Final project report to the Virginia Wine Board, FY-2012

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

PRINCIPAL INVESTIGATOR:	COLLABORATOR:
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Personnel
Ms. Kathleen Kablonski (supported by this grant)
Ms. Charlotte Oliver (MS student, supported by VT)

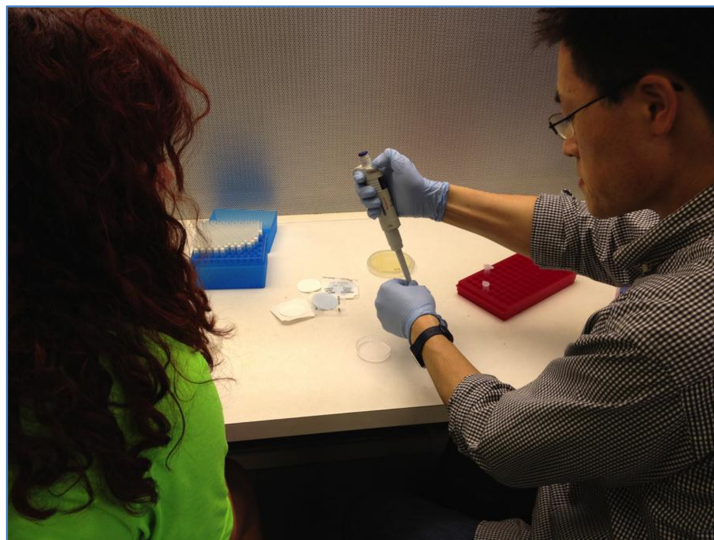
OBJECTIVES:

1. Transformation of *C. acutatum* and *C. gloeosporioides* isolates with the Green fluorescent Protein (GFP) reporter gene using *Agrobacterium tumefacience*-mediated transformation,
2. Confirm whether ripe rot pathogens can survive in woody tissue of grape,
3. Determine detailed berry infection conditions for ripe rot pathogens.

Progresses made
Objective 1)

Dr. Kang visited our lab May 3rd through the 5th, to help us set up the lab for genetic transformation of *C. acutatum* and *C. gloeosporioides* cultures (Fig. 1). He demonstrated methods of the ATMT (Agrobacterium tumefacience Mediated Transformation, described in the proposal) to the PI (Nita) and a lab assistant (Ms. Katie Yablonski).

Figure 1. Dr. Chang-Hyng Khang of University of Geogia (right) teaching ATMT to Ms. Yablonski (left)



In addition, our lab went through the IBC approval process in August to November of 2012 in order to safely handle recombinant DNA. Now our lab is certified as BL-1 facility, and all of our lab members went though various trainings. This legal step was necessary to handle recombinant DNA material such as GFP-transformed strain of ripe rot pathogens. We will keep working on improving transformation technics to create the isolate that is stable and biologically representative (i.e., it can cause infection). Once we identify the best candidate, we will commence our infection study to keep track of the movement of the pathogens within the grape tissues.

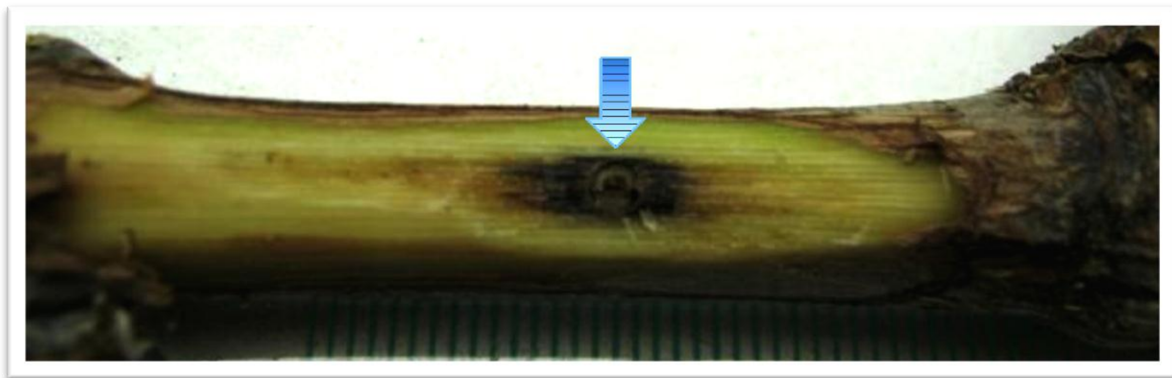
We also obtained a new fluorescent microscope (Nikon Eclipse Ci) through Virginia Tech's Equipment Trust Fund, which enable us to observe gfp-transferred fungal isolate within grape tissues. In addition, we have been working on light microscopy as well as Scanning Electron Microscopy techniques.

