Final report to the Virginia Wine board
3 July 2013

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

PRINCIPAL INVESTIGATOR:	COLLABORATOR:
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A) 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.

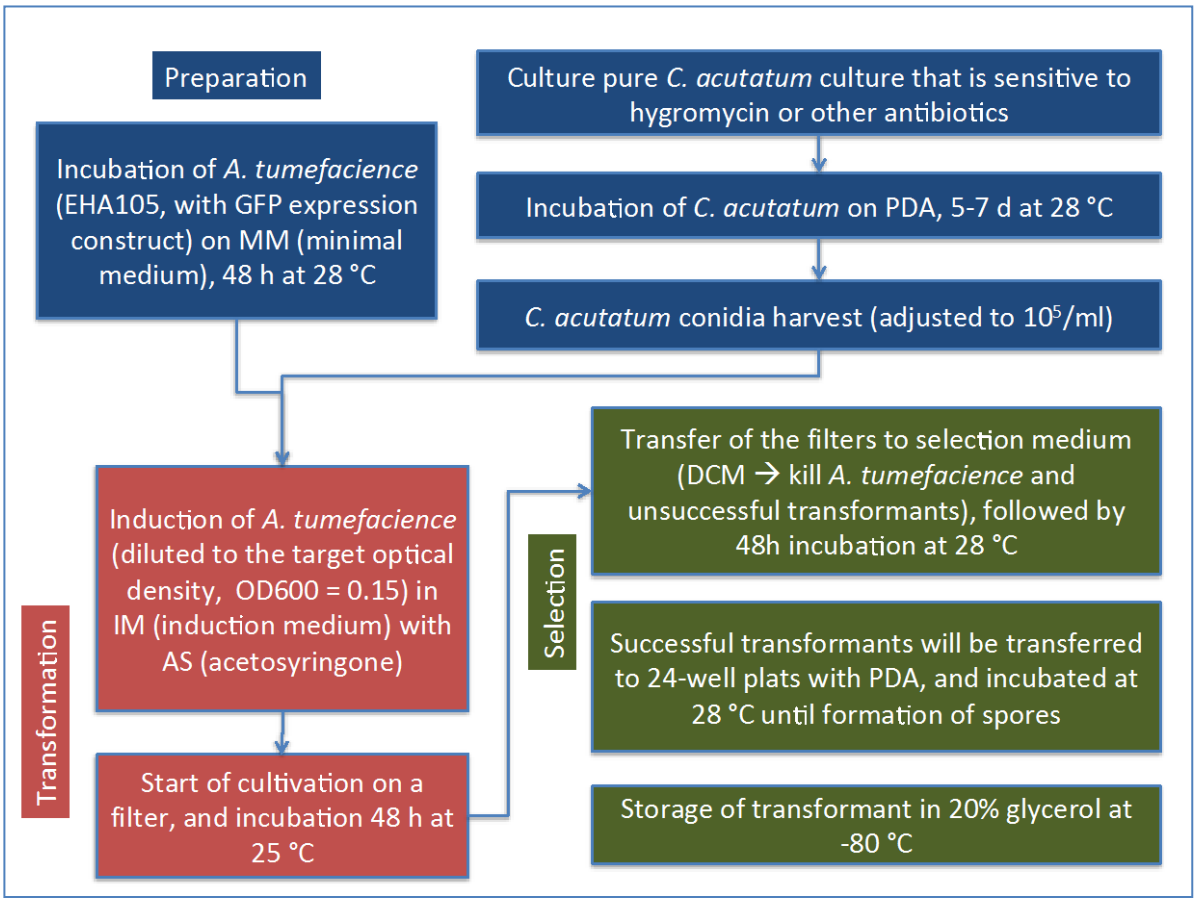
Current Progress

Objective 1) Transformation of *C. acutatum* and *C. gloeosporioides* isolates with the Green fluorescent Protein (GFP) reporter gene using *Agrobacterium tumefacience*-mediated transformation

