Final report to Virginia Wine Board 2014 FY
(14-1690-02)

PROJECT TITLE:
Investigating the lifecycle of ripe rot of grape caused by *Colletotrichum* species.

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OBJECTIVES:

1) Determine the optimal environmental conditions (temperature, wetness duration, relative humidity, and grape cluster developmental stage) for the two ripe rot pathogens;
2) Examine chemical management options;
3) Investigate their capability to survive in woody tissues;
4) Investigate how infection process takes place in the absence of symptom expression; and
5) Determine a baseline sensitivity of ripe rot pathogens to various QoI fungicides.

I. Activities Performed

Objective 1) Examine environmental conditions for infection by two ripe rot pathogens
We conducted a series of experiments using table grape, potted vine, and field-grown vine to determine the effect of environmental condition to the infection.

Preparation of detached berries: Several lab experiments were conducted using table grapes and detached wine grapes (cultivars Chardonnay and Petit Manseng) to examine the infection process. In 2014, a row of Chardonnay was maintained in AHS AREC using a seven-day application schedule of protectant fungicides, such as Dithane Rainshield and Microthiol, to control other grape pathogens and provide a clean source of Chardonnay grapes at the end of the season. Initially, the whole cluster was inoculated using an atomizer, but it was observed that this pathogen was very efficient at finding natural openings and wounds. An attempt was made to seal a natural opening of a table grape at the base of pedicel with wax and other substances; however, the fungus still can find wounds on the surface of the berry that are not visible at the time of inoculation. Therefore, the following method was developed. White table grapes were purchased at a local grocery store, then the clusters were rinsed in a sink then berries were detached from the rachis with the pedicel still attached to the berry. The pedicel and the attaching end of the berry were coated with a hot wax in order to reduce moisture loss and risk of infection through natural openings. Then, each berry was surface sterilized by submersing it for 1 min and 30 sec in 10% Clorox solution, then rinsed three times in distilled water. Berries were dried under a laminar flow hood (air will be filtered through a HEPA filter), and twelve berries were placed into a quail egg carton (eggcartons.com, Manchaug, MA) to provide support and prevent rolling.

Preparation of inoculum: Due to demand of spores, the technique for making spore suspensions was altered this year. Single-spore cultures of *C. acutatum* and *C. gloeosporioides* were grown on at 25°C quarter-strength potato dextrose agar, with a diurnal light cycle, for spore production. After 7-10 days, the surface was flooded (~3 mL) with distilled water and then brushed with a bent glass rod to suspend the spores; the suspension was then filtered through two layers of miracloth. The concentration of spores was adjusted using a hemacytometer to $5x10^5$ spores per mL. A drop of Tween 20 (200µL) is added to the diluted suspension to help break the surface tension before use.

Inoculation of detached berries: A drop of 5 µL spore suspension was placed onto the surface of ten sterilized berry. Two berries were inoculated with a 5 µL drop of sterile distilled water to serve as a control to measure natural ripe rot infections. The trays were placed in a sealed wet chamber in order to avoid the droplet evaporating. At 6, 12, 18, 24, and 30 hours after inoculation, one quail egg carton (containing twelve inoculated berries) was removed from the outer container and air-dried in a laminar flow hood until the berry skin surface is completely dry. Then the container was moved to a dry incubator that is maintained at 25°C. Daily observation of berries was made and visual assessment of disease incidence was made at 5, 7, 10, 14, and 17 days after inoculation. After 10 days of incubation, the berries were frozen at -20°C for 20 min to break down the berry skins and encourage symptom development.

Inoculation of potted vines: In the 2013 potted plant studies, 3 year-old plants of Cabernet sauvignon, Chardonnay and Merlot were used. At each of six growth stages (bloom, BB-size, pea-size, berry touch, veraison, and two weeks after veraison), three pots of Cabernet sauvignon, and two pots of Chardonnay and Merlot were inoculated with a 10^5 spore suspension that was prepared as described above using a hand atomizer to clusters until run-off. In 2014, inoculations were repeated using the same vines as in 2013 (Cabernet sauvignon, Chardonnay and Merlot) with the addition of Petit Verdot at the same six growth stages. The numbers of pots inoculated at each time point were reduced to one vine per cultivar due to limited flowering from winter damage. Due to the limited availability of vines, *C. acutatum* was only used to inoculate Cabernet sauvignon and Chardonnay clusters in both years. The clusters were bagged with a wet paper towel to increase humidity. The whole plant was then placed into an environmental growth chamber (Model E75L1, Percival Scientific Perry, IA) that was set to 25°C with a diurnal light cycle. After the 24-hr period, the bags were removed and each cluster tagged for future reference. Visual assessment of disease was made at the end of the season (late-October). Clusters were harvested by hand and individually bagged. A visual assessment of disease was completed on the day of harvest, then berries were cut from the rachis, placed in individual wells of quail egg containers, and incubated for 14 days at 25°C with a diurnal light cycle. Assessments were taken every 5, 7, 10, and 14 days.

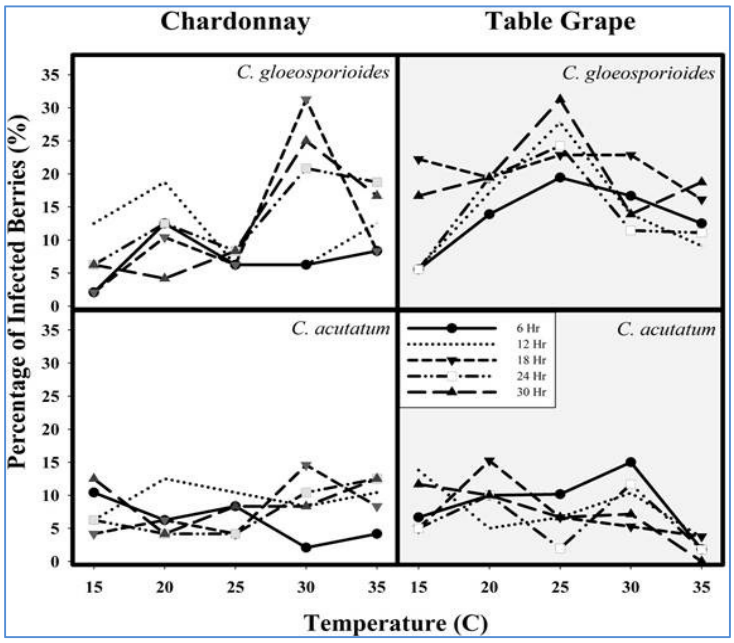
Inoculation of vines in the field: Sections of Merlot, Cabernet Franc, Cabernet sauvignon, and Chardonnay at AHS AREC were used. All vines have been trained in the vertical shoot positioning system, and each vine was trained to bear 20-24 clusters. In 2013, five clusters were randomly chosen on the vines of Cabernet Franc, Cabernet sauvignon, and Chardonnay and ten clusters were chosen on Merlot vines for each growth stage (bloom, BB-size, pea-size, berry touch, veraison, and two weeks after veraison). In 2014, six clusters were randomly chosen on Cabernet Franc and Cabernet sauvignon vines and 30 clusters were randomly chosen from six vines of Merlot and Chardonnay for each growth stage. For each cultivar, a separate cluster was also chosen to serve as a control to provide a sample of naturally occurring *Colletotrichum* in the field. A 10^5spore suspension was prepared as described above and was applied with a hand atomizer to clusters until cover in the afternoon (3-4 PM) to reduce the time that the clusters are in the bags in sunlight. After a period of 22-24-hrs (bags were removed early on overly bright, hot days), the bags were removed and each cluster flagged for future reference. A visual assessment of disease was done weekly during the season. At the end of the season, clusters were harvested by hand and individually bagged. The bagged clusters were placed in low relative humidity environmental growth chambers at 25°C with a diurnal light cycle to encourage ripe rot symptoms. Disease incidence and severity (percent of berries infected per cluster) were recorded after two weeks of incubation when the disease is most active and easily observed.

