~~Progress~~ Final report to Virginia Wine Board FY 2016

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

PRINCIPAL INVESTIGATOR:	COLLABORATOR:
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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 pathogen complexes~~s~~;
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. After several trials, the following method was developed. White table grapes were purchased at a local grocery store, then the clusters were rinsed in sink then 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.

To address the issue of decreasing pathogenicity, our lab modified the spore production plating technique. Single-spore cultures of *C. acutatum* and *C. gloeosporioides* were grown at 25°C on quarter-strength potato dextrose agar, with a diurnal light cycle. Agar plugs were removed from the agar plates and dried. The culture chips were transferred to a 2 mL

cryogenic vial (Simport,) and stored at -20°C. Weekly, agar chips were removed from storage and placed in potato dextrose broth on a shaker at 25°C with a diurnal light cycle. After 4-7 days, mycelia was transferred from the broth to quarter-strength potato dextrose agar and grown at 25°C with a diurnal light cycle, for spore production.

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. For each growth stage (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: The effect of *Colletotrichum* species complex, cluster developmental stage, cultivar, and their interactions on the mean cluster disease incidence and on the mean percentage of infected berries per cluster were analyzed using a generalized linear mixed model (PROC GLIMMIX, SAS, ver. 9.4, SAS institute, Cary, NC) for both studies. The GLIMMIX model utilized the logit link function for mean cluster disease incidence and identity for the mean percentage. When the effect of a factor or their interaction was found to be significant, the mean cluster disease incidence or the mean percentage of infected berries among treatments was compared using Fisher's least significant difference (LSD) method.

In addition, for the controlled-environment study, a pair-wise comparison of the mean cluster disease incidence and the mean percentages of infected berries per cluster between the visual assessment at and after the 14-day incubation was conducted. The effect of cultivar, assessment date, and their interaction on the mean cluster disease incidence, and the mean percentage of infected berries, were examined using a longitudinal linear mixed model (GLIMMIX). Cultivar, assessment date, and their interactions were considered as fixed factors, and each cluster was defined as a subject for a repeated measurement. Compound symmetry covariance structure was selected for both analyses, based on comparisons of several covariance structures with Akaike's Information Criterion. Once a significant effect of the assessment date was observed, the difference in the mean cluster disease incidence and the mean percentage of infected berries per cluster were compared between assessment dates for each cultivar using Student's *t*-test.

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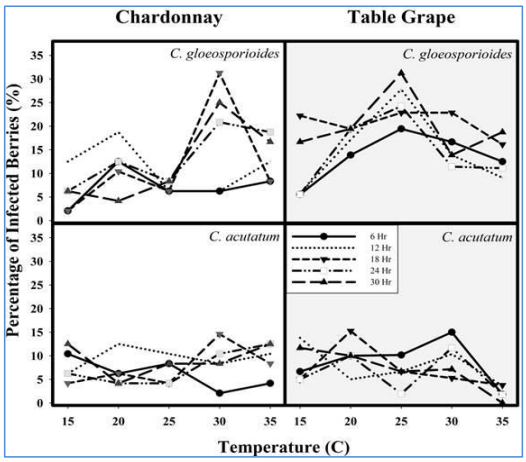
Results

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 the 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: Inoculated berries were rated twice, at harvest and 14 days after harvest in both 2013 and 2014. Using the combined data from the six cluster developmental stages, a longitudinal analysis was conducted to examine the effect of the incubation on the mean disease incidence and the mean percentage of infected berries per cluster. Consistent increases were observed in the mean cluster disease incidence and the mean percentage of infected berries per cluster after 14 days of incubation across cultivars in both years. The difference was significantly larger ($P \leq 0.05$) for many of the cultivar and *Colletotrichum* species combinations in 2013. Moreover, even if a very low level of symptom development was observed at harvest, the actual level of infection was often higher. For example, the mean cluster disease incidence was 0% at the time of harvest for *C. gloeosporioides* (Cg-) inoculated Merlot in 2013 and *C. acutatum* (Ca-) inoculated Cabernet sauvignon in 2014; however, Cg inoculations on Merlot resulted in 16% and Ca inoculations on Cabernet sauvignon resulted in 27% mean cluster disease incidence after the incubation. Therefore, analyses hereafter are based on the data collected after the incubation period.