- The goal of this objective is to create fungal isolates with a so-called reporter gene so that we can see the fungal hyphae in plant cells. The gfp-transformed fungal hyphae will glow under a fluorescent microscope. It will help us to observe their lifecycles better, and it is particularly important for ripe rot pathogens because we know so little about them.
- We have isolated candidate fungal isolates for both *C. acutatum* and *C. gloeosporioides*, and conducted an inoculation assay to fulfill Koch's postulates which are:
  - The microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organisms.
  - The microorganism must be isolated from a diseased organism and grown in pure culture.
  - The cultured microorganism should cause disease when introduced into a healthy organism.
  - The microorganism must be reisolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.
- We have been maintaining several isolates of *C. acucatum* from single spore isolation in order to establish a line of pure culture. These isolates will be the subject for the transformation
  - Single spore isolation of *C. gloeosporioides* will be made soon. We are currently investigating the best method to make the isolate sporulate on artificial medium

- We had a meeting with Dr. Khang, who recently moved to University of Georgia as a faculty member.
    - We discussed details of transformation techniques (Fig. 1) at the meeting
    - Currently we are in process of fine tuning the procedures for the transformation, which are; maintain pure culture, test for anti-biotic resistance level of pure isolates, and prepare *A. tumefacience* culture with GFP and other reporter genes.
- In 2013-14, we will focus on light microscopy as well as Scanning Electron Microscopy to see if these methods can yield results that can help us using gfp-transformant.

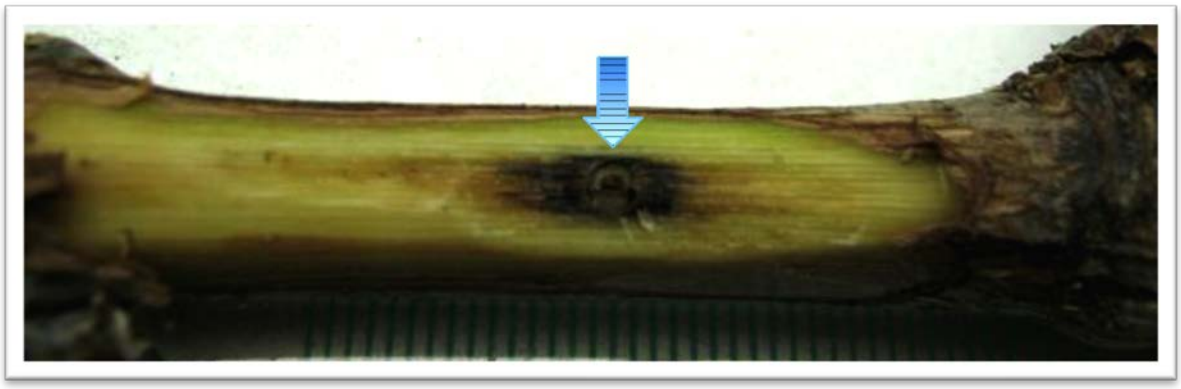
Figure 1. The protocol of *Agrobacterium Tumefacience*-Mediated Transformation (ATMT) on *Colletotrichum* species



Objective 2) Confirm whether ripe rot pathogens can survive in woody tissue of grape

- We conducted a preliminary experiment in the fall of 2012. The results 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*) four months after inoculation. 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) Determine detailed berry infection conditions for ripe rot pathogens.