Assessments of disease: Visual assessment of disease incidence and severity will be made weekly until the end of the season (when Brix level > 20-25). As needed, we conducted bioassay by isolating the fungus from the infected tissue to confirm infection. Cluster disease incidence was analyzed using a generalized linear mixed model (PROC GLIMMIX, ver. 9.4, SAS institute, Cary, NC) and disease severity was analyzed using a mixed linear model in SAS (PROC MIXED, SAS).

Results

Detached berry assays: In order to determine the effect of temperature and wetness, we used a wine grape cultivar, Petit Manseng to conduct three replications of experiment. Results, however, turned out that despite the fact that all treatment berries were inoculated with high concentration of spores, disease rarely developed. Only seven berries resulted in ripe rot out of 1,800 berries inoculated. We are suspecting that pH level of the berry was the factor. On the detached Chardonnay and table grape assays from 2014 had similar mean disease incidences with the range of 2.08 - 14.58% for *C. acutatum* and 2.08 - 31.25% for *C. gloeosporioides*. Few symptoms appeared on the grapes until the 17th day of observation when the final data was collected. The effect of wetness duration was not significant on wine ($P = 0.19$) or table grapes ($P = 0.60$) but there was a significant effect of isolate and temperature on both wine and table grapes ($P < 0.01$) however; the effect of temperature was not significant for *C. acutatum* ($P = 0.76$) on wine grape. There was a general peak in disease incidence at 30 °C for *C. gloeosporioides* on Chardonnay grapes but the peak was shifted lower to 25 °C on table grape. The lowest incidence for both Chardonnay and table grapes inoculated with *C. gloeosporioides* was at the lowest temperature at 15 °C on Chardonnay grapes. There were no obvious peaks in disease incidence for *C. acutatum* on both wine and table grapes but infection was observed at all temperatures for all wetness durations.

Figure 1. Effect of temperature and wetness duration on ripe rot disease severity on detached Chardonnay and table grape berries



Controlled environment study: Results from the 2013 studies showed cluster disease incidence ranged from 0.0% to 100.0% for both ripe rot species and was significantly impacted by cultivar ($P < 0.01$) for both *Colletotrichum* species but not the growth stage. Disease severity for each cluster stage varied from 0.0% to 65.0% for C. gloeosporioides-inoculated clusters and 0.0% to 54.8% *C. acutatum*-inoculated clusters with a two-way interaction between cultivar and growth stage ($P < 0.01$). Higher disease incidence and severity was observed on Chardonnay than on Cabernet sauvignon and Merlot for both

species. Peaks in disease severity were observed at pea-size and veraison on Chardonnay and Cabernet sauvignon, however; on Merlot, there were no peaks, but consistent, low amounts of disease severity throughout the season. As with 2012 results, earlier inoculation (from bloom to BB-size) resulted in lower disease severity than the inoculation after pea-size (Fig. 2).

Overall cluster disease incidence for the cluster developmental stages in the field was lower in 2014 than in 2013. Results from the 2014 studies showed cluster disease incidence ranged from 0.0% to 100.0% for both ripe rot species, same as in 2013, with no significant interactions between the growth stages or cultivars. Disease incidence was limited on Cabernet sauvignon and Merlot, with no observable symptoms on Petit Verdot. Disease severity varied from 0.0% to 66.7% for *C. gloeosporioides* inoculated-clusters and 0.0% to 23.8% *C. acutatum* inoculated-clusters. As in 2013, a two-way interaction between cultivar and growth stage ($P < 0.01$) was observed on *C. gloeosporioides* inoculated-clusters while there were no significant effects ($P > 0.05$) on *C. acutatum* inoculated-clusters. Clusters inoculated with *C. acutatum* exhibited low disease severity throughout the season with no significant differences between growth stages. (Fig. 3)

Figure 2. Effect of inoculation timing on ripe rot disease severity on Chardonnay, Cabernet sauvignon and Merlot in the 2013 controlled environment study.

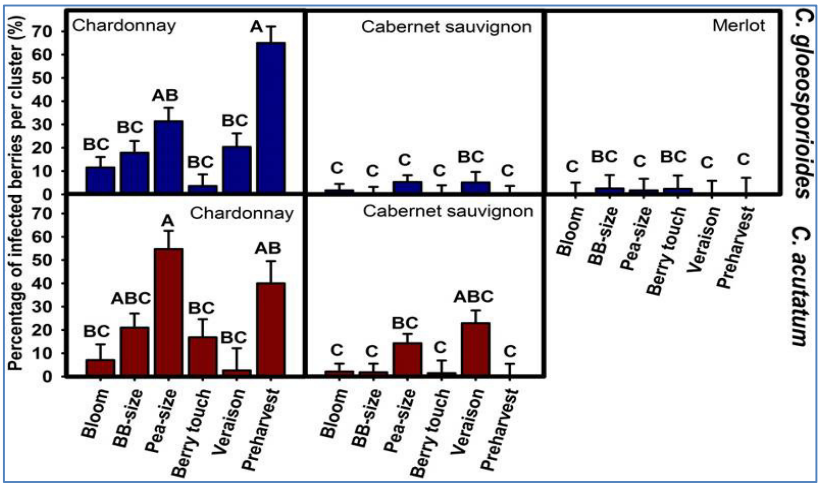
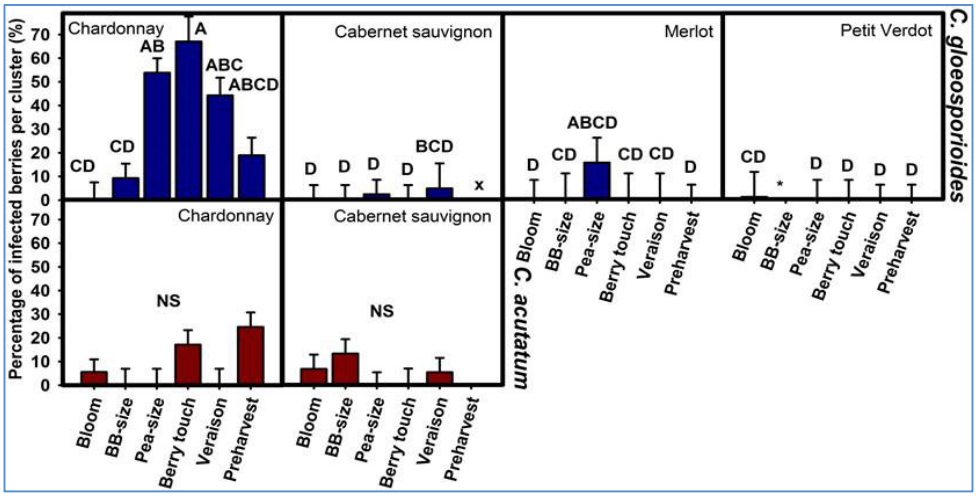