The mean cluster disease incidence among the four cultivars varied according to the developmental stage from 0.0% to 100.0% for both Cg-inoculated clusters and Ca-inoculated clusters (Fig. 2, A-E) in 2013. Higher overall mean cluster disease incidences were observed with Cg- and Ca-inoculated Chardonnay clusters (Fig 2, A and D). Inoculation of Merlot with Cg resulted in sustained levels of mean cluster disease incidence across cluster developmental stages, and only a period between BB-size and berry touch showed development of disease.

When differences among cluster developmental stages were examined, relatively lower mean cluster disease incidences were observed at berry touch with both Cg- and Ca-inoculated clusters among the three tested cultivars (Fig 2, A-E). However, the ANOVA results showed that there was no significant effect ($P > 0.05$) of pathogen species, cluster developmental stage, cultivar, and their interactions on the mean cluster disease incidence. The lack of significant

difference among cluster developmental stages was probably due to the high variability among samples at each stage (Fig. 2).

The mean percentage of infected berries per cluster across the three cultivars for each cluster developmental stage varied from 0.0% to 65.0% for Cg-inoculated clusters (Fig. 2, F-H) and 0.0% to 54.8% for Ca-inoculated clusters (Fig. 2, I and J) in 2013. Significant effects ($P \leq 0.05$) of cultivar, cluster developmental stage, and their interaction on the mean percentage of infected berries were observed. The significant interaction resulted from the differences in the susceptible cluster developmental stages among cultivars (Fig. 2). For example, two peaks in the mean percentage of infected berries per cluster were observed with Chardonnay (at pea-size and pre-harvest) and Cabernet sauvignon (at pea-size and veraison) (Fig. 2, F and I) while Merlot (Fig. 2, G) resulted in very low percentage of infected berries per cluster across all six cluster developmental stages.

In 2014, mean cluster disease incidence among the four cultivars for each cluster developmental stage varied from 0.0% to 100.0% for both *Colletotrichum* species (Fig. 3, A-E). As in 2013, Chardonnay clusters exhibited higher overall mean cluster disease incidence, and all cluster developmental stages resulted in some level of disease development (Fig 3, A and D). As with 2013, Merlot sustained low levels of mean cluster disease incidence throughout the season, with the exception of pea-size where it was 100% (Fig. 3, B). However, since only one cluster per cluster developmental stage was available for inoculation, Merlot was not included in the ANOVA. Similar to 2013, the ANOVA results showed that there were no significant effects ($P > 0.05$) of *Colletotrichum* species, cluster developmental stage, cultivar, and their interaction on the mean cluster disease incidence. With Cabernet sauvignon, inoculation at veraison resulted in a relatively higher mean cluster disease incidence for both Cg and Ca (Fig 3, C and E), which was also observed in 2013.

Overall, the mean percentages of infected berries for Ca-inoculated clusters in the controlled environment were lower in 2014 than in 2013. The mean percentage of infected berries per cluster among four cultivars for each cluster developmental stage varied from 0.0% to 66.7% for Cg-inoculated clusters (Fig. 3, F-H) and 0.0% to 23.8% Ca-inoculated clusters (Fig. 3, I and J) in 2014. Unlike 2013, there were significant effects ($P \leq 0.05$) of cultivar, *Colletotrichum* species complexes, the cultivar and *Colletotrichum* species complexes interaction, and the cultivar and cluster developmental stage interaction. As with 2013, Chardonnay sustained higher mean percentages of infected berries per cluster (Fig. 3, H and I). The results from Chardonnay clusters clearly showed the interaction between *Colletotrichum* species complexes and cultivar. For instance, on Cg-inoculated Chardonnay clusters, an increase in the mean percentage of infected berries was observed toward the middle of the season (pea-size to veraison) then a decrease as the season progressed (Fig. 3, F). In contrast, the overall level of disease development was very low with Ca-inoculated Chardonnay clusters with higher mean percentages observed at berry-touch and pre-harvest (Fig. 3, I). The interaction between cultivar and cluster developmental stage is best observed in the comparison of Ca-inoculated Chardonnay and Cabernet sauvignon (Fig. 3, I and J). On Chardonnay, the mean percentage of infected berries per cluster increased through the season to the highest percentage at the end during preharvest (Fig. 3, I). However, on Cabernet sauvignon, the mean percentage of infected berries per cluster was the highest during bloom and BB-size, and then decreased to a very low level for the rest of the growing season (Fig. 3, J).

