YEAR-END PROJECT REPORT
Virginia Wine Board, August, 2015

Title: Characteristics of Grapevine Yellows-susceptible vineyards and potential management strategies

Principal Investigators:

Dr. Paolo Lenzi (100% time on project)
Postdoctoral Research Associate
AHS Jr. AREC, Virginia Tech
Virginia Tech
Phone: (540) 869-2560 extn 42
lenzi@vt.edu

Dr. Tony K. Wolf (10% time on project)
Professor of Viticulture
AHS Jr. AREC
Virginia Tech
(540) 869-2560 extn 18
vitis@vt.edu

Type of Project: Research
Amount funded: $110,903

Overall Project Objectives

1. Identify phytoplasma alternative hosts in and around North American Grapevine Yellows (NAGY)-affected vineyards and attempt to identify the characteristics of vineyards that predispose them to increased risk of NAGY
2. Evaluate efficacy of potential Grapevine Yellows management practices

Summary

North American Grapevine Yellows (NAGY) was recognized in Virginia vineyards as early as 1987. Principal investigator T. Wolf began a collaborative research association with Dr. Robert Davis and his lab at the USDA/ARS in Beltsville Maryland and that partnership led to a greater understanding of the nature of the pathogens that cause NAGY in Virginia. An important finding from our work of the nineties was that the causal agents of NAGY – bacteria-like organisms called *phytoplasmas* – were unique to grapevine yellows diseases in North America. That is, they were not the same pathogens that cause the European variants of the disease, such as *Flavescence dorée*, or *Bois noir*. The missing information about NAGY was which vector or vectors were involved in phytoplasma transmission, and what other plants in the vineyard environment served as alternative hosts for the pathogens. Subsequent work with Dr. LeAnn Beanland between 2000 and 2004, funded by the Virginia Wine Board, identified *candidate* insect vectors, and their movement into and within NAGY-affected vineyards. Proving that a particular insect is an effective vector of NAGY phytoplasmas is a painstaking process. It requires an ability to collect, identify and manage candidate species from the field. It also requires robust detection methodology for the causal pathogens. And it requires a system that allows for testing the transmission capability of the candidate insect(s). Dr. Beanland provided some important groundwork in this regard during her 4-year appointment with Virginia Tech. In addition to providing preliminary evidence that several leafhoppers could transmit NAGY phytoplasmas, Dr. Beanland also showed that wild grapevines (*Vitis vulpina*) and black cherry (*Prunus serotina*) could serve as alternative hosts; however, it remained unclear whether additional insects and additional alternative hosts were involved with NAGY.

Following Dr. Beanland's departure we concentrated on other areas of research and had no active NAGY projects again until 2012. Following two seasons (2010 and 2011) of widespread and increased attrition of grapevines to NAGY in many vineyards, we were encouraged to renew research on NAGY management strategies. This research project started in July 2012 with the hiring of post-doctoral research associate, Dr. Teresa Stoepler. Dr. Stoepler expanded the vector studies which were begun in

2000, and conducted an extensive (30-vineyard) survey in 2013 of vineyards to determine the relationship of certain leafhopper species to the incidence of NAGY. Based on that survey, as well as artificial transmission trials conducted with leafhoppers in 2013 and 2014, Dr. Stoepler determined that at least seven leafhopper species were potentially capable of serving as vectors of the causal agent of NAGY (Table 1, Appendix).

Dr. Stoepler accepted a fellowship with the US Geological Survey in mid-2014, and a molecular biologist, Dr. Paolo Lenzi, was hired to continue the work proposed in our Fiscal Year-2015 project proposal. To that end, we conducted a series of transmission assays, with several modifications aimed at increasing the rate of transmission by candidate vectors. We also collected leaf samples from infected grapevine shoots to be tested during the winter and to determine the concentration of phytoplasma in susceptible and less susceptible species and varieties. Finally, we collected material from a range of *non-Vitis vinifera* plants that could be potential alternative hosts for phytoplasma, and constitute a possible inoculum source for NAGY infections.