Figure 3. Effect of inoculation timing on ripe rot disease severity on Chardonnay, Cabernet sauvignon, Merlot and Petit Verdot in the 2014 controlled environment study.



Field inoculation: In both years, regardless of *Colletotrichum* species, all cluster developmental stage with all tested cultivars resulted in some level of infection. Results from the 2013 study showed that disease incidence varied from 55.6% to 100.0% for *C. gloeosporioides*-inoculated clusters and 60.0% to 100.0% for *C. acutatum*-inoculated clusters with no significant effects of cultivar or growth stage on disease incidence ($P > 0.05$). For disease severity, there was a two-way interaction between cultivar and growth stage ($P < 0.01$) for both species of ripe rot.

When we compared the effect of timing of infection, there were some differences observed (Fig. 4). For Cabernet Franc, inoculation at Berry touch was significantly lower in disease severity than other timings, and there seemed to be two peaks of disease severity at BB-size and after veraison. The similar trend was observed for Chardonnay, where two peaks at bloom and at veraison (note: clusters on Chardonnay inoculated at preharvest were missing due to combination of rots and bird damage). Merlot, on the other hand, had low disease severity at bloom, and overall disease severity was lower. The difficulty of infection at bloom may attribute to the overall low disease severity. The results from Cabernet sauvignon should be considered as a supplemental because there were only two time points for Cabernet sauvignon due to loss of clusters (Fig. 4).

Overall, disease incidence and severity was lower in 2014 than in 2013 but the range varied from 73.3% to 100.0% for *C. gloeosporioides*-inoculated clusters with a significant effect ($P < 0.05$) of growth stage and 40.0% to 100.0% for *C. acutatum*-inoculated clusters with no significant effects of cultivar or growth stage. Disease severity varied from 1.3% to 14.4% for *C. gloeosporioides*-inoculated clusters and 1.0% to 25.1% *C. acutatum*-inoculated clusters. For *C. acutatum*, there was a two-way interaction of cultivar and growth stage ($P < 0.01$) but there was only an effect of cultivar ($P < 0.01$) for *C. gloeosporioides*. Similar peaks in disease severity were observed in 2014 as in 2013 at the beginning and end of the season but with far less exaggerated differences between the growth stages.

Figure 4. Effect of inoculation timing on ripe rot disease severity on Chardonnay, Merlot, and Cabernet Franc in the 2013 field study.

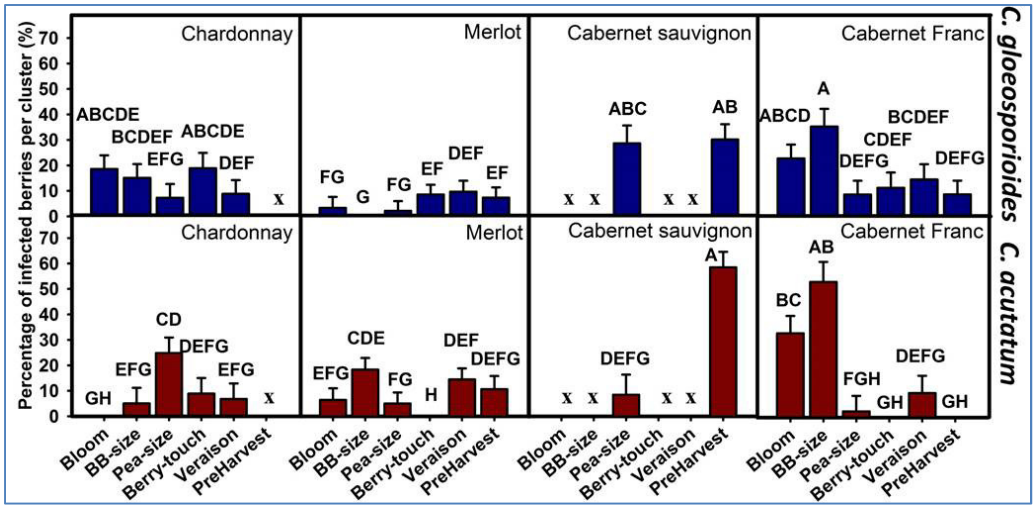
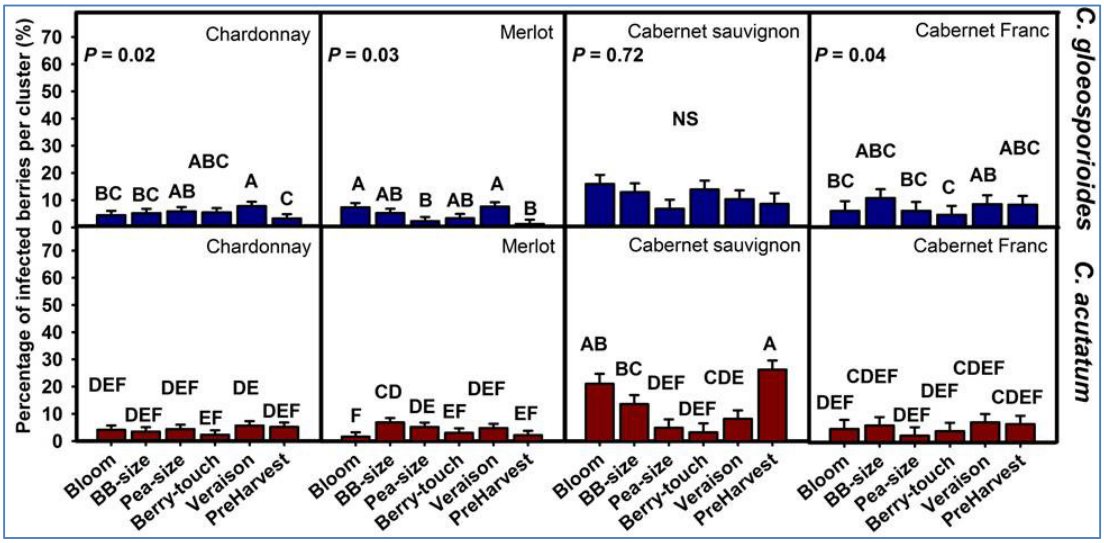


Figure 5. Effect of inoculation timing on ripe rot disease severity on Chardonnay, Merlot, Cabernet sauvignon, and Cabernet Franc in the 2014 field study.