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Figure 2. Effect of inoculation timing on ripe rot ~~disease severity~~disease incidence and percentage of diseased berries per clusters on Chardonnay, Cabernet sauvignon and Merlot in the 2013 controlled environment study. Three wine grape cultivars were shown vertically [Chardonnay (A, D, F, and I), Merlot (B and G), and Cabernet sauvignon (C, E, H, and J)]. Gray bars represent clusters inoculated with *C. gloeosporioides* and black bars represent clusters inoculated with *C. acutatum*, with standard errors as error bars.

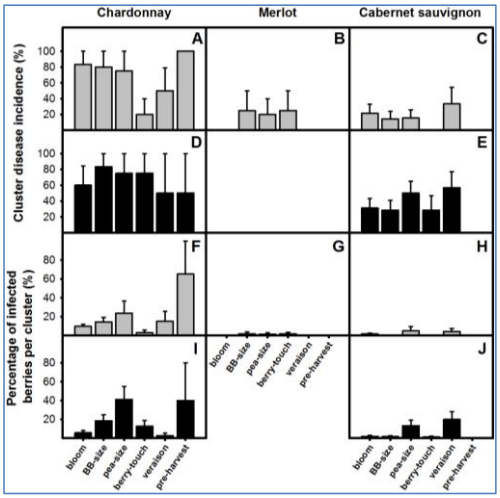
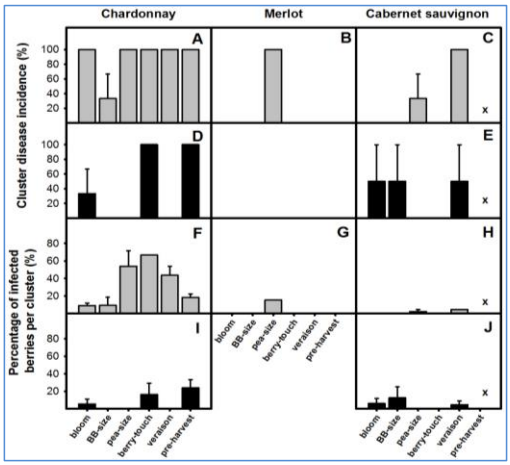


Figure 3. Effect of inoculation timing on ripe rot disease incidence and percentage of diseased berries per clusters on Chardonnay, Cabernet sauvignon and Merlot in the 2014 controlled environment study. Three wine grape cultivars were shown vertically [Chardonnay (A, D, F, and I), Merlot (B and G), and Cabernet sauvignon (C, E, H, and J)]. Gray bars represent clusters inoculated with *C. gloeosporioides* and black bars represent clusters inoculated with *C. acutatum*, with standard errors as error bars.



Field inoculation: In 2013, the mean cluster disease incidence across six cluster developmental stages across the four cultivars varied from 25.0% to 100.0% for Cg-inoculated clusters (Fig. 4, A-D) and 11.1% to 100.0% for Ca-inoculated clusters (Fig. 4, E-H). There were no data available for clusters of Cabernet sauvignon that were inoculated at bloom, BB-size, berry touch, and veraison; thus, data from Cabernet sauvignon in 2013 were not included in the ANOVA. As in the controlled environment study, many clusters had 100% mean cluster disease incidence regardless of pathogen species, cluster developmental stages, and cultivar (Fig. 4, A-H); therefore, no significant effects ($P > 0.05$) of cultivar, *Colletotrichum* species complexes, and cluster developmental stage and their interactions on the mean cluster disease incidence were observed. There were several cases where numerically lower mean cluster disease incidences were observed: Cg-inoculated clusters of Merlot from bloom to berry touch (Fig. 4, B); Ca-inoculated clusters of Chardonnay at bloom, Merlot at berry touch; and Cabernet Franc at pea-size and pre-harvest (Fig. 4, A, F, and H).

In 2013, the mean percentage of infected berries per cluster for each cluster developmental stage across the four cultivars varied from 0.0% to 35.2% for Cg-inoculated clusters (Fig. 4, I-L), and 0% to 58.6% Ca-inoculated clusters (Fig.

