Final report to the Virginia Wine Board – 2014 FY
(14-1689-02)

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

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OBJECTIVES:

1. Determine the association of viruses within a vine (mixed infection) and its potential effects
2. Development of mealybug management strategies
3. Determine the effect of grapevine leafroll virus infection
4. Development of better diagnostic methods for grapevine viruses

Reports for each objective

1) Determine the association of viruses within a vine (mixed infection) and its potential effects

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.

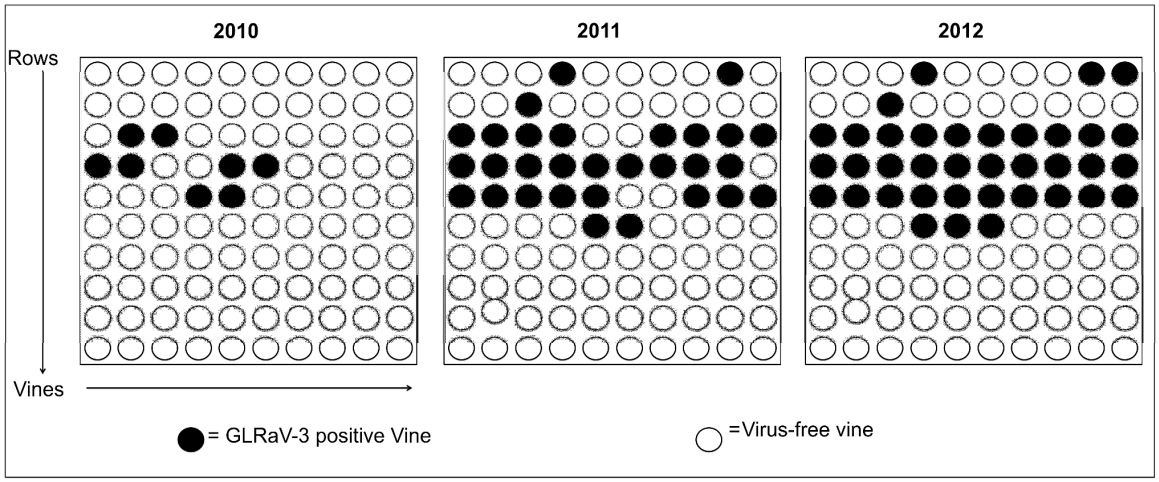
	GLRaV-2		GLRaV-3	
Age group	**LSMean[z]**		**LSMean[z]**	
Pre-1990	18.4%	A	71.4%	A
1990's	9.1%	B	38.6%	B
2000's	5.0%	B	12.2%	C

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.