Summary: On the detached berry studies, disease incidence of *C. gloeosporioides* increases with increased temperature and wetness duration. For both species of ripe rot, temperature was the more significant factor. This agrees with prior literature from experiments on other fruit crops. The range of temperature (25 – 30°C) is also similar to the other estimated temperature range of *C. gloeosporioides* from other hosts. As we found in 2012 to 2014, results confirmed that the infection could occur at any growth stage of the clusters (i.e., there is no critical period as in downy mildew, powdery mildew, and black rot); however, the intensity of disease symptoms differs greatly between inoculation times and cultivars. We observed two peaks of higher disease severity in the field in earlier and late in the season while disease severity was higher in later in the season in the hoop house. In both 2013 and 2014, a shade cloth was used from the beginning of the season to reduce the heat accumulation in the hoop house, yet, at bloom inoculation resulted in lower disease severity than later inoculation timings.

Also, there was strong cultivar effect observed. In both controlled environment and field study showed that Merlot is less susceptible to ripe rot than three other cultivars: Chardonnay, Cabernet sauvignon, and Cabernet Franc. Cabernet sauvignon was not significantly different from Merlot in the controlled environment study, but it was the most susceptible cultivar in the field. Petit Verdot requires further investigation since symptoms were not observed at any growth stage, however, during BB-size, the inoculated clusters completely aborted within a week of inoculation. The other notable cultivar is Petit Manseng. In this trial, we were not able to produce diseased berries. Even with repeated inoculation, we were able to achieve less than 20% disease incidence.

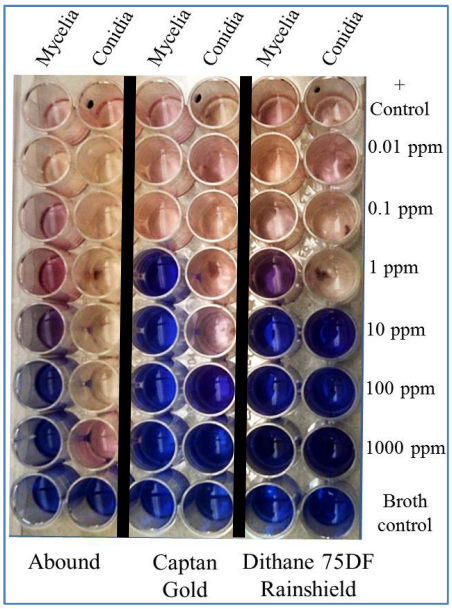
Future direction: Our results indicated that susceptible cultivars are susceptible throughout the season. This will make it very difficult to time our fungicide application for ripe rot management. Since we have a very limited tools available (EBDC, captan, and QoI are only group that has ripe rot on the label), we need to seek for other mode of action groups (Objective 2), and eventually test best candidates in the field.

Objective 2 and 5) Chemical management options for ripe rot pathogens, and determine the evidence of QoI fungicide resistance among VA isolates

We have been using two-step methods to reduce costs. The first step is the use of alamarBlue® (AB) in the fungicide amended culture plate [11], and then we will use more traditional fungicide amended media with potentially resistant isolates.

In order to develop a protocol for *Colletotrichum* species, we have conducted a preliminary study with AB assay to screen several modes of action groups using two of our isolates. At the beginning of the assay, we had some concern on the proposed filtering (which was adapted from the original paper (15)) since some of fungicides, such as Abound, left significant amount of residues on the surface of the filter. We were not certain that we could consistently achieve the correct end concentration after the filtering. In addition, the original paper used V8 as a medium, but we learned that it is very difficult to filter V8, and also it was difficult to obtain consistency with V8 media. After several trials, we made several modifications to the protocol to make it work with our isolates. We changed medium from V8 to 2% PDA and fungicide stocks were prepared with ethanol to reduce contamination.

Figure 6
AB plate test example from our test



Modified protocol: The single-spore isolate plates will be flooded by adding 3 mL of clarified, buffered 2% Potato Dextrose Broth (PDB). Then, the suspension will be filtered using two layers of Miracloth to remove mycelium. Then, 100 µl of a suspension of 10^5 conidia/ml (adjusted using a hemocytometer) or 100 µl of 2% PDB with aerial mycelium will be added to test wells of 48-well cell culture plate (Corning Costar), and stock fungicide solutions will be added to give final concentrations of each fungicide (0.0, 0.01, 0.1, 1.0, 10.0, and 100.0 µg/mL Note: for some of fungicides, rate up to 500 ppm were tested). AB dye (AbD Serotec) will be added as 10% of the final volume in the test wells [12]. Plates will be covered with sterile plastic plate covers, gently rotated horizontally to mix the well contents, then incubated in the dark at 25°C for 48 h. There will be negative control (200 µL of 2% PDB and 10% AB dye only), and positive control (100 µL of PDB, 100 µl of 10^5 conidia/ml, and 10% AB dye). A chemical control plate will be also prepared to ensure that the fungicides themselves did not reduce the AB dye (100 µL of stock fungicide, 100 µL of 2% PDB, and 10% AB).

A positive test result was recorded as a color change from blue to pink, which indicated that the dye had been reduced due to the presence of viable conidia (Fig. 5). A negative test result will be recorded as no color change or the dye remained blue, i.e., the dye was not reduced due to the absence of viable conidia/fungal growth. One mean inhibitory concentration (MIC) endpoint will be visually determined and defined as the lowest concentration of fungicide that prevented a color change from blue to pink (MIC- blue) after 48 h of incubation. Optimal incubation time was determined by monitoring the color of the negative control wells, and set to 48 hours.