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4, M-P). There were significant effects ($P \leq 0.05$) of cultivar, cluster developmental stage, and their interaction. The significance of cultivar and cluster developmental stage was well illustrated in the differences between the results from Chardonnay and Cabernet Franc (Fig. 4, I and M for Chardonnay, and L and P for Cabernet Franc). With Chardonnay, all cluster developmental stages resulted in similar degrees (15-20%) of infection with the exception that Ca-inoculation resulted in relatively higher mean percentage at pea-size inoculation. In contrast, there were higher mean percentages of infected berries observed at earlier stages (bloom and BB-size) on Cabernet Franc, then decreased as the season progressed. As in the controlled environment study, Merlot resulted in relatively lower mean percentages of infected clusters (Fig. 4, J and N).

The mean cluster disease incidences among pathogen species, cultivars, and cluster developmental stages in the field were much lower in 2014 than in 2013. It varied from 50.0% to 100.0% for Cg-inoculated clusters (Fig. 5, A-D), and 40% to 100% for Ca-inoculated clusters (Fig. 5, E-H). Similar to 2013, the majority of observed mean cluster disease incidence per cluster developmental stage was close to 100%, with a few exceptions (Fig. 4, A-H). Numerically lower mean cluster disease incidences were observed: both Cg- and Ca-inoculated Merlot at pre-harvest (Fig. 5, B and F); Cg-inoculated clusters of Cabernet Franc at berry touch (Fig. 5, D); Ca-inoculated clusters of Chardonnay at berry touch; Cabernet sauvignon at BB-size, berry touch, and veraison; and Cabernet Franc at pea-size (Fig. 4, E, G, and H).

Overall, the mean percentage of infected berries per cluster among the tested cultivars and six cluster developmental stages in the field was lower in 2014 than in 2013. In 2014, mean percentage of infected berries per cluster among four cultivars varied from 1.3% to 14.4% for Cg-inoculated clusters (Fig. 5, I-L), and 1.0% to 25.1% Ca-inoculated clusters (Fig. 5, M-P), with smaller differences among the cluster developmental stages than in 2013 (Fig. 4). There were significant effects ($P \leq 0.05$) of cultivar, cluster developmental stage, and their interaction on the mean percentage of infected berries (Table 2.5). In 2014, that interaction was obvious with Cabernet sauvignon, which was not included in the analysis in 2013. Higher mean percentages of infected berries were observed at bloom, BB-size, and pre-harvest with Ca-inoculated Cabernet sauvignon (Fig. 2.4, O), while with the other cultivars, differences among cluster developmental stages were not as large (Fig. 2.4). It should be noted that, as in 2013 and the controlled environment study, even clusters with low mean percentages of infected berries, resulted in high cluster disease incidences (Figs 2.1-2.4).

A common attribute between the two studies was the significant effect of cluster development stage on the mean percentage of infected berries (Tables 2.4 and 2.5). When the mean percentage of infected berries per cluster was compared across the six cluster developmental stages, consistently low development of disease was observed at berry touch. At berry touch, mean percentage of infected berries was often significantly lower ($P \leq 0.05$) than other cluster developmental stages (Fig. 2.5) with the one exception of the controlled environment study in 2014 (Fig. 2.5).

Figure 4. Effect of inoculation timing on ripe rot disease incidence and percentage of infected berries per cluster ~~severity~~ on Chardonnay, Merlot, Cabernet sauvignon and Cabernet Franc in the 2013 field study. Four wine grape cultivars were shown vertically [Chardonnay (A, E, I, and M), Merlot (B, F, J, and N), Cabernet sauvignon (C, G, K, and O), and Cabernet Franc (D, H, L, and P)]. Gray bars represent clusters inoculated with *C. gloeosporioides* and black bars represent clusters inoculated with *C. acutatum*, with standard errors as error bars. Data were not collected at certain cluster developmental stages due to a lack of clusters (x).