There are two components to Objective #2. In the first, we repeated in 2014 a seasonal insecticide program in three vineyard blocks represented by two different vineyards, both of which are in Fauquier County. The vineyard "blocks" represent a large enough planting of one variety that it can be divided into 2 sub-plots; one-half of which is repeatedly treated with insecticides and the other half which is not sprayed (control). Leafhoppers are monitored weekly in both sub-plots of all three vineyard blocks using posted, yellow sticky trap cards, as well as floor sweeps with an insect collection net. This experiment asks the question, "Can a seasonal insecticide program depress leafhopper (vectors) levels low enough to extinguish new infections?" Because NAGY symptoms may take a year to develop, treatments applied in 2014 required follow-up monitoring during 2015.

The second part of Objective #2 addresses a very practical question asked by growers who are losing grapevines to NAGY: "Can the disease be slowed or stopped by removing affected portions of a vine (e.g., a trunk+cordon unit) when the disease is first observed?" This too involves a multi-year approach to attempt to answer the question.

Objective 1: NAGY characteristics

NAGY transmission assays: In our previous transmission assays, transmission rates were sometimes very low and were inconsistent between years because these field-collected insects may not have fed on infected plants, or may have fed on infected plants too soon before collection to transmit phytoplasmas (insufficient time for phytoplasmas to replicate in insect tissues). This year, we aimed to increase the transmission rate, by having an acquisition and a latency period. Insects were randomly collected in vineyards with high incidence of NAGY infections, and were then caged to NAGY-symptomatic grapevine shoots in the fields, to force them to acquire phytoplasma. Then the insects were transferred to barley and clover plants and allowed to feed for up to three weeks, the time needed for phytoplasma replication within the insect bodies. After this latency period, insects were separated by species and transferred to healthy Chardonnay seedlings (highly susceptible plants) and to artificial transmission assays based on sucrose solutions. We performed a total of 65 transmissions, focusing on the seven insect species that tested positive in our previous tests (Table 1, Appendix). Preliminary results show that *Agallia constricta* and *Endria inimica* are likely to be vectors, since group III phytoplasma were detected in the sucrose solutions used to feed these insects. To be certain that transmission has occurred and that we can unequivocally say that a particular insect species is a competent NAGY vector, the recovered phytoplasmas in plants and sucrose solution must undergo a positive process of identification. Our USDA collaborators have recently published evidence (Davis, et al. 2015) that a unique phytoplasma was found in NAGY-affected grapevines in VA, MD, PA, NY, OH, and MO. We consistently recover this phytoplasma from infected grapevines in Virginia, but have had some

difficulties in reliably detecting the phytoplasma from sucrose solutions on which the candidate leafhoppers have fed. We are currently in the process of trying different approaches to consistently detect the phytoplasmas. We continue to monitor the Chardonnay seedlings for the presence of NAGY symptoms in the greenhouse. Usually, symptoms appear late in the season, especially in younger plants.

Alternative hosts: We also tested whether alternative host plant species (non-*Vitis vinifera*) serve as effective sources of phytoplasma inoculum, based on evidence of higher transmission efficiency of *Scaphoideus titanus* leafhoppers collected from wild grape compared to *V. vinifera* in New York. We collected samples from woody and herbaceous plants from floors and forests adjacent to high incidence vineyards. Preliminary results of molecular analysis carried out on leaf samples suggest the presence of phytoplasma in wild grape (*Vitis spp*.) and other plants, such as *Platanus occidentalis* (sycamore), Malus spp. (apple) and *Ulmus* spp. (elm). Alternative hosts will continue to be sampled for the remainder of the project (2015).

Phytoplasma titer in infected plants: We were also interested in determining the concentration (titer) of phytoplasma in infected grapevines. Our hypothesis is that a relatively high concentration of phytoplasma could be responsible for severe symptoms in susceptible varieties such as Chardonnay, compared to relatively tolerant varieties such as Cabernet Sauvignon. PCR is a valuable tool to test the presence of phytoplasma DNA in symptomatic samples. All the samples were tested by PCR, using primers specific for the 16S ribosomal gene. We then sequenced the DNA of positive samples and were able to determine that they are infected by phytoplasma belonging to group III (X-disease group, subgroup 16SrIII-I).