Table 1 shows the results from our preliminary experiments. EC50 (Effective concentration with 50% inhibition) was determined by estimating the intercept and slope using a generalized linear model in SAS (PROC GENMOD, ver. 9.4, SAS institute, Cary, NC) where logit was used as a link function. Then, effective concentration with 50% (EC50) and 80% (EC80) of inhibition (i.e., no change in color) were then estimated using a nonlinear mixed model (PROC NLMIXED in SAS), using the estimated intercept and slope from the generalized linear model. Based on the assay, we did not find significant difference ($P < 0.05$) between two isolates (*C. acutatum* and *C. gloeosporioides*), and many of fungicide resulted in less than field rate EC50. However, one of fungicides that showed an excellent efficacy with our table grape assay (*data not shown*), Mettle, did not work with AB assay. At the same time, we had to use a buffer (sodium bicarbonate) to increase the pH of Mettle treated well, so, it might have affected its efficacy. As with any other *in vitro* tests, tests using a live plant tissue should be conducted to validate true efficacy.

The poor performance by Endura was also shown with our table grape assay, thus, SDHI group might not have efficacy against *Colletotrichum acutatum* and *C. gloeosporioides*.

Table 1. Estimated EC50 values for each fungicide based on AB assay: + = exceeded the field ppm, '*' = different' formulation of copper

Fungicide	Rate per Acre	PPM^{X} in field	Mycelia EC50	Mycelia EC80	Spores EC50	Spores EC80
Abound®	12 fl oz	215.3	608.63 +	9260.60	355.64 +	767.97
Captan Gold™ 80 WDG	2 lb	1922.2	47.99	129.31	11.48	22.19
Champ® Dry Prill	3 lb	2775.2	88.05	152.31	33.97	61.18
Cueva®*	1 gal	2000.0	97.01	98.53	39.37	75.29
Dithane® 75DF Rainshield	3 lb	1333.5	21.37	43.24	6.53	10.40
Endura®	8 fl oz	420.5	1087.66 +	1518.61	3357.09 +	6348.51
Property®	5 fl oz	70.3	829.05 +	1251.05	63275.00 +	822967.00
Mettle® 125 ME	4 fl oz	36.0	44.60 +	70.95	26.11	46.84
ProPhyt®	5 pt	3382.9	194.91	353.80	66.36	90.64
Topsin® M 70 WDG	1 lb	841	490.55	871.20	255.83	456.05
Additives						
Ethanol			1030.18	1056.05	-1.00	-1.00
SHAM			1000.00	1024.80	-1.00	-1.00
Sodium bicarbonate			188.89	283.78	69.52	117.67

Future direction: Once some of isolates are identified to be less sensitive to the fungicide, traditional mycelium growth and spore germination tests will be conducted to confirm their lack of sensitivity to fungicides. Plates of ¼ PDA will be amended with stock fungicide solution to give final concentrations of 0.0, 0.01, 0.1, 1.0, 10.0, 100.0, and 1,000.0 µg/mL for each fungicide + SHAM (100 µg/ml).

Mycelium growth: A 5 mm diameter agar block will be cut from the advancing edge of an actively growing culture on ¼ PDA (using a 100 mm Petri dish) and placed in the center of the dish, mycelia-side down, on the surface of the amended PDA. Plates will be incubated in the dark at 25°C for 4-6 days. The radial diameter (perpendicular measurements in millimeters) will be recorded for each colony. The corrected diameter (mean radial diameter minus the length of the agar block) will be used to calculate percent relative growth (%RG = [mean diameter of colony/mean diameter of colony on non-amended agar] × 100) and percent relative growth inhibition (%RGI = 100 – %RG) compared with the non-amended controls. At each experiment, three plates will be used per isolate, the experiment will be conducted twice, and the mean corrected colony diameters will be used in all calculations.

Germination rate: Four 5 µL of spore suspension with 10^6 spore/ml will be placed on to aforementioned amended (and non-amended) ¼ PDA. Then spore germination rate (and formation of appressorium) will be determined using microscope (40x and 100x objectives, Nikon Eclipse Ci, Nikon, Inc.). The observation will be made at 6 hours after inoculation, and 25 spores will be examined per drop (i.e., 100 spores will be examined per isolate per run). Percent germination and relative germination inhibition rate will be determined. The assays will be conducted three times. Data from mycelium growth and spore germination rate will be analyzed using linear (PROC REG) or non-linear (PROC NLIN) or other methods such as beta model [13] regression to determine EC_{50} (Effective Concentration to inhibit 50% of sample) and EC_{90}. In 2014, 36 isolates were selected based on their genetic similarities (please see the section below), host (some are from apples and strawberries), and geographic locations.

A first run of mycelium growth test has been conducted in Oct-Nov. 2014 using four isolates selected based on the G143A mutation test (described below). Due to contamination issues, which seem to be attributed to our fungicide stock, we repeated the test in Jan. – May 2015. Currently we are in the

middle of data collection and analysis from the first set of experiments. Germination rate tests will be conducted once we figure out the source of contamination.

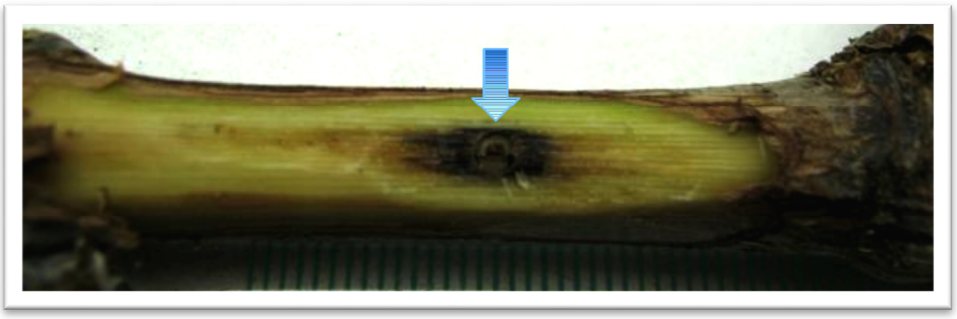
G143A mutation in samples: We completed all of 2013 isolates, got 292 *Colletotrichum* isolates (99, Ca and 193, Cg). Since high levels of resistance are indicative of a target site mutation, we sought to characterize the cytochrome b gene to determine if there were mutations that were specific to the resistant isolates. Amplification of an internal portion of the cytb gene using primers RSCBF1 and RSCBR2 produced a single fragment, of ≈285 bp in size. Cloned cytb gene fragments derived from a QoI-resistant and -sensitive isolate of grape ripe rot. Positive PCR-product bands were obtained from all *C. acutatum* isolates (99/99), but 36 from 193 *C. gloeosporioides* isolates. The PCR product will be needed to be treated using a mutation-specific restriction enzyme ItaI (=Fnu4HI) for confirmation of G143A mutaiton. The PCR products from resistant isolates carrying the mutated sequence GCN at position 143 could be digested by the restriction enzyme, whereas those from sensitive isolates remained undigested.