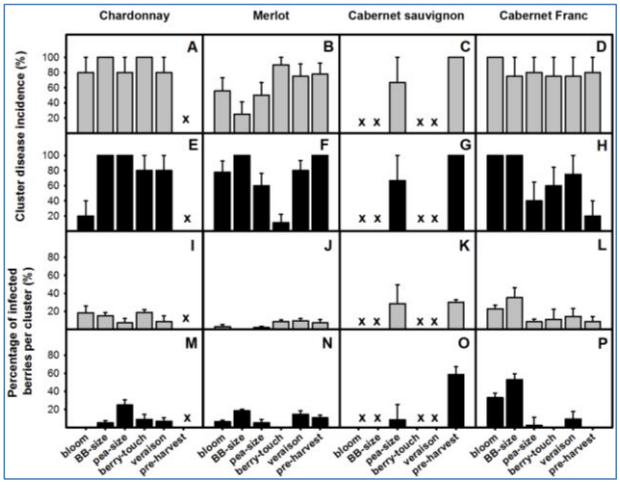

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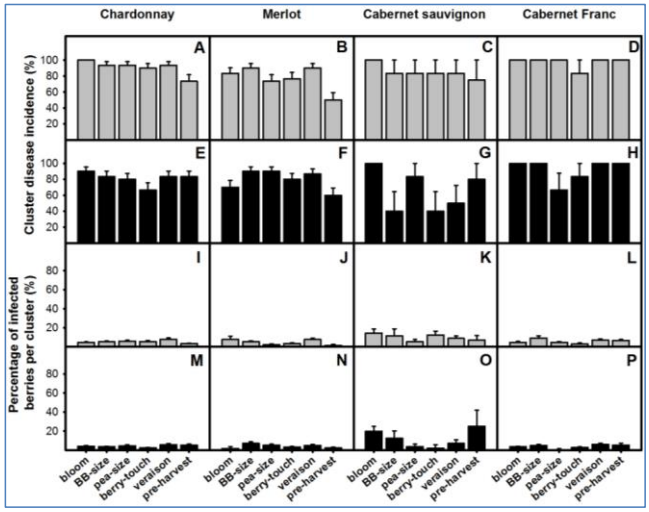
Figure 5. Effect of inoculation timing on ripe rot disease incidence and percentage of infected berries per cluster on Chardonnay, Merlot, Cabernet sauvignon and Cabernet Franc in the 2013 field study. Four wine grape cultivars were shown vertically [Chardonnay (A, E, I, and M), Merlot (B, F, J, and N), Cabernet sauvignon (C, G, K, and O), and Cabernet Franc (D, H, L, and P)]. Gray bars represent clusters inoculated with *C. gloeosporioides* and black bars represent clusters inoculated with *C. acutatum*, with standard errors as error bars.



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 or powdery mildew); 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.

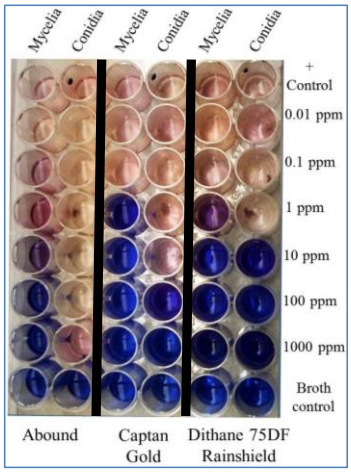
Future direction: This project has been experimentally wrapped up and we are currently working on a publication.

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 quite a bit 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 or mycelia (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/mycelial 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 probably does 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

QoI sensitivity assay using fungicide amended medium. Currently, a limited number of chemical options are available for controlling ripe rot. Among the fungicides recommended for management of ripe rot are the QoI group, or strobilurins, which prevent the production of ATP in the fungal cell. While these fungicides are effective, they are commonly associated with fungicide resistance due to the specificity of their mode of action, meaning a single mutation can result in a resistant population. A common, but not exclusive, source of insensitivity is mutation of the Cytochrome β gene at codon 143 (*Ishii et al., 2001*).This research examines the prevalence of QoI insensitivity among 283 *Colletotrichum* isolates collected throughout Virginia during 2013. Isolates were screened using a QoI amended media and later data was compared with results from PCR-RFLP.