Quantitative PCR (qPCR) is an even more sensitive technique that allows us to detect the presence of DNA at smaller concentrations. We used this technique to determine the titer of phytoplasma DNA in grapevine samples. The amount of Yellows DNA was normalized to a plant gene, to make sure that the final results were not affected by different amounts of DNA. During the 2014 season, we collected various leaf samples from infected and healthy Chardonnay, Riesling, Tannat and Cabernet Sauvignon plants. Infected symptomatic samples were compared and qPCR analysis showed that the difference in phytoplasma titer in the four different varieties is not statistically significant (Fig.1). In our samples, for instance, Riesling vines contained the highest phytoplasma concentrations, even though we usually observe that Chardonnay is the most severely affected variety. In conclusion, it appears that phytoplasma titer is not directly related to the susceptibility of the different varieties to NAGY.

In order to characterize and better understand the spreading of NAGY disease, we aimed at determining the titer of phytoplasma in different plant organs. Infected vines usually show only one or a few symptomatic shooting stems, carrying symptomatic leaves. Since the bacteria are phloem-limited, they are theoretically able to move throughout the plant through the vascular tissues. The spreading of phytoplasma within the plant is currently poorly understood. Studying the presence of bacteria in asymptomatic tissues, and understanding how quickly bacteria colonize new organs is, therefore, important. Symptomatic NAGY-infected leaves were compared by qPCR with asymptomatic leaves collected from adjacent shoots on the same infected vine. Results showed that phytoplasma are definitely more abundant in symptomatic leaves, and nearly undetectable in asymptomatic leaves of infected vines (Fig. 2).

The same technique was used to compare the titer between infected leaves and the infected shoot stems. Similarly to the previous findings, results showed that phytoplasma concentration is barely detectable in the infected shooting stems, compared to the leaves (Fig. 3).

Finally, root samples of infected Riesling, Cabernet Sauvignon and Chardonnay vines were collected and tested, but no phytoplasma was detected in any sample (not shown). These results are particularly interesting, because they strongly correlate the symptoms to the actual presence of the the bacteria, and because they suggest how slowly phytoplasma move and replicate in organs distal from the infected areas.

In an effort of understanding how phytoplasma concentration builds up in infected vines from year to year, in season 2015 we collected leaf samples from grapevines that tested positive in 2014. Again, qPCR was used to determine the relative concentration. The experiment was carried out on Chardonnay vines and showed that the relative amount of phytoplasma is higher in 2015, compared to 2014 (Fig 4). These findings demonstrate that phytoplasma titer increases from year to year, suggesting either that the microbes are able to replicate during the winter, or that their population doesn't drop during the winter. Taken together, our results seem to indicate that the actual presence of phytoplasma is linked to the manifestation of symptoms. We did not find a significant titer difference between more susceptible and less susceptible varieties, suggesting that susceptibility has more to do with the defense mechanisms of the different varieties, rather than the replication rate of phytoplasmas.

Objective 2: Grapevine Yellows management practices evaluation

Insecticide programs targeting vectors: Dr. Tony Wolf designed and managed a season-long insecticide spray program in 2014 to determine whether this approach effectively reduced leafhopper populations. This study was conducted in three vineyard blocks at two cooperating vineyards in Fauquier County, and repeated similar spray programs conducted in those same vineyards during 2013. Weekly leafhopper samples using both sticky traps and sweep netting were compared between insecticide-treated and paired control (non-treated) blocks. We found that season-long spraying does effectively reduce leafhopper abundance, as illustrated by the data of Figure 5 (Linden Chardonnay); however, it remains to be determined whether this leads to a concomitant reduction in the incidence of new NAGY infections. Our hypothesis here was that if we could suppress leafhopper populations for the entire season, we would expect to see a significant reduction in the incidence of NAGY in the insecticide-treated blocks, relative to the unsprayed control blocks, in the subsequent year. This crude "shotgun" approach to leafhopper management ignores the specifics of *which species* and *what timing* might be important, and may well be unsustainable; however, it allows us to ask a general question about whether insect (vector) management may aid NAGY management. We showed again that multiple (6 to 7) insecticide sprays could be used to depress populations of leafhoppers (Fig. 5 and 6). Fortunately for the vineyards, but unfortunately for our research evaluations, the 2014 season produced no new NAGY-diseased vines in any of the three test blocks at either vineyard. We will need to monitor these vineyards in 2015 to determine the outcome of our spray programs in 2014.