Objective 3) Woody tissue infection by ripe rot pathogens

We have inculcated a total of 15 vines with cultures of *C. acutatum* and *C. gloeosporioides*. The cane was purchased from the foundation planting service to make sure its cleanliness. After rooting, we drilled a hole (2 mm diameter, 5 mm in depth), and inoculated the woody tissues as described in the proposal. The inoculation took place in the month of September 2012. Since the development of fungal colony within woody tissues often takes time, we waited four months for the development of the fungi inside of the woody tissue. The visual assessment of disease was performed after dissecting the wood, and re-isolate the pathogen was made from the wood.

The results showed that these fungi were capable of surviving in the woody tissue for more than 4 months, indicating long-term survival of these pathogens. The results also showed that there were development of necrotic lesions at the site of infection (sign of fungal activity) (Fig. 4), and we were able to re-isolate fungal isolates (both *C. acutatum* and *C. gloeosporioides*). The results indicated that these pathogen can survives in woody tissue, but also able to cause disease. We have expanded this research in 2014 to investigate longer-term effect of woody tissue infection. Specifically, we will increase the number of plant and time that we will wait for the assessment. By waiting for 6 – 12 months, we are hoping to see the development of spore baring structures on woody tissues. The inoculate took place in May 2014, and observations were planned in the summer of 2015. However, due to the severe winter in 2015, we have lost nearly 100% of potted vine in the high tunnel. The experiment will be repeated in 2015-2016. We built a raised bed system in the high tunnel to reduce the risk of winter coldness.

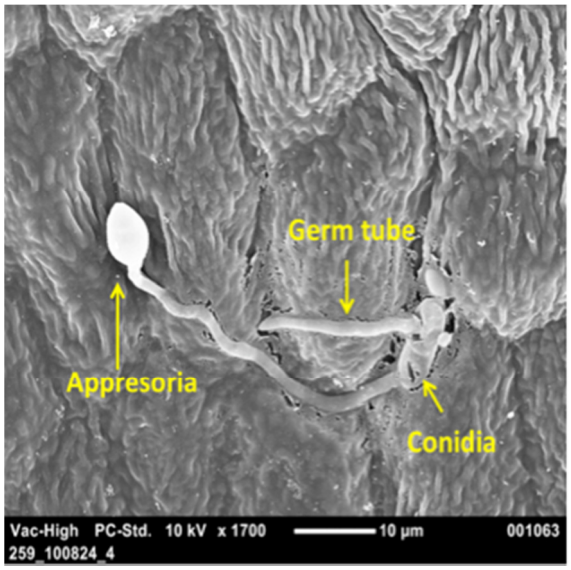
Close-up of woody tissue inoculation. The point of inoculation is shown with an arrow and you can observe dark necrotic vascular tissues developed from it.



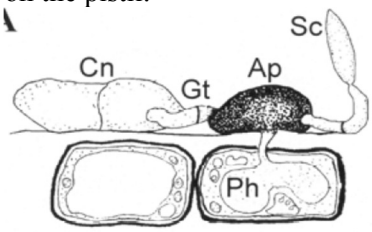
Objective 4) Infection by ripe rot pathogens without symptom development

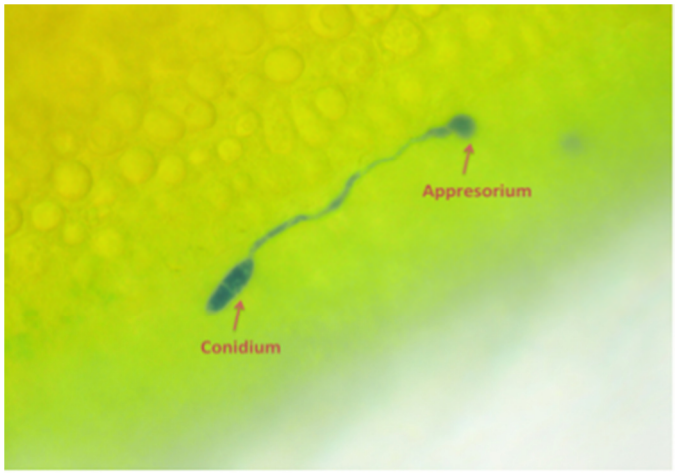
We used light microscopy and scanning electron microscope (SEM) to investigate various stages of infection process during infection period. Infection by spores on leaf tissues, flower tissues, and young berry tissues were examined. For flower tissues, the invasion of flower part, especially in pistil was examined. For young berry tissues, inoculation was made at bloom, BB-size, Pea-size, and bunch closure. The preliminary results indicated that (1) *C. acutatum* and *C. gloeosporioides* can cause infection on the flower tissues, leaf tissues, and young berry tissues (bloom, bb-size, pea-size, bunch closure), (2) The germination of the conidia, development of the germ tube, and formation of appressoria generally occurred in 24 h after inoculation, and (3) The leaf age may affect the timing and extent of conidial germination and appressorium formation. We will investigate more details of the infection and fungal establishment processes in 2014-2015.

Colletotrichum species develop many specialized infection structures, including germ tubes (Gt), appressoria (Ap), and hyphae; a short, immature hypha that emerges from a germinating spore. The dark ball-like structure in the diagram is the appressorium, which helps to infect host plant for initial penetration



The following images are showing infections on leaf and pistil tissues, confirmed with our study. You can visualize germ tube developed from a conidium, and appressorium that formed at the end of the germ tube, indicating potential invasion to grape epidermal cells. The picture on the right shows germination on leaf surface, and the picture on the left shows germination on the pistil.



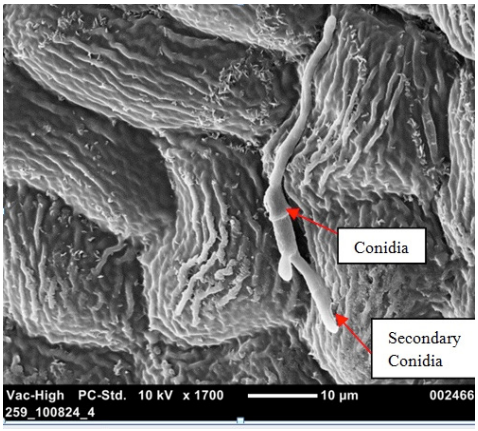


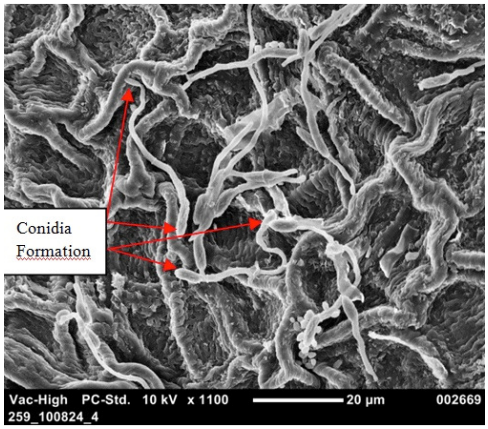
We used light microscopy and scanning electron microscope (SEM) to investigate various stages of infection process during infection period. Infection by spores on leaf tissues, flower tissues, and berry

tissues were examined. For flower tissues, the invasion of flower part, especially in pistil was examined. For berry tissues, inoculation was made at bloom, BB-size, Pea-size, bunch closure, veraison and two weeks after veraison.