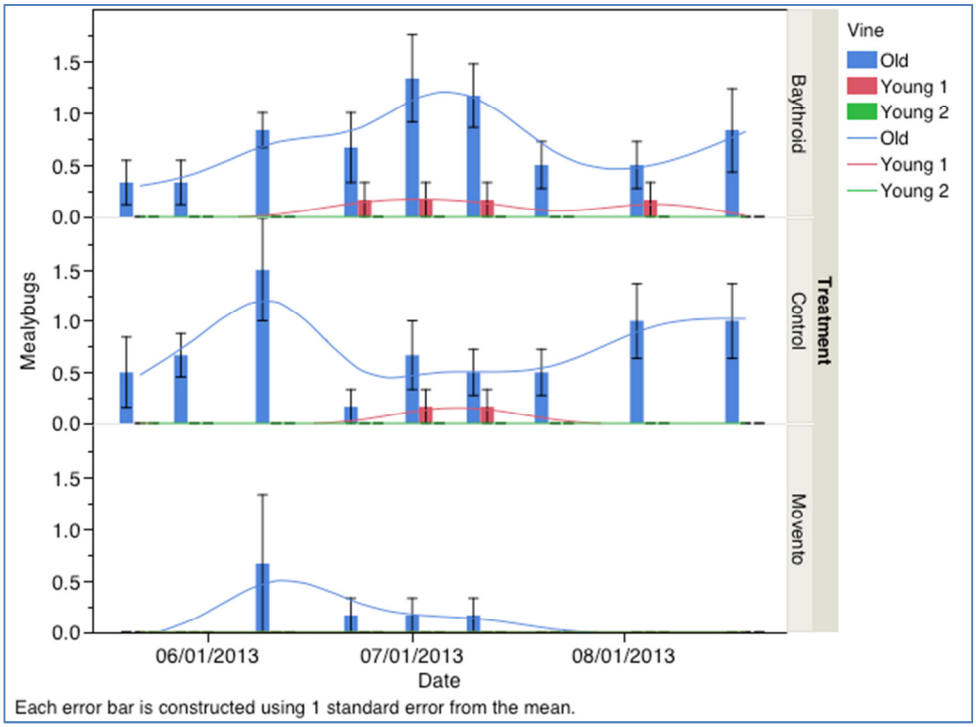
We have also conducted survey for mealybug species. In 2012, mealybugs were collected from 7 different vineyards in the state and species identification of these insects is currently underway. We have now shown that the Gill's mealybug, grape mealybug, and striped mealybug are present in vineyards in 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.

Objective 2) Development of Mealybug Management Strategies

At AHS AREC, we have been conducting two insecticide trials. At Cabernet sauvignon vineyard, which was planted in 1990 and nearly all vines are infected with GLRaV-3, we replanted all but one vine per panel with a new Cabernet Franc vines. The idea here is to monitor movements of the virus and the vector (mealybug). Initially, contact insecticide programs were tested during 2009-2011, and we found out that application of contact insecticide (Baythroid) can actually increase the population of mealybugs in the treated vines.

In 2012, all young Cab Franc vines were replaced with new Cab Franc vines, and a new field study has been started. With the new trial, we included three insecticide treatments 1) Assail at bud break then Baythroid at bloom, 2) Movento (spirotetramat) at bud break and at bloom, and 3) no spray check. The experiment was repeated in 2013 and the results can be seen in Figure 2. We found that the Baythroid treatment was not significantly different from the control treatment in respect to mealybug numbers and, as before, the older vines in the planting still maintained the highest numbers of mealybugs. Overall counts in 2012, 2013, and 2014 were lower than in 2011, suggesting possible climatic or seasonal changes in mealybug populations.

Figure 2: Efficacy of insecticide treatment on to mealybug population over the course of 2013 season.

