

**Final report to the Virginia Wine Board, FY-2013  
#13-1748-02**

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

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<b>PRINCIPAL INVESTIGATOR:</b>	<b>COLLABORATOR:</b>
<b>Mizuho Nita, PhD</b> Grape Plant Pathologist AHS Jr. AREC; Virginia Tech 595 Laurel Grove Road Winchester, VA 22602  Phone: (540) 869-2560 Ex33 FAX: (540) 869-0862 Email: <a href="mailto:nita24@vt.edu">nita24@vt.edu</a>	<b>Chang-Hyun Khang, PhD</b> Assistant Professor Department of Plant Biology University of Georgia 1603 Miller Plant Sciences 120 Carlton Street Athens, GA 30602  Phone: 706-542-0461 FAX: 706-542-1805 Email: <a href="mailto:ckhang@plantbio.uga.edu">ckhang@plantbio.uga.edu</a>
<hr/> <b>Personnel</b> Ms. Amanda Bly and Ms. Sabrina Hartley (supported by this grant) Ms. Charlotte Oliver (MS student, supported by VT)	

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**OBJECTIVES:**

- 1) Determine the optimal environmental conditions (temperature, wetness duration, relative humidity, and grape cluster growth 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.

**Progresses made**

**Objective 1 Determine the optimal environmental conditions (temperature, wetness duration, relative humidity, and grape cluster growth stage) for the 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 (variety, Petit Manseng) to examine the infection process. 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 washed for 1 min 30 sec in distilled water. Berries were dried under a transfer hood (air will be filtered through a HEPA filter), and placed on to a tray that supported the berries to 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 quarter-strength potato dextrose agar for a week at 30°C with a diurnal light cycle for spore production. After 7-

10 days, the surface was flooded (~5mL) with distilled water and then brushed with a bent glass rod to suspend the spores; the suspension was then filtered through four layers of cheesecloth. The concentration of spores was adjusted using a hemacytometer to  $1 \times 10^5$  spores per ml. A drop of Tween 20 (200 $\mu$ L) is added to the diluted suspension help break the surface tension before use.

**Inoculation of detached berries:** A drop of 5  $\mu$ l spore suspension was placed onto the surface of the surface sterilized berry, and then placed in a sealed the 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 30°C. Daily observation of berries was made and visual assessment of disease incidence was made at 5, 7, 10, and 14 days after inoculation.

**Inoculation of potted vines:** In the 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 spore suspension that was prepared as described above using a hand atomizer to clusters until run-off. Due to the limited availability of vines, *C. acutatum* was only used to inoculate Cabernet sauvignon and Chardonnay clusters. 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 30°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).

**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. Five clusters were randomly chosen on the vines of Cabernet franc 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). For each variety, a separate cluster was also chosen to serve as a control to provide a sample of naturally occurring *Colletotrichum* in the field. A spore suspension was prepared as described above and was applied with a hand atomizer to clusters until run-off in the afternoon to reduce the time that the clusters are in the bags in sunlight. After the 24-hr period, the bags were removed and each cluster tagged for future reference. The visual assessment of disease was done weekly. Disease incidence and severity (percent of berries infected per cluster) were recorded at the end of the season 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. The datasets were examined using PROC MIXED of SAS 9.2.