For the fungicide-amended media assay, petri dishes containing PDA amended with 100 ppm azoxystrobin (Abound, 22.9% a.i. azoxystrobin, Syngenta Crop Protection) and 1,000 ppm SHAM (salicylhydroxamic acid) were inoculated with each of the isolates using a mycelial plug and then incubated at 25^{o}C. The presence or absence of growth with exposure to azoxystrobin amended media was examined after 3 and 6 days. PCR-RFLP was utilized to detect a point mutation (G143A) of the cytochrome β gene (Ishii et al., 2001). The primers GCCBF1 and RSCBR2 were used to amplify a fragment of 120 base pairs. This fragment was then digested with a restriction enzyme *Fnu4HI* (aka *Satl)* which cuts the fragment at codon 143 if the mutation is present, creating two fragments 65 and 55 base pairs in size. Gels did reveal fragments fitting this description, although some also had an additional banding.

The preliminary results showed that after six days of incubation, 28% of isolates grew on the fungicide-amended media, and thus showed signs of insensitivity. The PCR-RFLP results indicated that 16% of the isolates expressed signs of G143A mutation. The higher percentage with the fungicide-amended media assay was expected, since there is more than one kind of mutation that can lead to insensitivity, while the PCR-RFLP assay was designed to test for only one mutation.

Future direction: Currently, we are preparing the last run for the AB assay. Once the last experimental run is completed, results will be published.

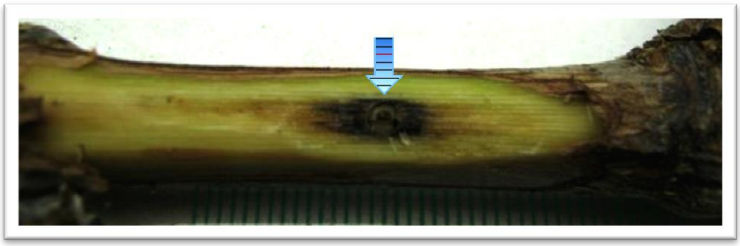
Objective 3) Woody tissue infection by ripe rot pathogens

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 not only 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 to be made in 2015; however due to the severe winter in 2014, we lost nearly all the potted vines by the beginning of 2015.

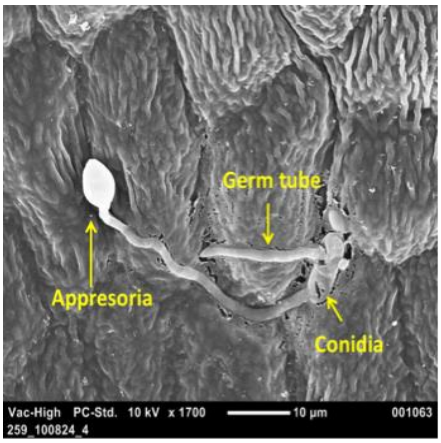
Close-up of woody tissue inoculation. The point of inoculation is shown with an arrow and you can observe dark necrotic vascular tissues developed from it.



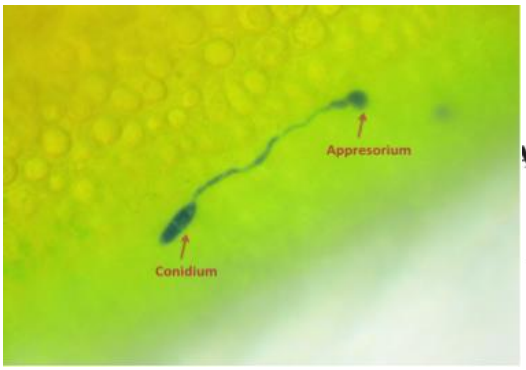
Objective 4) Infection by ripe rot pathogens without symptom development

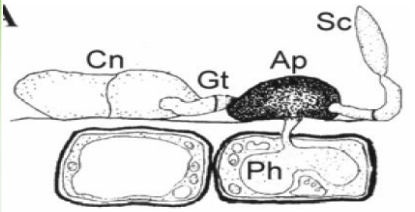
We used light microscopy and scanning electron microscope (SEM) to investigate various stages of infection process during infection period. Infection by spores on leaf tissues, flower tissues, and young berry tissues were examined. For flower tissues, the invasion of flower part, especially in pistil 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.