- To fully understand how the pathogen grows, we have started a series of three different experiments: detached berry assays, field inoculations and potted plant inoculations.
- The detached berry assays have been performed to find the optimum growth conditions of the pathogen and its fungicide sensitivities on a smaller scale and with a shorter incubation time.
    - For the growth conditions:
        - 45 table grapes were removed from the rachis with the pedicle still attached. The grapes were then disinfested in 10% bleach solution to reduce the post-harvest pathogens and remove resides for a minute and a half and then rinsed with sterile water.
        - The berries were allowed to dry and then the pedicle ends were waxed before placing the berries into trays in sealable containers.
        - Inoculations were performed by placing 20uL of a $1x10^5$ /ml spore suspension of either *C. acutatum* or *C. gloeosporioides* which were dropped onto the surface of the berry.
        - 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 (Fig. 3)
    - For the fungicide sensitivity:
        - A list of more than 20 commercial products was compiled based on common vineyard applications and prior work.
        - For each fungicide tested, 40 berries were prepared the same as described above and then sprayed with a mixture of the fungicide similar to what would be used in the field.
        - The berries were then dried overnight and then inoculated the same as above and placed in a moist condition for 24hrs at 28°C.
        - After 24 hours, the containers are allowed to dry and then left at 28°C to be observed for 14 days.
- The potted plant infections:
    - A varying number of clusters on 2 Petit Verdot plants, 5 Cabernet sauvignon, 2-3 pots of Chardonnay and two pots of Merlot were inoculated with *C.*

gloeosporioides and , clusters on 5 Cabernet sauvignon and 2-3 pots of Chardonnay with *C. acutatum.*

- The spore suspension was prepared as above and sprayed with a hand atomizer onto the clusters
- Clusters were bagged with a ziploc bags containing a wet paper towel to increase the relative humidity, and then the pots were placed in a growth chamber where temperature was maintained at 28°C with 12 hours light and dark for 24 hours

- Berries were inoculated at bloom, bb-size, pea-size, berry touch, bunch closure, veraison, and late maturity.
- Then inoculated vines were evaluated for development of disease over the course of the season

- For the field inoculations:
  - Ten clusters of Merlot, and 5 clusters of Cabernet sauvignon, Cabernet franc, and Chardonnay were inoculated per species at the same berry maturities as described above.
  - Clusters were bagged in a Ziploc with a wet paper towel for 24hours and then the bag was removed, allowing the clusters to remain on the vine to mature.

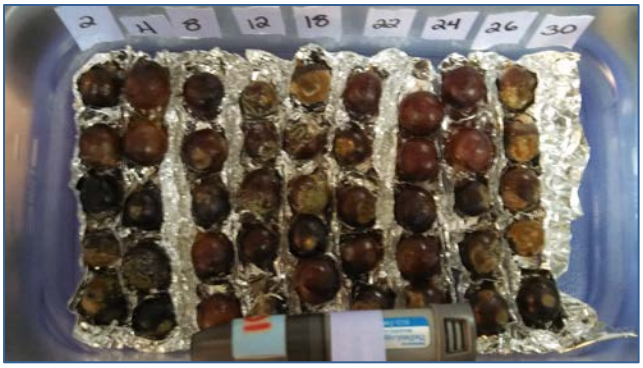


Figure 3. An image of a *C. acutatum* moisture assay container after 14 days of incubation.

Summary and future objectives

- In the fall and spring semesters, our Masters student (Ms. Charlotte Oliver) completed 23 credit hours of classes such as on Plant Pathogenic Agents, Pesticide Usage and Plant Clinic Experience.
- The table grape trials have begun to shed light on the differences between the two pathogens. From the current tests, we have found that *C. acutatum* tends to favor cooler conditions than *C. gloeosporioides* which was found to flourish at 35C. *C. acutatum* also tends to be more aggressive than *C. gloeosporioides*, causing more berries to be heavily infected with fewer incubation days.
- 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.
- For the field and potted plant inoculations, all of the results will be collected just before and during harvest when the fungi begin to show symptoms on the clusters.

More information about both of these pathogens has come to light recently in new studies suggesting that the species is a complex mix of multiple forms of the pathogen. Thus, our research will bring new findings to actual management options to this important disease

complex. In 2013 season, the detached berry assays have been conducted to lay the ground work for a more intense potted plant series of infections that will mirror what was done with the detached berries. This will provide a more controlled assessment of the pathogens' infection conditions than either the detached berries or field inoculations. The field inoculations will also be repeated to provide a comparison between normal vineyard conditions and the controlled conditions of a lab. The fungicide assays will continue through this summer and into the fall to cover all of the products desired with repeated experiments to create a clearer view of which products provide the best control for both pathogens and if the sensitivities of the pathogens vary.