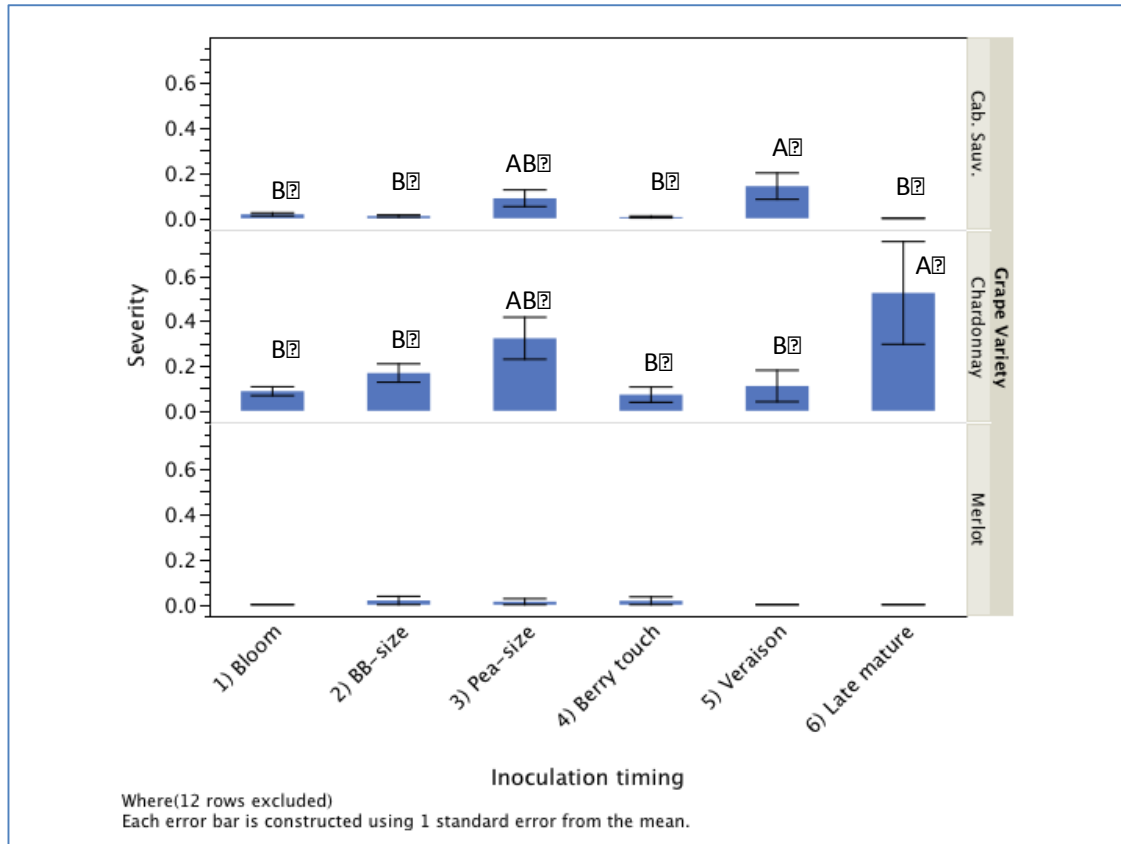
## Results

**Detached berry assays:** In order to determine the effect of temperature and wetness, we used a wine grape variety, 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. We will repeat the experiment with table grape and more susceptible wine grape (e.g., Chardonnay) in 2014 season. Also, we are currently conducting detached berry experiment using table grapes.

**Controlled environment study:** Results from 2013 study showed disease incidence was significantly affected by variety ( $P < 0.0001$ ) and species ( $P < 0.0012$ ) effect, but effect of the timing of infection was not significant ( $P = 0.1$ ). Disease severity, was affected by all three factors ( $P < 0.001$  for all). *C. acutatum* resulted in significantly higher disease incidence and severity ( $P < 0.05$ ) than *C. gloeosporioides*. Chardonnay resulted in significantly higher disease incidence and severity ( $P < 0.05$ )

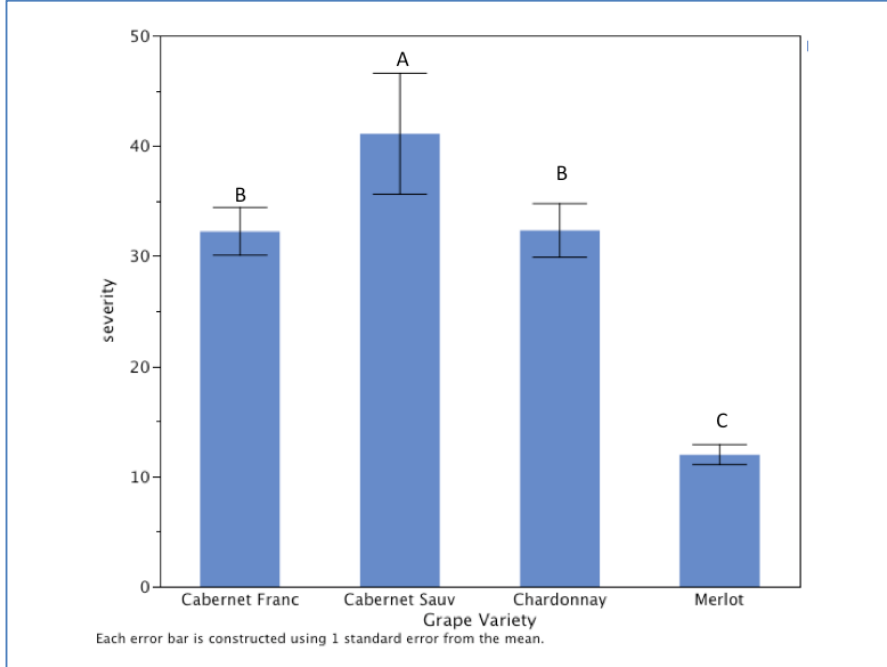
than Cabernet sauvignon and Merlot. As with 2012 results, earlier inoculation (from bloom to BB-size) resulted in lower disease severity than the inoculation after pea-size, but in terms of disease incidence, there was no significant difference among timing. When we compared disease severity for each variety, there was a peak at veraison for Cabernet sauvignon, and a peak at late harvest for Chardonnay (Fig. 1).