Objective 2

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. 2), 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 will expand this research in 2013-14 to investigate longer term effect of woody tissue infection.

Figure 2. 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 3

Materials and Methods

One of the issues we realized was inconsistent behaviors of *Colletotrichum* species we used in our experiment. The *C. acutatum* were able to produce spores on an artificial media; however, *C. gloeosporioides* often time does not produce spores. In order to address this issue, growth media trials for sporulation and table grape infections were performed. In the media trials, a series of eight media types were made from fresh fruits, such as grapes and strawberries, or following conventional media recipes such as potato dextrose agar. Single spore colonies were transferred to the center of the media under

sterile conditions and were then wrapped with parafilm to limit the loss of moisture while it incubated. Plates were incubated inside a surface sterilized moist chamber on a bench top (~28C) to receive approximately 12hrs of light a day. After a 1.5 weeks of incubation, the plates were flooded with 2 mL of sterile deionized water and then the surface of the colony was scratched with a sterilized scalpel. The resulting suspension was pipetted off and filtered through cheesecloth to remove debris. A single chamber in a hemacytometer was loaded with 12µL of the concentrated solution to get comparable a spore count for all eight of the media types for both species of *Colletotrichum*.

Several lab experiments were conducted using table grapes 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.

Culture of *C. acutatum* was grown on malt-extract agar for spore production. A single-spore culture will be grown on the medium for 3 weeks. After observing conidia developing from its fruiting structure (acervuli), the media plane will be flooded with approximately 20 mL distilled water for 2-3 min. The surface will be blushed with a paint blush to make spore suspend in the water, and the suspension will be filtered through four layers of cheesecloth. The concentration of spore will be adjusted using a hemacytometer to 10^5 spores per ml.

A drop of 20 µl 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. The berries were left in a moist condition for 2,4,8,12,18,22,24,26, or 30 hours at 15, 20, 25, 30, or 35°C. At each time point, 5 berries were removed, dried and then placed in a dry chamber to be observed for 14 days Daily observation of berries will be made and visual assessment of disease incidence was made at 7 days after inoculation.

In the greenhouse study, a small number of potted grapevines of three varieties (Cabernet sauvignon, Chardonnay, and Petit Verdot) with fruit clusters were inoculated with a culture of *C. acutatum* in the 2012 season. The timing of inoculation will be based on cluster growth stage. At each growth stage, there were two vines per variety used. Infections were made at bloom, bb-size, pea-size, bunch closure, veraison, and 2-weeks after veraison. Prior to the inoculation, these vines and a container with 500 ml of distilled water will be placed in an environmental growth chamber (Model E75L1, Percival Scientific Perry, IA) with a set temperature (25C in this experiment) 10-12 hours prior to the inoculation in order to make both leaves and water for spore suspension to reach target temperature. The spore suspension was prepared as described earlier in the table grape procedure and adjusted to 10^5 spores/ml. It was sprayed onto the clusters with a hand atomizer to run-off, and then the clusters were covered with a zip-lock bag with a piece of wet paper towel to maintain the humid conditions. The vines were then returned to the growth chamber, and kept under 25C with light for 24 hours for infection. After the infection period, zip-lock bag was removed from the cluster, and the vines were maintained in the hoop house where vines are protected from precipitation. Visual assessment of disease incidence and severity will be made weekly until the end of the season (when Brix level > 20-25). The datasets will be examined using PROC MIXED of SAS 9.2, considering cluster growth stage and variety as fixed factors and pot as a random factor.