Removal of affected vine parts/organs as a tool to manage NAGY: As proposed, trials were initiated in two vineyards during 2013 to survey and remove NAGY symptomatic portions of vines when symptoms became apparent. We did this to determine if this severe pruning delayed or arrested symptom development in the subsequent year (2014). Although it is difficult to explain how removal of an affected cordon or trunk might arrest the development of a systemic pathogen, we had anecdotal evidence that the severe pruning can be used to effectively prolong the life of affected vines *of some varieties, but not others*. Approximately 60 vines were either heavily pruned in this fashion, or were left untreated during the 2013 season. These vines were closely monitored for NAGY symptoms and for vine development in 2014, and will be followed, if still alive, into 2015. A summary of our results are presented in Table 2 for three of the varieties evaluated. The vines involved in this study were followed throughout the 2014 season, and will be monitored in the coming, 2015 season. Although it's of a preliminary nature, we have seen some situations where vines that were severely pruned – such as the removal of one or both cordons and much of the corresponding trunks – remained apparently free of NAGY symptoms for over a year. For example, 7 of 10 Tannat vines have responded in this fashion (Table 2). On the other hand, 7 of 18 Cabernet Sauvignon vines at Willowcroft Vinyards appeared to "recover", or failed to show NAGY symptoms in 2014, despite showing symptoms in 2013 and not having removed affected organs from those vines when the symptoms were first observed.

6. Conclusions and Discussion

The transmission studies facilitated with this VAC project have yielded data that strongly implicate two leafhopper species, *Agallia constricta* and *Endria inimica* as vectors of the phytoplasmas that cause NAGY. Both of these species have been shown to be competent vectors for different pathogens, including viruses. Our results do not rule out the potential that other leafhoppers are also competent vectors, and the lack of positive transmission results with grape plants leaves open the possibility that while capable of transmitting phytoplasmas into sucrose solution, the insects might not actually be able to transmit phytoplasmas into phloem tissue of grapevine. The ability of a leafhopper to release the phytoplasma upon feeding is a positive indication of its ability to be a vector, because phytoplasmas have to overcome many barriers within the insect body before reaching the salivary glands where they can proliferate. However, this condition is not a sufficient proof that an insect is a vector. Some insects, in fact, feed on plants using the cell rupture feeding strategy, which is the least efficient method for pathogen transmission. In this method, the insect punctures multiple cells in the mesophyll and then feeds on the liquefied cells. In this case, acquisition and inoculation are less efficient than in the salivary sheath strategy, in which the insects find the phloem and ingest directly from it. We have no evidence of the feeding behavior of *Agallia* and *Endria* species on grapevines. The fact that grapevines are not their preferred host might suggest their inability to efficiently reach the phloem. This hypothesis is confirmed by studies found in literature, which discuss how host plant species can influence the transmission competence. Nevertheless, even though these two species do not preferentially feed on grapevine, they could occasionally transmit causal phytoplasmas through incidental feeding. Given the large numbers of *Agallia* and *Endria* found in northern Virginia vineyards, the field rates of disease transmission are presumably extremely low. Transmission rates could, however, increase when environmental conditions, such as drought or destruction of vineyard floor vegetation resulted in movement of foraging leafhoppers into vine canopies. While the lack of observed transmission to susceptible indicator plants has been one of the more frustrating aspects of the project, those results are consistent with the hypothesis that natural rates of transmission are also very low.

Appendices

Impact Statement

North American Grapevine Yellows is a lethal, insect-transmitted disease of grapevines caused by phytoplasmas (bacteria-like organisms). NAGY is a statewide threat in Virginia, but is particularly severe in the Blue Ridge and Piedmont regions where the highest vineyard densities occur. The goal of our research is to increase understanding of this complex disease and to inform management practices to mitigate vine losses. We anticipate that our research will identify vectors, which may allow temporally-targeted insecticide sprays. We may also identify important alternative hosts of the causal agents of the disease, which might allow removal of the alternative hosts from the vineyard environment. Our preliminary results also suggest that removal of affected organs (e.g., cordons or trunks) from less susceptible varieties may extend the productive lifespan of such vines.

Publications and presentations

Davis, RE, EL Dally, Y Zhao, I-M Lee, W Wei, TK Wolf, L Beanland, DG LeDoux, DA Johnson, JA Fiola, H Walter-Peterson, I Dami and M Chien. 2015. Unraveling the etiology of North American Grapevine Yellows (NAGY): Novel NAGY phytoplasma sequevars related to '*Candidatus Phytoplasma pruni*'. Plant Dis. 99:1-11.