**Figure 1.** Effect of inoculation timing on ripe rot disease severity on Cabernet sauvignon, Chardonnay, and Merlot in a controlled environment study.

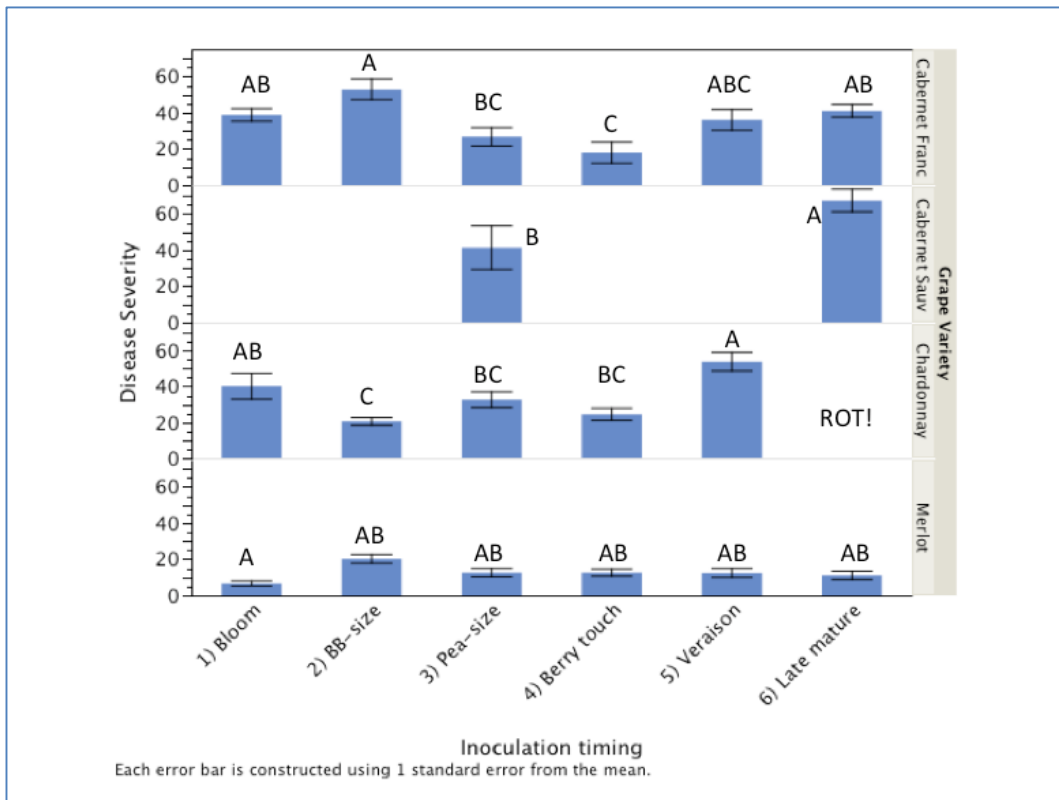


**Field inoculation:** Results from 2013 study showed that significant variety ( $P < 0.0001$ ), Application timing ( $P < 0.0005$ ), and isolate ( $P < 0.0015$ ) effects on the development of the disease (disease severity = percentage of infected berries over total number of berries on the cluster). The difference in the isolate was simply between untreated control and two isolates of *Colletotrichum* we used. We did not find significantly different disease severity between *C. acutatum* and *C. gloeosporioides*. Differences among varieties showed that Merlot resulted in significantly lower disease severity ( $P < 0.05$ ) than other varieties. Disease severity on Cabernet sauvignon was significantly higher than that of Chardonnay or Cab Franc (Fig. 2). However, the results from Cabernet sauvignon should be considered as a supplemental because there were only two time points for Cabernet sauvignon due to lost berries.

**Figure 2.** Variety effect on the development of ripe rot in the field, berries are inoculated at the different time point during the season.



**Figure 3.** Effect of inoculation timing on ripe rot disease severity.



When we compared the effect of timing of infection, there were some differences observed (Fig. 3). 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 late mature 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.