A sections of Merlot and Chardonnay vineyards at AHS AREC was used for field experiments. All vines have been trained in the vertical shoot positioning system, and each vine will be trained to bear 20-24 clusters. Within a variety, ten clusters will be randomly selected and sprayed with spore suspension

(10^5 spores/mL) of *C. acutatum*, prepared similarly to the above suspensions but a drop of Tween 20 was added to reduce the surface tension. The suspension was then applied with a hand atomizer to clusters until run-off. After inoculation, each cluster was bagged for 24 hours to simulate an infection event. After the 24-hr period, the bags were removed and each cluster was tagged for future reference. Disease incidence and severity (% of berries infected per cluster) was recorded.

Results

There is a difference in between the two species of *Colletotrichum* in terms of spore production in artificial medium. For *C. acutatum*, malt extract agar provides the most conducive for the production of spores. However, for *C. gloeosporioides*, quarter-strength potato dextrose agar is the best medium for spore production. Also, we found contamination in our culture that required a clean-up using several antibiotics. Now we established clean cultures and stable spore production method.

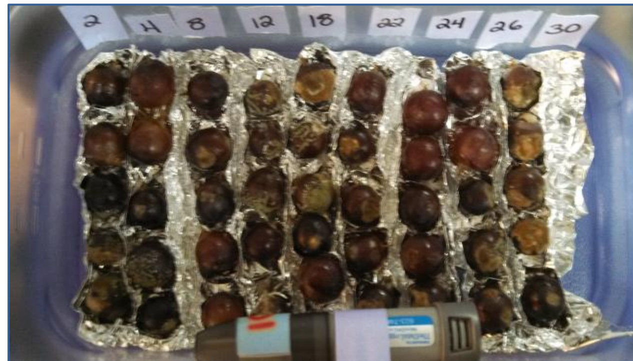
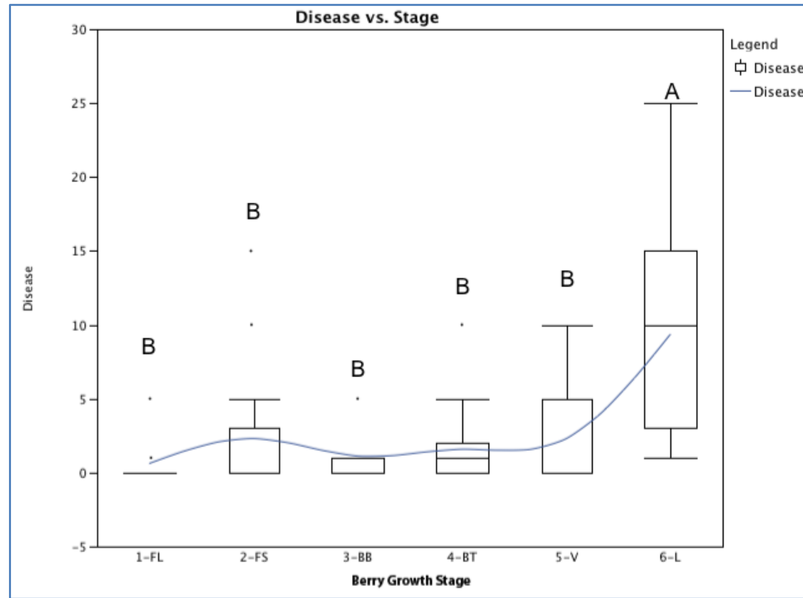


Figure 3 Detached-berry assay, berries are showing various degree of infection at 14-day after inoculation

In the table grape studies, we found a difference between two species on their optimal temperature range. *C. gloeosporioides* was found to flourish at 35C, while *C. acutatum* thrived around 30C. We also found that *C. acutatum* tended to be more aggressive than *C. gloeosporioides*, causing more berries to show higher disease severity with a fewer days after inoculation (Fig. 3). The tests we have done need to be repeated several more times to confirm the trends that have been found. The fungicide assays, potted plant and field inoculations are currently in progress.

