLRaV-3 RNA recovery from NPN Membranes

Macerate in:	Wash in:	Extraction method*	Success Rate** (# samples correctly identified as positive/total known positive samples tested)
GEB	No trt	Punch	0/48
GEB	Triton X-100	Punch	16/48
GEB	Triton X-100	2ul solution	24/48
GEB	FTA reagent	Punch	0/48
GEB	FTA reagent	2ul solution	0/48
GEB	GES+beta-M+incubation	Punch	39/48
GEB	GES+beta-M+incubation	2ul solution	48/48
GEB	GES	Punch	8/48
GEB	GES	2ul solution	8/48
ELISA buffer	No trt	Punch	0/48
ELISA buffer	Triton X-100	Punch	0/48
ELISA buffer	Triton X-100	2ul solution	22/48
ELISA buffer	FTA reagent	Punch	0/48
ELISA buffer	FTA reagent	2ul solution	0/48
ELISA buffer	GES+beta-M+incubation	Punch	40/48
ELISA buffer	GES+beta-M+incubation	2ul solution	48/48
ELISA buffer	GES	Punch	4/48
ELISA buffer	GES	2ul solution	8/48
Water	No trt	Punch	0/48
Water	Triton X-100	Punch	0/48
Water	Triton X-100	2ul solution	0/48
Water	FTA reagent	Punch	0/48
Water	FTA reagent	2ul solution	0/48
Water	GES+beta-M+incubation	Punch	0/48
Water	GES+beta-M+incubation	2ul solution	0/48
Water	GES	Punch	0/48
Water	GES	2ul solution	0/48

* Membrane punch used directly in PCR, or 2ul Membrane punch solution used in PCR. Negative controls used for all membrane reactions

**GLRaV-3 Membrane Testing results on FTA cards using previously reported protocol works well macerating initially in GEB or ELISA buffers (wash in FTA reagent always) but does not work with water maceration.

Objective 4) Determine transmission of GLRaV-3 by the Gill's mealybug

Both grape mealybugs (*Pseudococcus maritimus*) and Gill's mealybugs (*Ferrisia gilli*) were commonly found in VA vineyards. The grape mealybug is a common transmitter of GLRaV-3 and has a wide host range including grapevines, figs, apples, and citrus crops. There is no knowledge yet as the ability of Gill's mealybugs to transmit the viruses associated with GLD.

A large colony of Gill's mealybugs was established initially on a single, virus-free Cabernet franc grapevine planted in a 5-gallon pot in a greenhouse. The colony was started by taking 54 large adult female Gill's mealybugs from underneath the bark and along the lower canes of grapevines at a local vineyard in Orange County, VA known to have large seasonal populations of this insect 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 the virus free vine located inside of a separate fine mesh 6'x6'x6' insect cage. On June 14, 2015, when clear evidence of 1st instar birth might be occurring soon, all propagated plants were tested for GLRaV-3 using the methods described previously to confirm these vines as negative or positive for GLRaV-3. One vine that contained only one shoot and was also positive for GLRaV-3 was selected as the "acquisition vine". 1st instars of the initial population sampled promptly emerged on June 16, 2015.

24 clean propagated Cabernet franc vines (all containing one or two shoots total) were separated into six groups of four, each group being assigned a "feeding time" of 1h, 2h, 6h, 12h, 24h, and 48h. The total of 72 1st instar Gill's mealybugs were placed onto the acquisition vine and allowed to feed for 24 hours in the cage. Following the acquisition period, the appropriate number of either five or one mealybug instars were transferred to each of the 24 healthy vines. Mealybugs were removed by hand after the transmission feeding times had expired at 1, 2, 6, 12, 24, and 48 hours. 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). Samples were then tested for species confirmation using a previously reported mealybug species identification multiplex PCR assay (Daane et al. 2011) as well as for GLRaV-3 presence using the same methods stated in our previous study. Vines involved in transmission were petiole tested for GLRaV-3 using a one tube one step RT-PCR method used previously at the end of August 2015 and end of September 2015 as it has been reported that two months following transmission, GLRaV-3 can be detected using common molecular tools.

When examining the presence of GLRaV-3 in these insects following transmission times, 0, 0, 0, 1, 8, and 7 out of 12 mealybugs/time period were positive for GLRaV-3 for time periods 1, 2, 6, 12, 24, and 48h respectively. Three total vines tested at the end of August 2015, about 2 months post-transmission assay, were positive for GLRaV-3 under UV trans-illumination following RT-PCR and gel electrophoresis: one GLRaV-3 positive for the 24-hour and two positive for the 48-hour feeding time period. All three vines that were positive were from vines that had 5 mealybugs/vine. Further studies need to be done to analyze specifics on efficiency of acquisition and transmission however.

Education and other opportunities: The graduate student, Mr. Taylor Jones, joined our program in Fall of 2010, and graduated with his MS degree in 2012. He continued on to his PhD with this project, maintaining a high GPA (3.74), presented his PhD research proposal in March 19th 2014, and he passed his PhD candidacy exam on November 19th 2014. He is expecting to graduate in Spring 2016 semester.

Extension and outreach: The progress has been reported as multiple oral and poster presentations in 2013, 2014, and 2015 at the VVA winter technical meeting, the national American Phytopathological Society meeting (2013), and the Cumberland Shenandoah fruit worker conference (2014). Also, resultes from our studies has been diectly and indirectly reported to our stakeholders through IPM workshops, vineyard meetings, and newsletter articles.
Publications: Part of the objective 1 was written as a journal article, and accepted in European Journal of Plant Pathology in December 2014

Jones, T. J., Rayapati, N., and M. Nita. (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.

Part of the objective 2 has been also prepared as a journal article and accepted in European Journal of Plant Pathology in February 2015

Jones, T. J. and M. Nita (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.

Presentations in 2014
Jones, T., and Nita, M (2014) "An update on grapevine viruses in Virginia and vector management strategies" Cumberland-Shenandoah Fruit Worker's Conference 4 December 2014
Jones, T., and Nita, M (2014) "Examination of grapevine viruses in VA and vector management strategies, PhD research proposal" PPWS Departmental Seminar 19 march 2014

Presentations in 2015
Jones, T., and Nita, M (2015) "An update on grapevine viruses in Virginia and vector management strategies" Virginia Vineyards Association Annual Winter Meeting. February 2015
Jones, T., and Nita, M (2015) "Grapevine Viruses: An Introduction to Recognition and Management". NJ Rutgers IPM Workshop. March 2015.
Jones, T., and Nita, M (2015) "NPN Membrane Grapevine Virus Sampling Technique for Efficient Nucleic Acid Storage and Testing." 90th Cumberland-Shenandoah Fruit Workers Conference. December 2015.

III. Future Project Plans

1. Survey economically significant and newly discovered grapevine viruses among commercial vineyards in VA.: completed, working on publication.
2. Evaluate foliarly-applied insecticides for mealybug management: completed, working on publication
3. Development of membrane-based sampling method, and suitable qRT-PCR method for testing GRBaV: completed, working on publication
4. Determine transmission of GLRaV-3 by the Gill's mealybug: completed, working on publication.

IV. Funding Expended To Date

We have utilized more than 50% as of 29 Feb 2016