**Summary:** As we found in 2012, 2013 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 varieties. 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. Interestingly, the trend was similar in 2012 where we suspected heat in the hoop house as a cause of low disease severity in early part of the season. This year, 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 variety effect observed. In both controlled environment and field study showed that Merlot is less susceptible to ripe rot than Chardonnay. Cabernet sauvignon was not significantly different from Merlot in the controlled environment study, but it was different from Merlot in the field study. The other notable variety 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:** The hoop house has been protected with a shade cloth since the mid-season, and we will continue the protection in 2013 season. We will use the same type of shade cloth to cover the inoculated vines during the inoculation process to reduce the heat accumulation in the bag as well. By protecting the vines from overheating, we are expecting to see more disease development throughout the season. We will repeat the experiments in 2013 season to show the reproducibility, and in addition, we will test several candidate fungicides so that we can find the best material to protect susceptible grape clusters from the ripe rot infection.

For the 2014 season, the field experiments will be repeated and expanded to include a section of the Cabernet Sauvignon vineyard. We will also expand the growth chamber experiments to include temperature and wetness duration into the current experiment on growth stages. The temperature range and wetness durations will be based off of detached table grape assays that will be done this winter. Also,

modifications to the plastic and shade cloth for the hoop house will be made. The shade cloth helped with the heat issue but the internal temperature of the hoop house still reached temperatures above 35°C multiple days over the summer. Another factor is



**Figure 4** The hoop house with the new cuttings with new leaves and the full irrigation system in place

that we feel that the shade cloth may be effecting the ability of the fungi to initiate the symptom development by reducing the quantity of light available since it is generally accepted that the fungi require strong light for sporulation.

**Preparation for 2014 season:** In the 2013 season, approximately a thousand Chardonnay cuttings were potted in preparation for experiments in the spring of 2014 (Fig. 4). Approximately 25 cm long cuttings were taken from experimental vineyards in AHS AREC, the bottom part of the cutting was dipped into a rooting hormone (Rootone, 1-Naphthaleneacamide 0.20%, Thiram 4.04%, GardenTech, Palatine, IL), and then the cutting was placed into a container containing vermiculite. The container with the cuttings was placed on a heating pad to retain the soil temperature to be 25°C. After 6-8 weeks, each cutting was planted in a plastic pot. The pot was filled with a mixture of top soil, peat moss, and perlite in a 7-2-1 mixture, and Osmocote (N-P-K 14-14-14, Scotts Miracle-Gro, Marysville, OH) at the beginning and end of growing season. Since such a large number of plants are required for these experiments, an automated irrigation system was installed during the middle of the growing season. This system delivers water directly to the soil surface of each pot to reduce the incidence and spread of diseases and is easily modified to change as the number of plants rises and falls. Note: Unfortunately, the severe winter in 2014 damaged many vines in the hoop house, and resulted in termination of this particular experiment. We will plant another set of vines in 2015 so that we can continue our objectives in 2016.

## 2) Screening of fungicides for ripe rot control

Detached berry fungicide assays were performed to screen 10 commercially available fungicides using detached berries of wine grape (Petit Manseng) and table grape (Autumn King). Prior to the experiment, each berry was surface sterilized by washing with tap water, dipped into 10% Clorox solution for 1.5 min, and then rinsed with sterilized water for three times. Then the pedicel end of the berry was sealed with hot wax to prevent berries from desiccation. In each experimental run there were 4 replications per treatment, and 3 berries per replication. Experimental factors included the 10 commercially available fungicides (Table 1), a *Colletotrichum* inoculated check and water inoculated check. Fungicides were applied using an airbrush, and inoculation was made with a drop of either *C. acutatum* or *C. gloeosporioides* spore suspension (adjusted to  $5 \times 10^5$  spores per ml of water). There were at least 4 hours between the application of the fungicide and the inoculation. After inoculation, berries were kept under high humidity condition for 24 hours, then dried for incubation. Disease assessment was made at 3, 5, 7, 10, 12, and 14 days after inoculation. Each species of *Colletotrichum* (*acutatum* and *gloeosporioides*) were analyzed separately using ANOVA (linear mixed model, SAS 9.3).

Disease incidence varied from 0 to 43% and 0 to 36% on table and wine grape, respectively. The effect of Fungicide treatment was significant with *C. acutatum* on wine grape; however, it was due to the fact that inoculated check resulted in high disease incidence. All the other treatment resulted in near 0% disease incidence. With *C. gloeosporioides*, none of treatment was significantly different, and there was very few berries with symptoms. Thus, the wine grape of choice, Petit Manseng, seemed to be very resistant to ripe rot. For table grape, treatment effect was significant on both *C. acutatum* and *C. gloeosporioides* (Table 2).