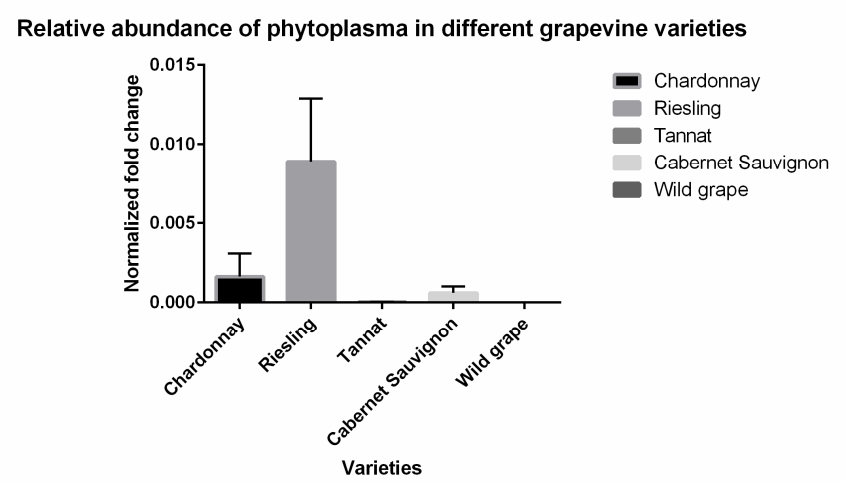

Fig. 1 Relative amount of NAGY phytoplasma in Chardonnay, Riesling, Tannat and Cabernet Sauvignon infected vines. Asymptomatic infected wild grape (Vitis spp.) was also tested. Phytoplasma titer was evaluated by qPCR using 16S specific primers. Results were normalized to the host 18S gene. Error bars indicate standard error.

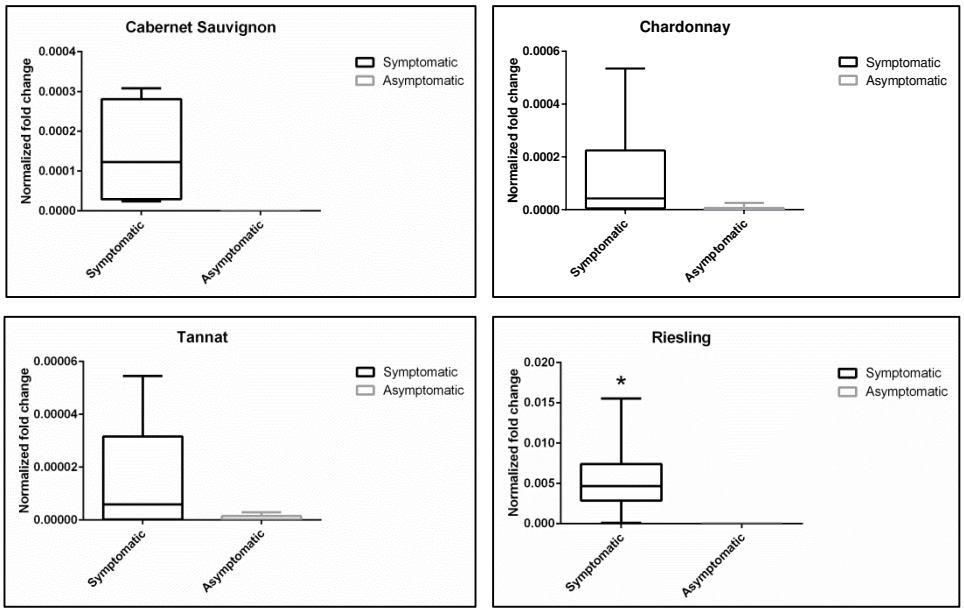

Fig. 2 Relative amount of NAGY phytoplasma in symptomatic and asymptomatic leaves of Chardonnay, Riesling, Tannat and Cabernet Sauvignon infected vines. Phytoplasma titer was evaluated by qPCR using 16S specific primers. Results were normalized to the host 18S gene. Error bars indicate standard error.