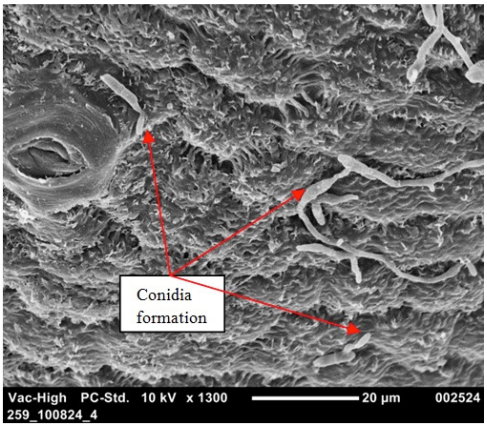
The preliminary results indicated that (1) *C. acutatum* and *C. gloeosporioides* can cause infection on the flower tissues, leaf tissues, and berry tissues (bloom, bb-size, pea-size, bunch touch, veraison and two weeks after veraison), (2) Conidial germination, appressorial formation and secondary conidiation of both CA and CG were observed 24 h after inoculation on the surface of flowers, leaves and berries at different stages of maturity, and (3) The penetration of cuticle could be observed on the flower and pre-mature (veraison) and mature (two weeks after veraison) berries, but not on the leaf and young berry surfaces (bb-size, pea-size, bunch touch).

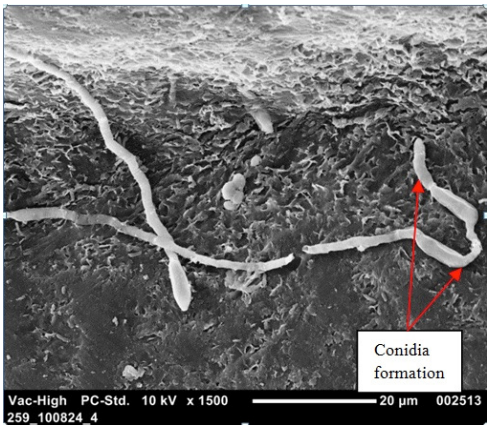
For leaf experiments, the raw data showed that the number of initially unmelanized appressoria on leaf surfaces decreased beginning 6 h after inoculation as melanization occurred. Appressorial pores were first observed at approximately the same time that melanization began. The number of appressoria with pores increased over time, and most had pores after 24 and 48 hr after inoculation. Secondary conidia were produced by primary conidia that germinated with phialides instead of germination tubes and by phialides forming on elongated germination tubes. Secondary conidia were first observed on conidial phialides 6 hr after inoculation, and on hyphal phialides 24 hr after inoculation. Both conidial and hyphal phialides produced multiple secondary conidia that detached and accumulated near the phialides. We confirmed that leaf infection can result in spore formation, and that may be a significant source of inoculum for flower and fruit infections.



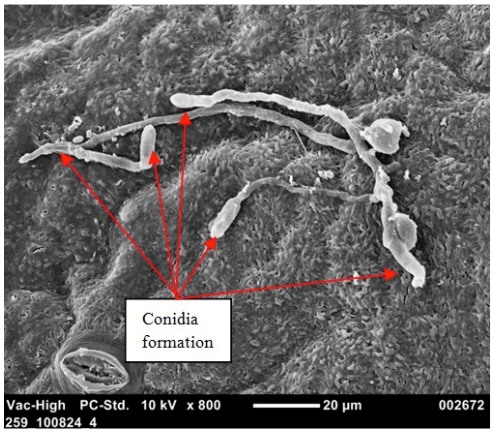


Cg-secondary conidia produced by primary conidia Ca-secondary conidia produced by hyphal phialides

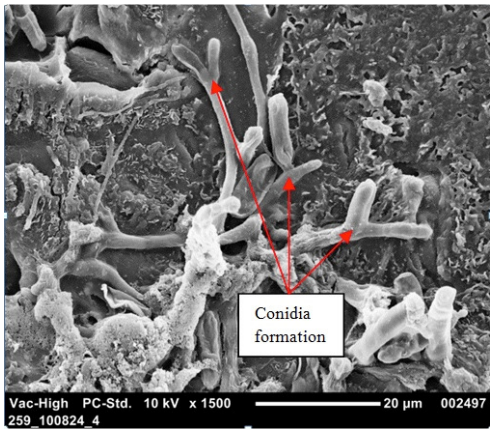




Ca- secondary condia on the flower surface Ca- secondary condia on the berry surface

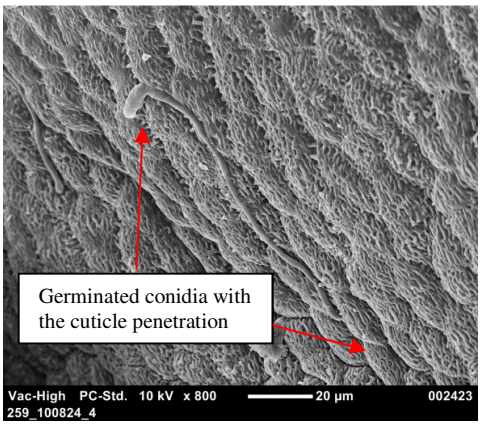


Cg- secondary condia on the flower surface



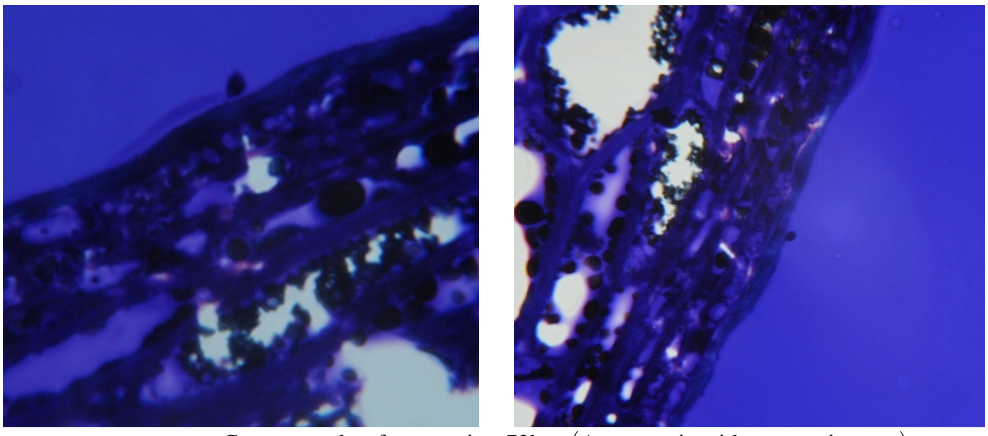
Cg- secondary condia on the berry surface