Preliminary results demonstrated how the different species, *Colletotrichum acutatum*, and *Colletotrichum gloeosporioides*, react differently to the same fungicide (Fig. 5). Response to the treatment depended not only on the fungicide, but also on the fungal species. Insensitivity to QoI fungicides was also demonstrated in our results. Initial findings suggest that DMI group fungicides have significant control of *Colletotrichum acutatum* as well as *Colletotrichum gloeosporioides*.

The experiment has been run a total of 4 times thus far, and the results of the first two runs were discussed in this report. The last two experiments were just finished and we are still working on the data. Once we identified potentially effective fungicide groups, we will examine whether there are variations in efficacy among chemicals in the same mode of action.

**Table 1.** A list of fungicides to be examined with detached berry assays.

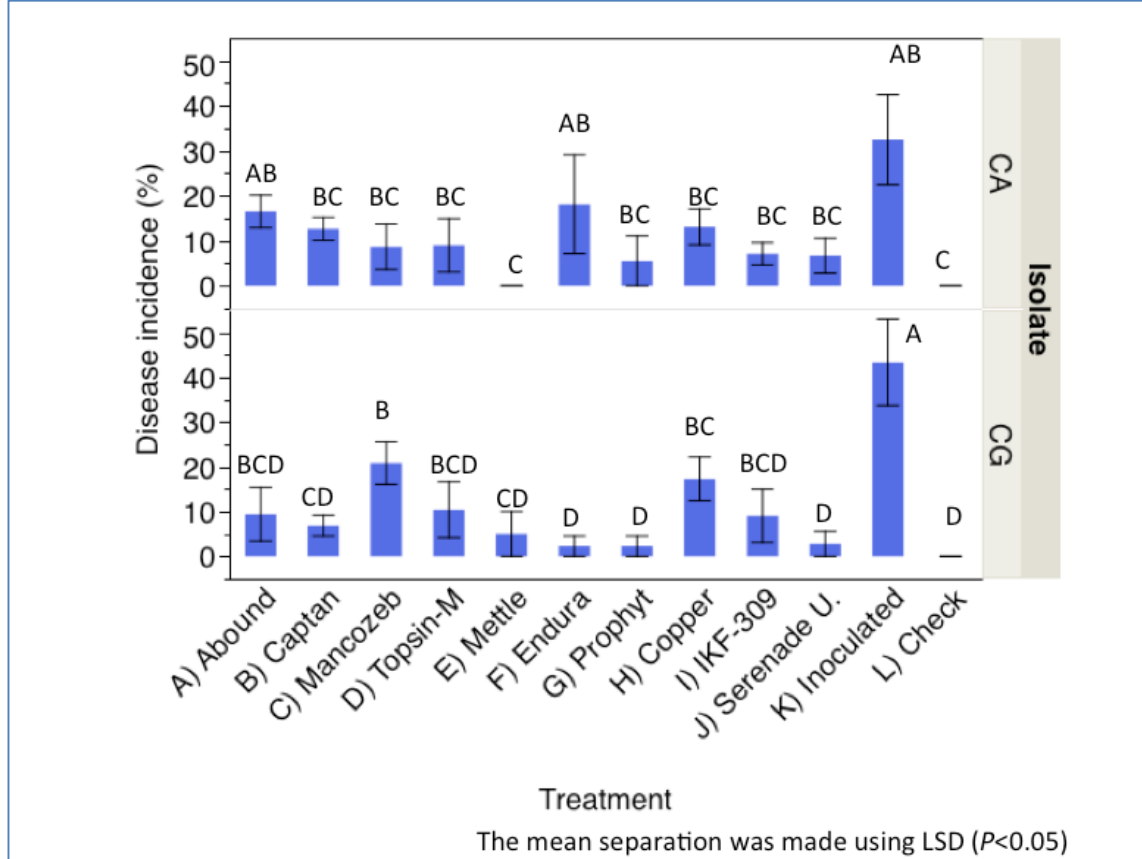
Commercial name	Active ingredient(s)	FRAC code (group name)	Comment
Penncozeb	mancozeb	M3 (dithiocarbamates, EBDC)	Currently recommended for ripe rot
Captan	captan	M4 (phthalimides)	Currently recommended for ripe rot
Basic copper	copper	M1 (inorganic)	
Rally	myclobutanil	3 (DMI)	Not many data are available for DMI against ripe rot pathogens
Inspire Super	difenoconazole + cyprodinil	3 (DMI) + 9 (AP)	
Abound	azoxystrobin	11 (QoI)	Currently recommended for ripe rot
Flint	trifloxystrobin	11 (QoI)	Currently recommended for ripe rot
Pristine	pyraclostrobin + boscalid	11 + 7 (QoI + SDHI)	Currently recommended for ripe rot
Endura	boscalid	7 (SDHI)	
Luna Experience	fluopyram	7 (SDHI) + 3 (DMI)	
Topsin-M	thiophanate-methyl	1 (MBC)	Similar to benzimidazoles that is effective against <i>C. gloeosporioides</i>
Prophyt	phosphorous acid	33 (phosphonates)	Some anecdotal comments on Colletotrichum control
Regalia	Extract of <i>Reynoutria sachalinensis</i>	P5 (plant extract)	Host plant defense inducer. Regalia treatment in a 2011 field trial showed lower incidence of general late season rots
Serenade MAX	<i>Bacillus subtilis</i> QST 713	44 (microbial)	Often recommended for Botrytis management