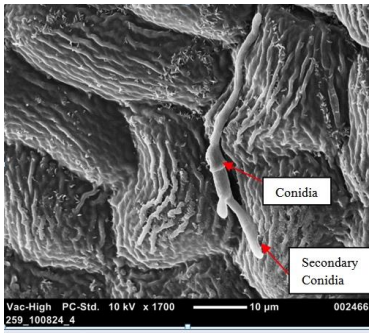


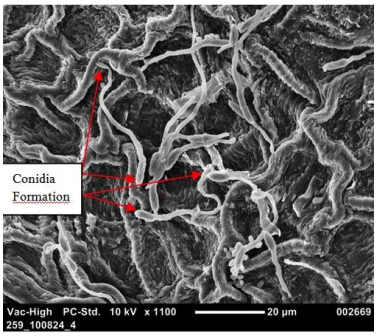


We used light microscopy and scanning electron microscope (SEM) to investigate various stages of infection process during infection period. Infection by spores on leaf tissues, flower tissues, and berry tissues were examined. For flower tissues, the invasion of flower part, especially in pistil was examined. For berry tissues, inoculation was made at bloom, BB-size, Pea-size, bunch closure, veraison and two weeks after veraison.

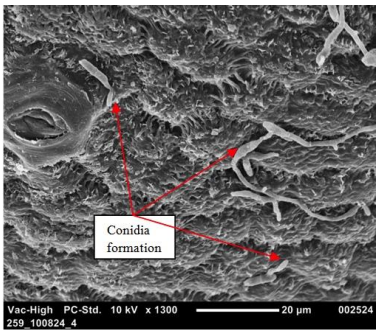
The preliminary results indicated that (1) *C. acutatum* and *C. gloeosporioides* can cause infection on the flower tissues, leaf tissues, and berry tissues (bloom, bb-size, pea-size, bunch touch, veraison and two weeks after veraison), (2) Conidial germination, appressorial formation and secondary conidiation 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.

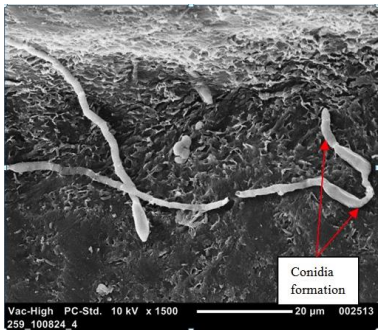




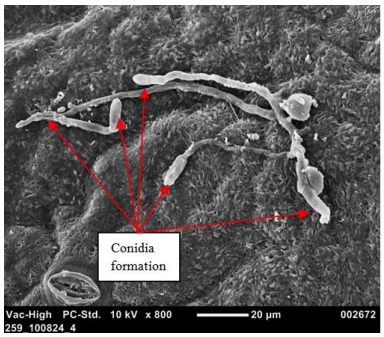
Cg-secondary conidia produced by primary conidia Ca-secondary conidia produced by hyphal phialides



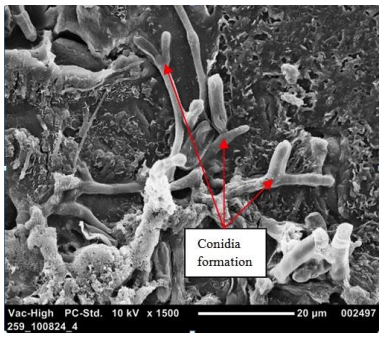
Ca- secondary condia on the flower surface



Ca- secondary condia on the berry surface

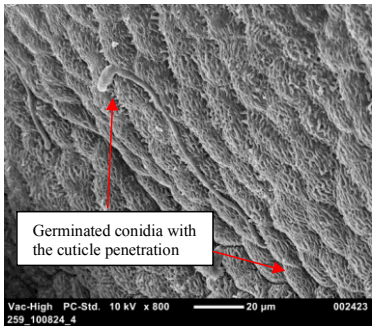


Cg- secondary condia on the flower surface



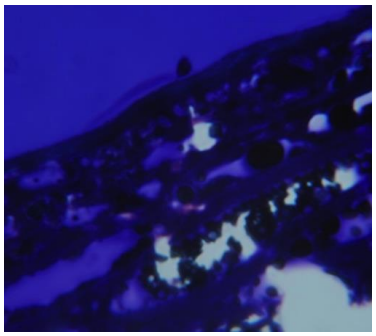
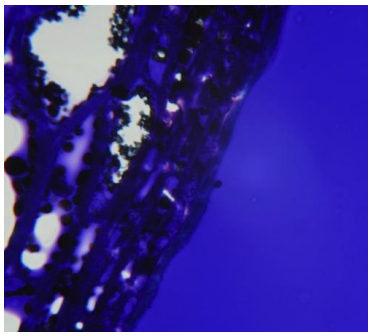
Cg- secondary condia on the berry surface

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.