In order to better evaluate various stages of infection process during infection period, the semi-thin cross sections (1-2 µm thick) were cut with ultra-microtome and attached to glass slides for optical microscopy. Based on the images of flower, leaf and berry tissues taken by the scan electron microscope (SEM) and light microscope, we found penetration pegs or infectious hyphae penetrated the cuticle of the bloom and the later growth stage berry samples (veraison and two weeks after veraison), and for the leaves and unripe berries (BB-pea size, berry touch), Ca and Cg are capable of survival on the surface as germinated conidia forming appressoria, with frequent secondary conidiation, but without the penetration of the cuticle. I addition, the trend of infection of both pathogens is more likely to move from pedicel to ovary on the flower tissues over time.

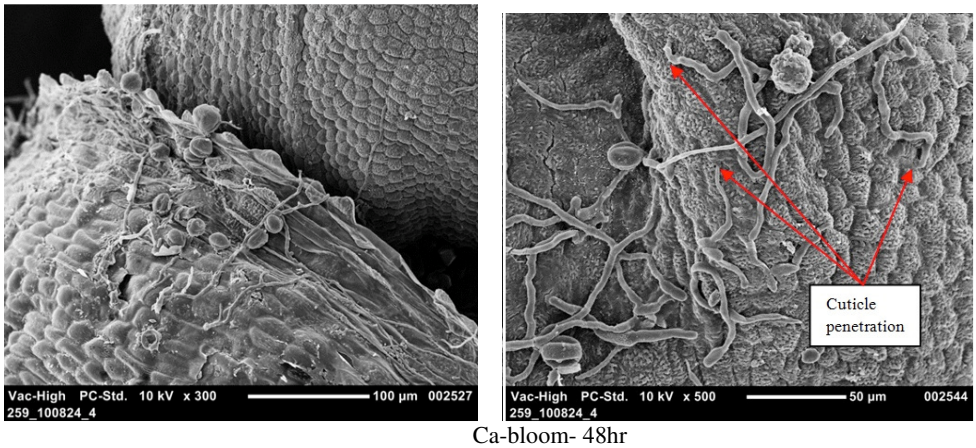


Cg-bloom- 48hr (SEM)

Cg-bloom-72hr (light microscope)



Cg-two weeks after verasion-72hr （Appressoria with penetration peg）



Ca-bloom- 48hr

Extension and Education

We presented two oral reports at Cumberland Shenandoah Fruit Worker's Conference in December 2013 and additional two during 2014, as well as three presentations during the Virginia Vineyard Association's annual meeting in February of 2013, 2014, and 2015. Results were discussed in two VA IPM workshops and two vineyard meetings in 2013, three IPM meeting in VA, one MD grower's meeting, and one PA IPM workshop in 2014. In addition, one presentation was made at our science society's regional meeting, where my student Ms. Oliver received a student research award, and another presentation was made at the national meeting in Aug. 2014.

Presentations provided by our group in 2013-15

Oliver, C. and Nita, M. "Characterizing the Infection conditions and potential control methods of Ripe rot of grape, *Colletotrichum acutatum* and *C. gloeosporioides*" at the Virginia Vineyard Association Annual meeting, 7 February 2015

Oliver, C. and Nita, M. "A quick fungicide efficacy screening for ripe rot pathogens, *Colletotrichum acutatum and C. gloeosporioides*, using alamarBlue® dye", Cumberland-Shenandoah Fruit Worker's Conference, 4 December 2014

Oliver, C. and Nita, M. "Characterizing the infection of ripe rot of grape, caused by *Colletotrichum acutatum* and *Colletotrichum gloeosporioides*", Cumberland-Shenandoah Fruit Worker's Conference, 4 December 2014

Nita, M., Hartley, S., and Oliver, C. "Screening of Fungicides for the Control of Ripe Rot on Grapes" American Phytopathological Society National Meeting, 5 Aug. 2014

Oliver, C. and Nita, M. “Characterizing the infection conditions of grape ripe rot (*Colletotrichum acutatum* and *Colletotrichum gloeosporioides)* on wine grape clusters”, American Phytopathological Society Potomac Division meeting, 13 March 2014

Nita, M. “Back to Basics III, Phomopsis, Ripe Rot, and Bitter Rot” at the Virginia Vineyard Association Annual meeting, 2 February 2013

Nita, M. “Updates of Grape Disease Management” at the Virginia Vineyard Association Annual meeting, 2 February 2014

Nita M., and A. Bly "Effect of relative humidity on germination of *Colletotrichum acutatum* and *C. gloeosporioides*" 89th Annual Cumberland-Shenandoah Fruit Worker Conference, Winchester, VA, December 5, 2013.

Nita M., and S. Hartley. "Screening of fungicides for management of ripe rot of grape" 89th Annual Cumberland-Shenandoah Fruit Worker Conference, Winchester, VA, December 5, 2013.

A master’s student, Ms. Charlotte Oliver, has been working on this project (her GRA has been provided by other grants). She has completed 23 credit hours of classes such as on Plant Pathogenic Agents, Pesticide Usage, and Plant Clinic Experience so far. She will be focusing on the objectives 1 and 2 for her program. In 2013, she completed her coursework for her Master’s and presented her first departmental seminar. During June of 2015, she defended her Master’s thesis and we are expecting completion of her degree during the Fall 2015 semester. She will continue her PhD studies under my supervision, working more on ripe rot complex and potentially expand into other grape pathogens and has begun her PhD coursework during the Fall 2015 semester.

II. Problems and Delays

We found out that this fungal species are prone to suffer from contamination issues, which lead to poor production of spores. We spent considerable amount of time on 2014-15 to fix this issue. In addition, the experiment in the objective 3 was failed due to the severe winter.

III. Future Project Plans

Objectives 1: This part of the study will be completed very soon.
Objectives 2 and 5: We will repeat the experiments in 2015 to show the reproducibility.
Objective 3: It will be repeated in 2015-2016.
Objective 4: We will continue investigating ripe rot infection process using light and SEM microscopy techniques in 2015 season. We will screen more transformed isolates to select several *C. acutatum* and *C. gloeosporioides* isolates that contain GFP reporter gene in the 2015.

IV. Funding Expended To Date

We have utilized about 100%.