**Table 2.** ANOVA on detached berry analyses using wine and table grape. An asterisks next to the *P*-value indicates significant effect of fungicide treatment

Wine Grape			
Species	Source	<i>F</i> -value	<i>P</i> -value
<i>C. acutatum</i>	Treatment	3.5	<0.0001*
<i>C. gloeosporioides</i>	Treatment	1.0	0.5
Table Grape			
Species	Source	<i>F</i> -value	<i>P</i> -value
<i>C. acutatum</i>	Treatment	2.4	0.003*
<i>C. gloeosporioides</i>	Treatment	5.9	<0.0001*



**Figure 5.** Effect of fungicide treatment on ripe rot disease incidence on detached table grape, letters next to the bar indicate there was a significant difference between treatments ( $P < 0.05$ ).



### 3) Woody tissue inoculation

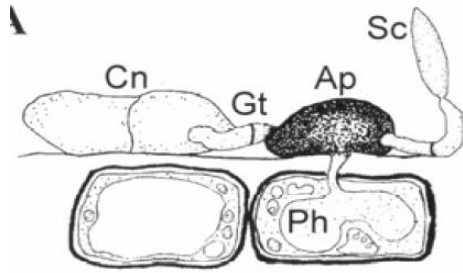
Results showed that there were development of necrotic lesions at the site of infection (sign of fungal activity), and we were able to re-isolate fungal isolates (both *C. acutatum* and *C. gloeosporioides*) four months after inoculation. The results indicated that these pathogen cannot only survives in woody tissue, but also able to cause disease. We will expand this research in 2013-14 to investigate longer-term effect of woody tissue infection, and vines are planted in January 2013.

### 4) Investigation of infection processes

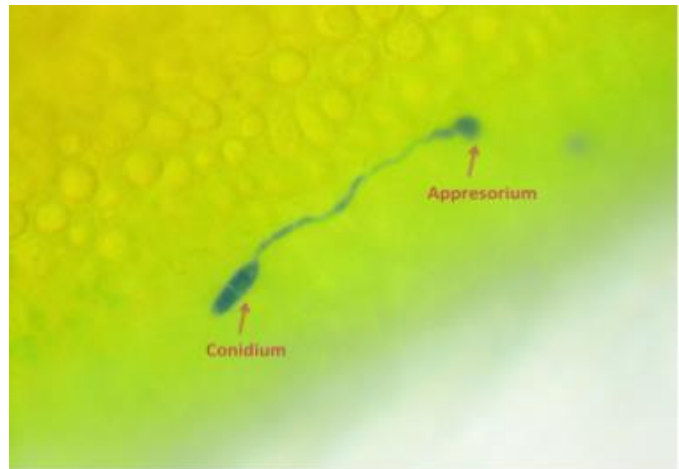
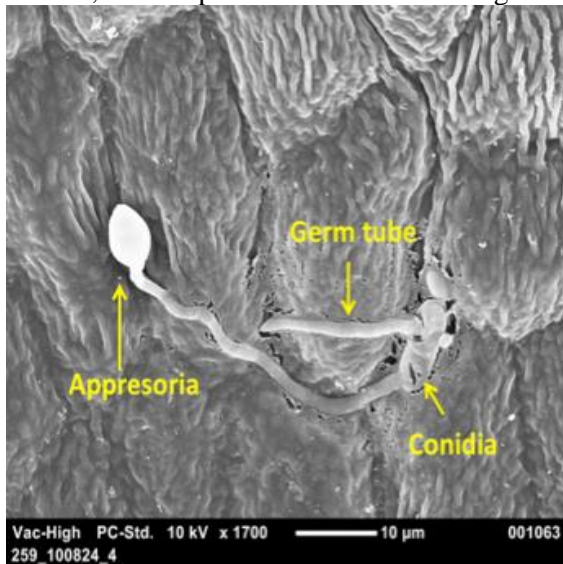
We used light microscopy and scanning electron microscope (SEM) to investigate various stages of infection process during infection period. Infection by spores on leaf tissues, flower tissues, and young berry tissues were examined. For flower tissues, the invasion of flower part, especially in pistil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