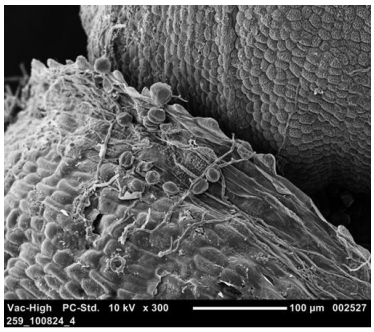


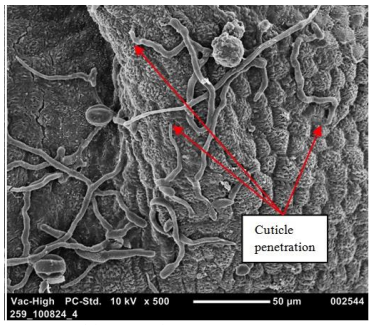
Cg-bloom- 48hr (SEM)

Cg-bloom-72hr (light microscope)

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





Ca-bloom- 48hr

To further explore the infection process of *Colletotrichum* species in 2016, berries were inoculated at BB/pea-size, berry touch and veraison. Berries were harvested after 24 hrs, 72 hrs, 1 week and 2 weeks after inoculation (5x10^5 spores/mL). Additionally, leaf inoculation experiments were also repeated (3, 6, 12, 18, 24, 48 and 72 hrs). By increasing the harvesting intervals, the location of the mycelia after infection and within the berry tissues can be observed. Samples collected during the growing season were immediately fixed and prepared for imaging. All samples were stained with an osmium tetroxide and uranyl acetate (1%) solution, dehydrated and stored in 100% ethanol for SEM or embedded in Embed 812 resin for light microscopy and transmission electron microscopy (TEM). Samples will be imaged during Fall/Winter 2016/2017 at the OSU Molecular and Cellular Imaging Center (MCIC) in Wooster, Ohio.

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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. Our lab also presented a poster at our national meeting in Pasadena, CA in Aug. 2015.

Presentations provided by our group in 2013-16~~5~~

Amanda Bly and Mizuho Nita (2015) "Screening for QoI Resistance Among Several *Colletotrichum* Species Associated with Ripe Rot of Grape Found in VA Vineyards", Cumberland-Shenandoah Fruit Worker's Conference, 3 December 2015

Oliver, C. and Nita, M. "Investigation of wine grape cultivar and cluster developmental stage susceptibility to grape ripe rot caused by two fungal complexes, *Colletotrichum acutatum,* and *C. gloeosporioides,* and evaluation of potential controls" Thesis Defense, Blacksburg VA, 12 June 2015

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 Spring 2016 semester. She has continued into her PhD studies under my supervision, working more on ripe rot complex and potentially expanding into further fungicide sensitivity studies. She began her PhD coursework during the Fall 2015 semester in which she completed 12 credit hours of additional coursework such as Experimental Design and Academic Integrity. She ~~should~~ will complete the coursework for her PhD in the Fall of 2016.

II. Problems and Delays

We found out that this fungal species are prone to suffer from contamination issues. Unfortunately, contamination tends to lead poor production of spores. We have been spending more than expected time to constantly cleaning up our fungal cultures. Unfortunately, we lost the experiment in the objective 3. We will repeat it in 2016.

III. Future Project Plans

Objectives 1: Preparing for a publication
Objectives 2 and 5: We will repeat the AB assay to complete the objectives
Objective 3: We will repeat the experiment in 2016
Objective 4: We will continue investigating ripe rot infection process using light and SEM microscopy techniques in 2016 season. We will screen more transformed isolates to select several *C. acutatum* and *C. gloeosporioides* isolates that contain GFP reporter gene in the 2016.

IV. Funding Expended To Date

We have utilized 100%~~about 50%, with an exception of wage and tuition, as of 29 December 2015~~.