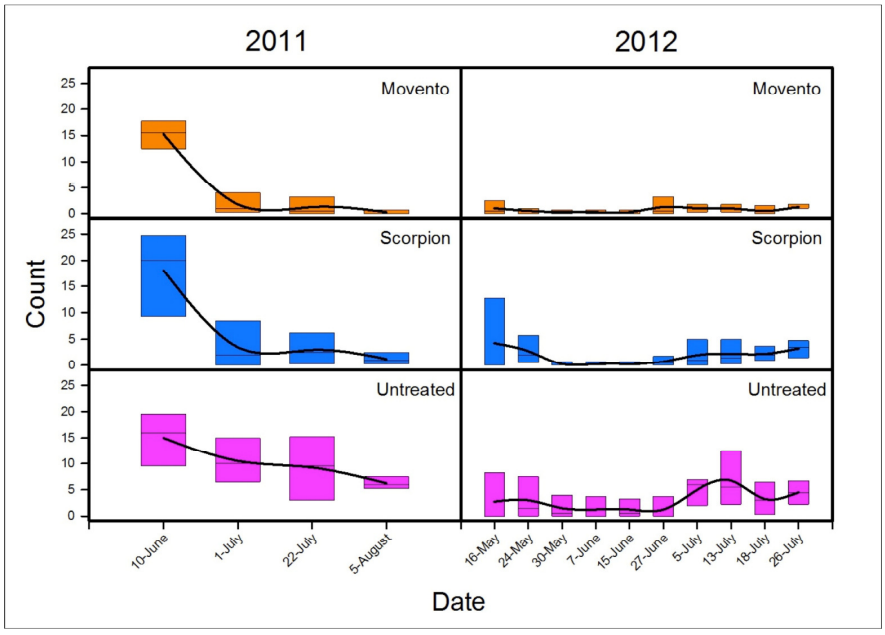


There is another trial at the AREC. We have been using Merlot vineyard where is located right next to Chardonnay vineyard where we found a few new infection by GLRaV-3 in 2010. Since none of Merlot vine was infected in 2011, we have been using this vineyard to monitor the movement of GLRaV-3 and mealybugs in new vineyard. We have used four insecticide programs 1) Lorsban High applied at dormant, 2) Lorsban low rate applied in-season, 3) Baythroid applied in-season, 4) Movent applied in-season, and 5) untreated check. As of 2013, there are some infected vines and a few (only one or two females) mealybugs observed, but no major outbreak has been observed.

In addition to the AREC vineyard, we have been conducting two studies at two commercial vineyards at Orange, VA. At the first location, three treatments were tested: 1) Scorpion (dinotefuran); 2) Movento (spirotetramat); and 3) no sprayed check. Unlike AREC vineyard, application was made after observation of mealybugs in canopies.

Due to different insecticide treatments applied, the Orange vineyard trial yielded different results from the AREC vineyard. In 2011, mealybug counts ranged from 0 to 26 and an average count per vine across date and treatments was 7.1 (Figure 3). When effects of date, treatment, and their interaction were examined using ANOVA, there was no statistically significant ($P < 0.05$) interaction between date and treatment. Both date and treatment significantly ($P < 0.05$) affected the number of mealybug on vines. The differences in dates were basically the declining trend in number of mealybugs following application of insecticide treatments. There was a significant difference of mealybug counts ($P < 0.05$) between treated and non-treated vines; however, spirotetramat and dinotefuran were not significantly different.

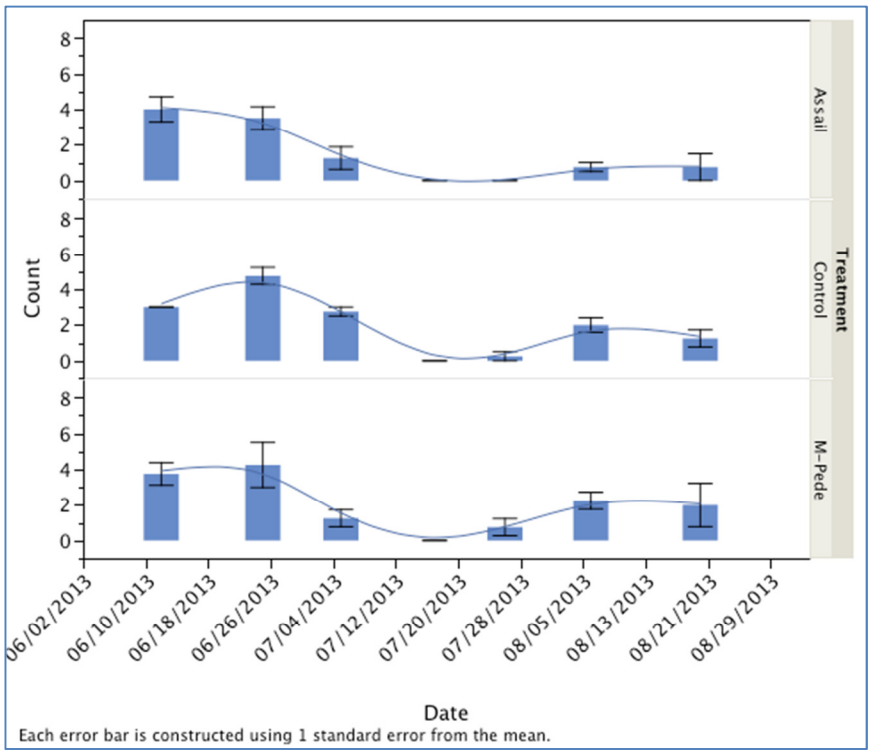
Figure 3. Seasonal changes of mealybug counts on the vines treated with Movento, Scorpion, and an untreated check in 2011 and 2012. The middle line of the box is the mean number, and bottom and top are 25% and 75% data range, respectively. Both Movento and Scorpion were applied using a backpack sprayer onto foliage twice in mid-June in both years.