*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 is the appressorium, which helps to infect host plant for initial penetration





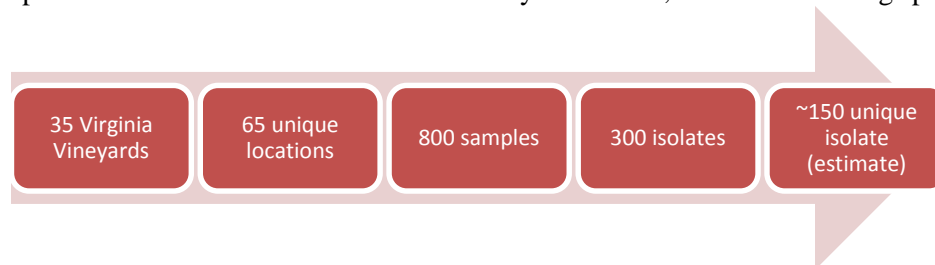
The following images are showing infections on leaf and pistil tissues, confirmed with our study. You can visualize germ tube developed from a conidium, and appressorium that formed at the end of the germ tube, indicating potential invasion to grape epidermal cells. The picture on the right shows germination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



We will be investigating more on the infection process to determine 1) whether leaf infection can result in spore formation, 2) how infection on flower parts result in rot, 3) whether difference between *Colletotrichum* species can be observed (= infection efficiency, etc.).

### 5.1) Investigation on QoI resistance

Originally, we were planning to focus only on detection of QoI resistance mutation gene among our ripe rot pathogens found in VA; however, recent study showed that what we believed as *C. acutatum* and *C. gloeosporioides* are actually two species complex with potentially 20+ species for each complex. The identification of species is very crucial for our disease management since some of species may exhibit different sensitivity to fungicide as we demonstrated in the objective 2. Also this may explain some of inconsistent results of fungicide efficacy tests in the past. Thus, we are also planning to identify species of *Colletotrichum* found in VA vineyards. Thus, we are conducting species identification study.



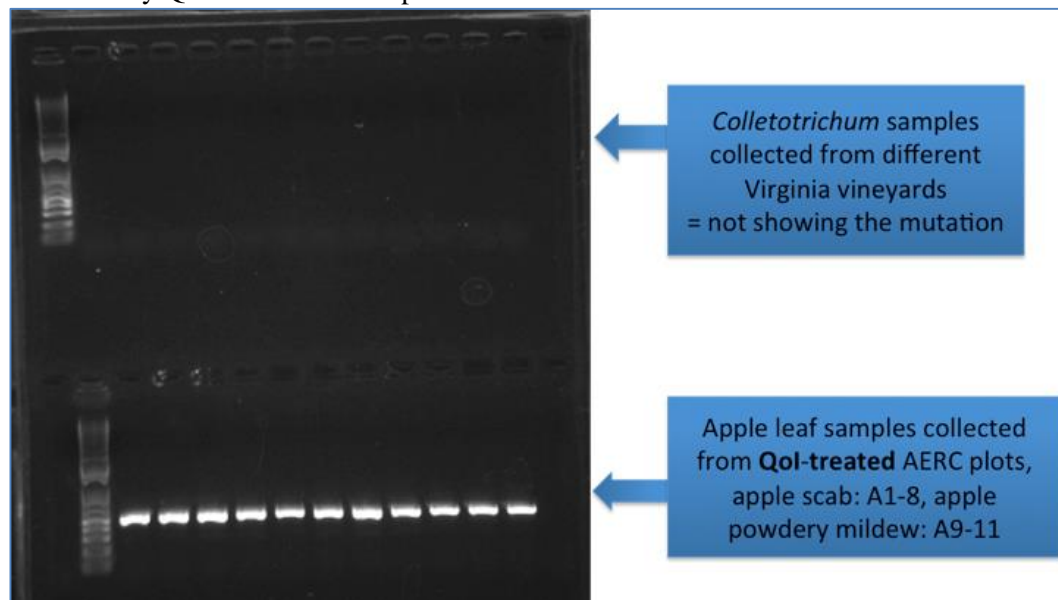
In 2012-13 seasons, our lab collected over 800 collect symptomatic ripe rot grape berries from 35 VA vineyards. In the lab, *Colletotrichum* species were isolated from infected grape berry skins. Then the tissue was placed onto ¼ PDA amended with antibiotic. Two consecutive transfers onto the antibiotic ¼ PDA were made to reduce contamination. Once clean colony is established, single spore isolation was made. The pure culture was stored on dry agar and into 20% glycerol solution for long-term storage. As of December 2013, we are estimating about 300 of our collections were either *C. acutatum* or *C. gloeosporioides* based on colony morphology and spore characteristics.