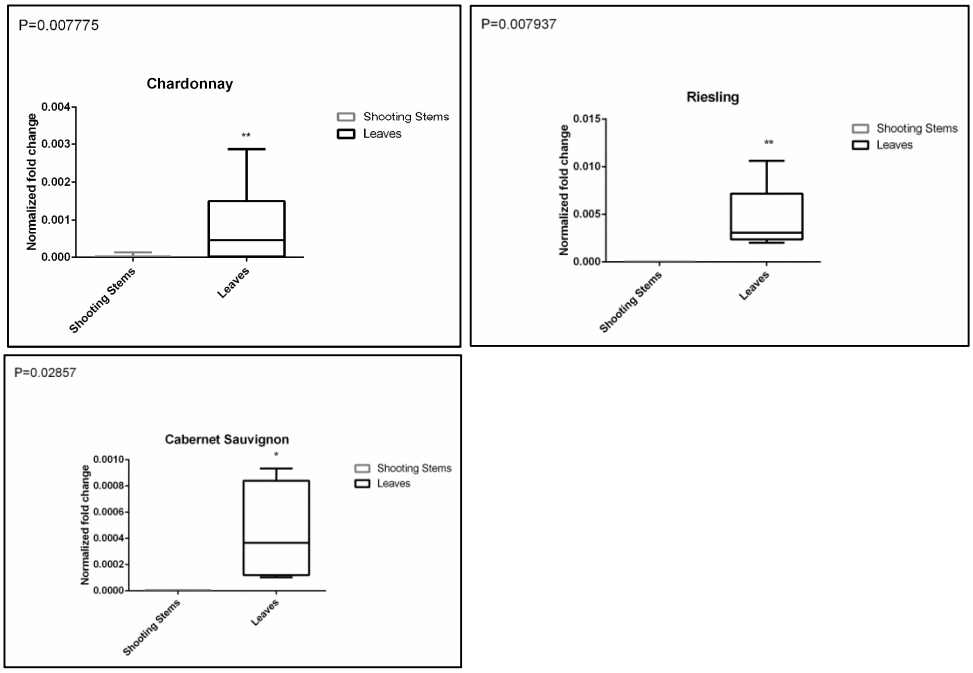

Fig. 3 Relative amount of NAGY phytoplasma in symptomatic leaves and shoot stems of Chardonnay, Riesling and Cabernet Sauvignon infected vines. Phytoplasma titer was evaluated by qPCR using 16S specific primers. Results were normalized to the host 18S gene. Error bars indicate standard error.

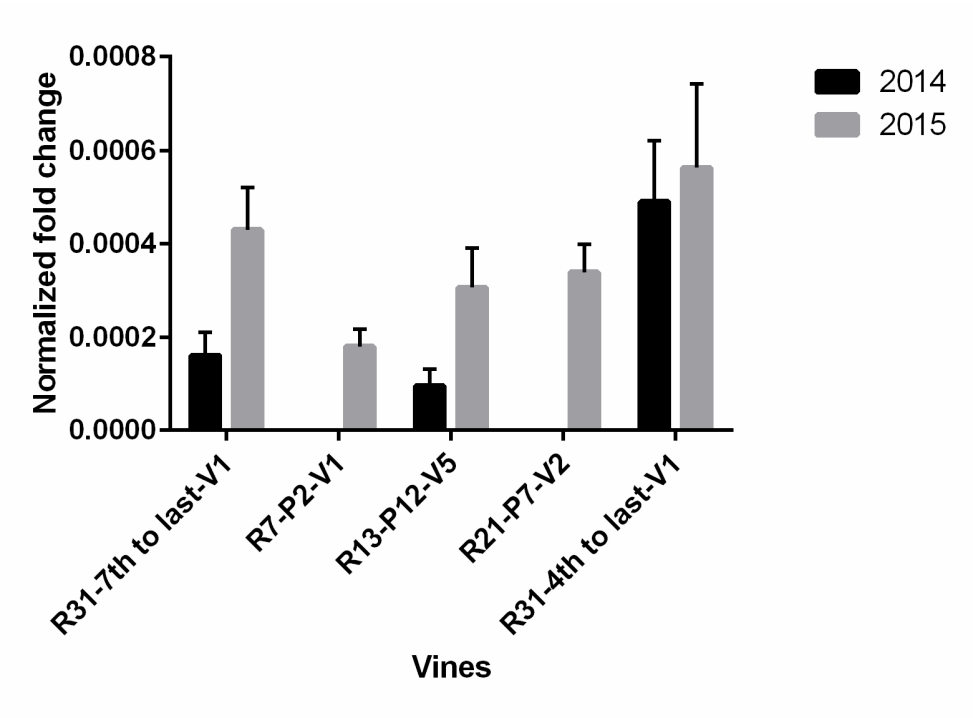

Fig. 4 Relative amount of NAGY phytoplasma in Chardonnay symptomatic leaves collected in 2014 and 2015 seasons. Phytoplasma titer was evaluated by qPCR using 16S specific primers. Results were normalized to the host 18S gene. Error bars indicate standard error.