In 2012, the overall population of mealybugs was lower than 2011 (Fig. 3). The range of mealybugs counted per vine varied from 0 to 17, and an average count per vine across date and treatment was 1.9. As in 2011, there was no interaction between date and treatment for mealybug counts, but both date and treatment were significant ($P < 0.05$). The difference in dates was due to small peaks at the beginning of the trial and a peak that happened after 5 July. Although the difference between spirotetramat and dinotefuran was small (~1.2 mealybugs per vine), it was significant ($P < 0.05$), and vines treated with dinotefuran harbored lower number of mealybugs per vine. In 2013, the initial mealybug population was counted, and we found that there was significant difference among treatments. Vines treated with Movento resulted in significantly lower initial mealybug population ($P < 0.05$) than Scorpion-treated vines. This indicates a potential carry-over effect from Movento application, which is a systemic insecticide.

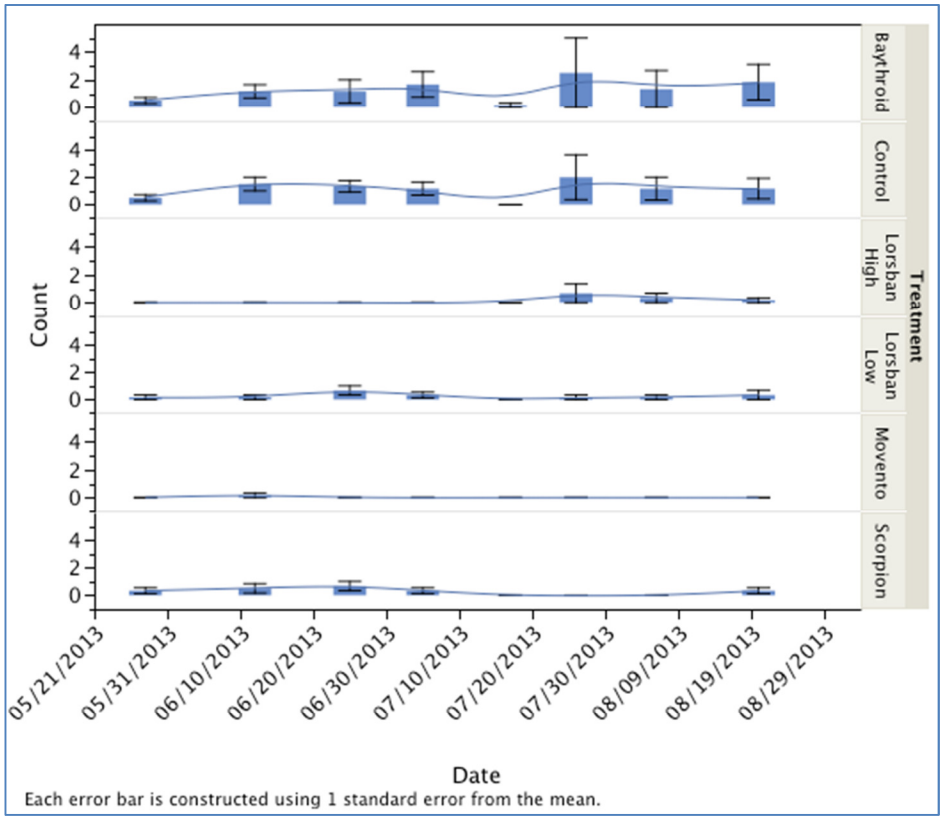
In 2013, a new trial was initiated at the same plot to examine the effects of Assail, M-Pede, and a control (no spray) (Fig. 4). Overall mealybug counts were low throughout the season, and we did not find significant differences among treatments; however, we can observe the same bimodal trend as in AHS AREC result. When we compared the effect of treatment, there was no significant difference ($P < 0.05$) among treatments. This trial was repeated in 2014; however, the mealybug population at the location was very low, probably reflecting a very cold winter condition.