*Colletotrichum* species were identified using both morphology-based verification and DNA sequence-assisted identification. Based on the observations on colony morphologies among isolates from different locations (grower/vineyard/sample site) and crops, we are estimating to have approximately over 100 unique isolates in our samples. DNA sequence-assisted identification will be conducted by using a species-specific internal transcribed spacer region 1 (ITS1) primer and the conserved universal primer ITS4 encoded in the 28S ribosomal subunit in pairs. To confirm species identification, PCR products will be sequenced directly in both directions at the University of Kentucky Advanced Genetic Technologies Center.

### 5.2) Examination of fungicide QoI resistance of ripe rot pathogens in VA vineyards

Another issues we may encounter are fungicide resistance issue with these pathogens. Currently QoI and Captan are recommended for late season management, but as many of you are aware, QoI fungicides has a history of fungicide resistance issue. We used the PCR primers to amplify pathogen cytochrome b gene fragments from total DNA to look for G143A mutation, which is common among QoI resistant fungal pathogens. To test the primers, we did some preliminary evaluations on QoI-resistant apple scab and apple powdery mildew that collected from QoI-treated AERC plots. The results showed that the mutated cytochrome b gene was successfully amplified from resistant isolates (Fig. 6). We have processed other isolates using molecular approaches and sequence analysis to confirm G143A mutation, and found out that nearly 20% of our isolates were resistant to QoI. Using these potentially resistant isolates,, a bioassay using fungicide amended media to determine the level of resistance in 2014-15. In addition, we are also planning to conduct a bioassay to screen for captan resistance.

**Figure 6.** Demonstration of the primer to detect G143A mutation, which is very common among QoI-resistant fungal isolates. Bands on lower half of the picture indicate the isolates of apple scab pathogen from heavily QoI treated orchard possess this mutation.



### **Extension and Education**

We presented two oral reports at Cumberland Shenandoah Fruit Worker's Conference in December 2013, as well as the VVA meeting in February 2013. In addition, results from fungicide assay was presented at American Phytopathological Society's Potomac division meeting and Annual meeting in 2014. Also, results were discussed in various extension meetings. With supports from USDA's SCRI block grant, Virginia Tech's College of Agriculture and Life Science, and Department of Plant Pathology, Physiology, and Weed Science.

A master's student, Ms. Charlotte Oliver, has been working on this project. 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 2 and 3 for her program. In 2013, she completed her coursework for her Master's and presented her first departmental seminar. We are expecting that she will finish her degree in Winter 2015. She indicated that she is planning to continue for her PhD study under my supervision, working more on ripe rot complex and potentially expand into other grape pathogens. She recently won a student oral presentation competition at American Phytopathological Society's Potomac Division meeting.

We also invited a post-doctoral scholar (Dr. Binbin Lin) with a support from Virginia Tech's College of Agriculture and Life Sciences. She has been working on the histology, species identification, and QoI resistance projects.

### **III. Future Project Plans**

- 1) Determine the optimal environmental conditions (temperature, wetness duration, relative humidity, and grape cluster growth stage) for the two ripe rot pathogens; Continue table grape assays (Jan-March 2014), initiate potted vine assay (summer 2014)
- 2) Examine chemical management options; Continue fungicide assay (Feb-March 2014)
- 3) Investigate their capability to survive in woody tissues; Initiate inoculation (April 2014)
- 4) Investigate how infection process takes place in the absence of symptom expression; Investigate leaf and berry skin infection (March-April 2014), histology study using field samples (Summer 2014)
- 5) Determine a baseline sensitivity of ripe rot pathogens to various QoI fungicides. Screening of fungal species (April – June 2014), QoI resistance gene screening (April – June 2014)

### **IV. Funding Expended To Date**

We have utilized all the original allocation.