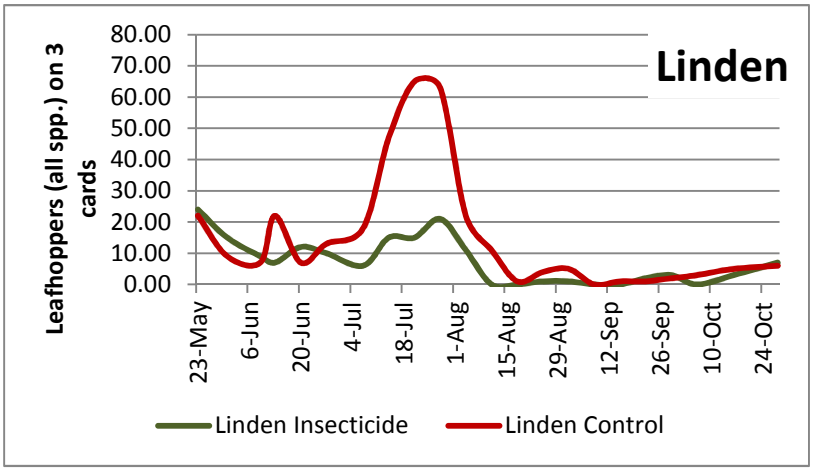

Figure 5. Total number of leafhoppers collected in insecticide-treated block (green line) or in non-treated control block of Chardonnay at a northern VA vineyard in 2014. The insecticide program depressed but did not eliminate leafhoppers from the treated portion of the vineyard.