Figure 4: Effect of insecticide treatments on mealybug population, Orange, VA Chardonnay 2013



In addition to the Chardonnay vineyard trial, we are conducting another study nearby vineyard, which is also located in Orange County, VA. Here, we have been testing 1) Lorsban High applied at dormant, 2) Lorsban low rate applied in-season, 3) Baythroid applied in-season, 4) Movent applied in-season, 5) Scorpion applied in-season, and 6) untreated check. Although 2012-13 seasons have been low mealybug seasons, the total number of mealybugs counted during the season showed that Baythroid treatment and untreated check resulted in higher number of mealybugs than other treatments, indicating once again the negative impact of in-season broad-spectrum insecticide application to mealybug populations (Fig. 5).

Figure 5. Effect of insecticide treatment on mealybug population, Orange, VA, 2013.



Summary, the 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) Determine the effect of grapevine leafroll virus infection

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.

Results

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.

Objective 4) Development of better diagnostic methods for grapevine viruses

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 are shown in Table 5. We were able to trap viral RNA in a nitrocellulose membrane, and recover them using either ELISA or PCR buffer solution. Our results also showed that we could increase the probability of recovering RNA by washing the paper with buffer. We will repeat our experiments in 2015 to confirm our method will work on other viruses including grapevine red block virus. Current results show that through this method, GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4, RSPaV-1, GVA, GVB, and Red Blotch all can be detected with a high rate of success (almost 100%) using the method of macerating in either GEB or ELISA, followed by washing in GES and beta-Mercaptoethanol and incubated for ten minutes, followed by RT-PCR. Current research has proved difficult in using these membranes in a Tissue Blot Immuno Assay and using the membranes in Real-Time PCR assays is currently being tested.

In addition, we have been seeking the way to detect not only presence and absence, but also quantity of grapevine red blotch DNA and GLRaV-3 RNA. It will help us to understand when these viruses will be more prominent within a vine. This information can help us determine the best timing for insecticide application.

Table 5. 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.

Education and other opportunities: The graduate student, Mr. Taylor Jones, who joined our program in Fall of 2010, and graduated with his MS degree in 2012, is currently taking courses and maintaining a high GPA (3.74) for his PhD work. He is currently involved in working on sample diagnosis for multiple viruses as well as the objectives listed above. He presented his PhD research proposal in March 19th 2014, and also he passed his PhD candidacy exam on November 19th 2014.

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. In addition, part of the objective 1 was written as a journal article, and accepted in European Journal of Plant Pathology in December 2014. Part of objective 2 has been also prepared as a journal article, and submitted in August 2015.

Presentation 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

Presentation 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.

III. Future Project Plans

1. Determine the association of viruses within a vine (mixed infection) and its potential effects: we will revisit some of vines with mixed infection, and obtain more information in 2015: Almost finished.
2. Development of mealybug management strategies: finished
3. Determine the effect of grapevine leafroll virus infection: Continue nutrient/alternative trials in 2015 season
4. Development of better diagnostic methods for grapevine viruses: Testing more virus-membrane combinations in 2015: Almost finished.

IV. Funding Expended To Date

We have utilized 100%