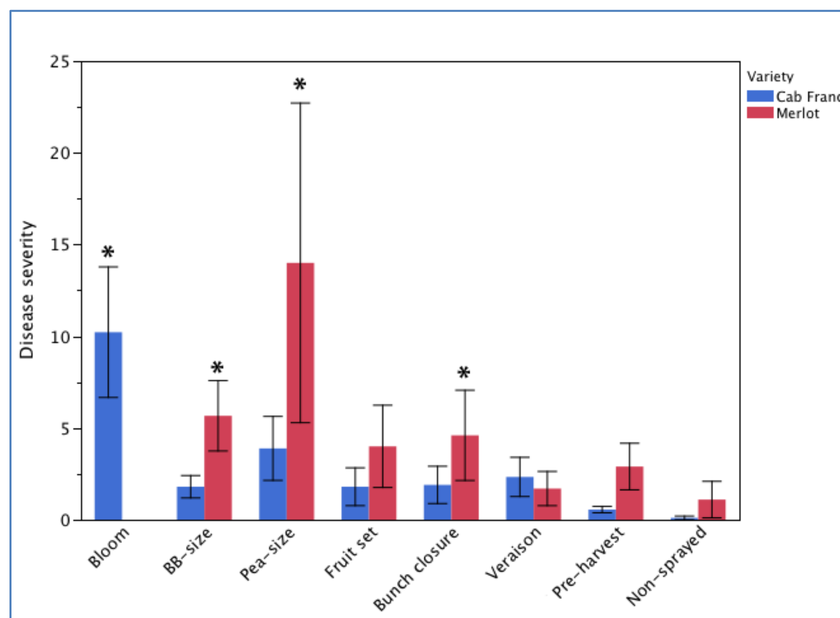
The greenhouse study results were analyzed using a linear mixed model (JPM 10, SAS institute, Cary, NC), where inoculation time ($p<0.0001$) was significant but variety ($p=0.16$) was not. All of the early inoculation disease severities were not significantly different from each other. Figure 4 shows that fungal invasion is possible at almost all cluster stages.

Figure 4. Changes in disease severity based on the timing of inoculation on cultivar Cabernet sauvignon grown two years in a pot, different letter indicates significant difference ($P < 0.05$) (FL=bloom, FS=Fruit set, BB=BB-size, BT=Berry-touch, V=Veraison, L=2-wk after V)



The field dataset was also analyzed using the linear mixed model and both variety (p=0.013) and inoculation date (p=0.002) were significant. The Fig. 5 shows the two peaks at bloom and BB-size to bunch closure, which are significantly higher in disease severity than the background infection level, indicating once again that the infection can happen anytime between bloom and harvest, and early season infection can result in high disease severity at the end of the season.

Figure 5. Changes in mean disease severity over different inoculation timings. Blue bar represents the variety Cabernet franc, red bars represent Merlot, and standard errors are shown as whiskers. An asterisk indicates that disease severity at that inoculation timing is significantly (p<0.05) different from the untreated check (listed as "Baseline" in the figure).



Summary: Results from 2012 suggests that the infection can 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 higher disease severity in the field earlier in the season while disease severity was higher in later in the season in the hoop house. The lack of disease development later in the season in the field condition can be explained with heat build-up in inoculation bags over the course of the day. The lack of disease development earlier in the season in the hoop house can be explained by a lack of shade cloth in the early season. Both of the pathogen is known to stop its biological activities when temperature hits above 35C.

Future direction: Obviously, the errors need to be fixed. 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.

Extension and Education

We presented oral reports at Cumberland Shenandoah Fruit Worker's Conference in November 2012, as well as the VVA meeting in February 2013. 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, now we have a master's student (Ms. Charlotte Oliver) who 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.