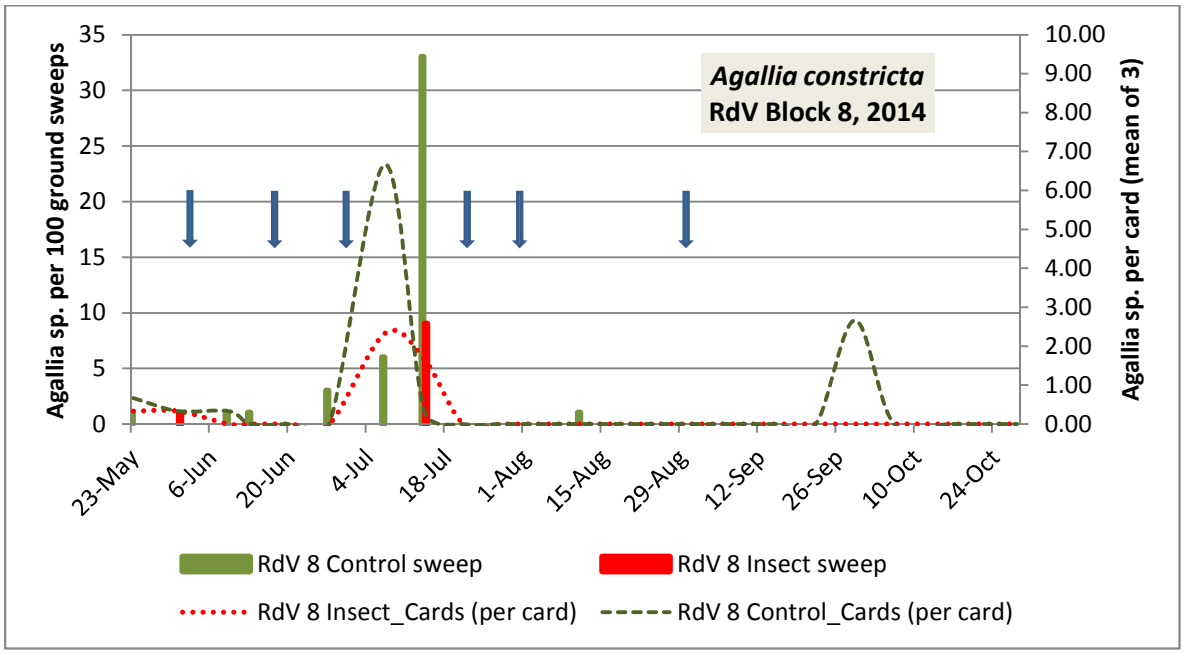

Figure 6. Seasonal abundance of *Agallia constricta* in a vineyard block of one vineyard surveyed and used in a test of insecticide suppression of leafhoppers during the 2014 season. The green bars reflect leafhoppers collected from vineyard floor sweeps in the half of the block that was used as a control (no insecticide), the green dashed line is the mean number (n=3) of *Agallia* leafhoppers found on sticky cards in the control half of the block, the red bars are insects found in ground sweeps of the insecticide half of the block, and the red dashed line is the mean number (n=3) of *Agallia* leafhoppers found on the sticky cards in the insecticide portion of the block. The blue arrows are the dates that the insecticide-treated half of the block was sprayed with an insecticide (6 times in 2014).

Table 1. Candidate insect vectors (Order Hemiptera) of Group I and III phytoplasmas identified in 2012-2013 sucrose transmission assays. Insects were collected from commercial vineyards in Virginia and fed a 5% sucrose solution in individual tubes. The sucrose solution/saliva mixture was subsequently tested for phytoplasmas with nested polymerase chain reaction (PCR).[1] Species abundance ranking is based on season-long sweep net samples of 72 species of leafhoppers in 27 mid-Atlantic vineyards in 2013; 1= most, 65 = least abundant).

Species	Species abundance rank	Subfamily	Sucrose media N positive/tested 2012	2013	NAGY-phytoplasma Group
Family Cicadellidae - Leafhoppers					
Agallia constricta	1	Agallinae	0 / 5	4 / 585	III-A
Graphocephala versuta	4	Cicadellinae	2 / 40	0 / 91	III-A
Exitianus exitiosus	6	Deltocephalinae	2 / 49	0 / 102	III-A
Endria inimica	10	Deltocephalinae	1 / 14	0 / 61	I-B
Amblycellus curtisii	15	Cicadellinae	2 / 5	0 / 25	I-B
Coelidia olitoria	32	Coelidiinae	0 / 0	4 / 24	III-A
Scaphytopius magdalensis	65	Deltocephalinae	1 / 2	0 / 1	I-B

[1]Species that were tested but did not yield any positive results in either 2012 or 2013 were omitted from this table (N = 38 species). In 2012, all insects were collected and tested during September only. In 2013, although insects were tested throughout the growing season (May – Oct.), only insects collected late in the season (late July – late Sep. 2013) yielded positive results.

Table 2. Status of vines in late-2014 that showed symptoms of NAGY in July 2013 as a function of either (a) removing affected organ(s) or (b) simply monitoring the progress of symptoms without attempting to excise the affected organ(s). Two vineyards and data from two varieties are presented.

Williams Gap, **Cabernet Sauvignon**	
27	Total vines monitored since July 2013
11	Decapitated vines (7/2013) that have remained apparently healthy, July 2013 – October 2014
11	Decapitated vines (7/2013) that were removed by vineyard owner in December 2013 (unclear if vine had succumbed to NAGY however).
1	Decapitated vines (7/2013) that still showed NAGY symptoms in 10/2014
4	Decapitated vines (7/13) that are of questionable status (might be NAGY positive, or could be other issues with vine)
Williams Gap, **Tannat**	
10	Total vines monitored since July 2013
7	Decapitated vines (7/2013) that have remained apparently healthy, July 2013 – October 2014
3	Decapitated vines (7/13) that are of questionable status (might be NAGY symptoms)
Willowcroft, **Cabernet Sauvignon**	
18	Total vines monitored since July 2013
2	Decapitated vines (7/2013) that have remained apparently healthy, July 2013 – October 2014
1	Decapitated vines (7/2013) that subsequently died
7	Vines that expressed some NAGY symptoms in 7/2013, but appeared healthy in August and October of 2014 *without any intervention*
2	Vines that expressed some NAGY symptoms in 7/2013, and subsequently died
4	Vines that expressed some NAGY symptoms in 7/2013, and still had some symptoms in August and October of 2014