FY2012 Final report to the Virginia Wine board
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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. Investigate on ripe rot disease cycle and determine whether modifications of the current management strategies can reduce the risk of future outbreaks.

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

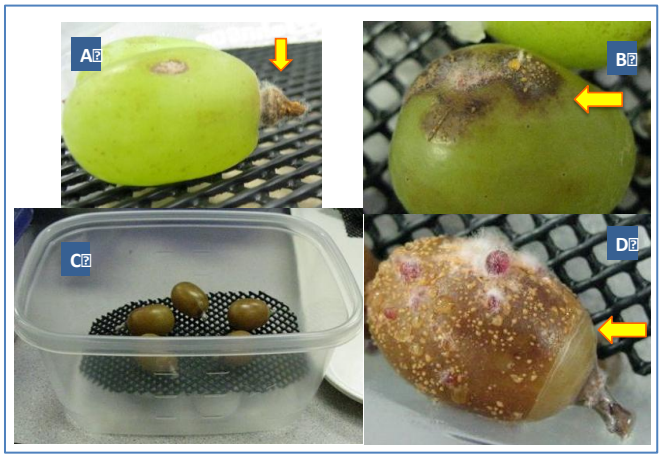
- The goal of this objective is to create fungal isolates with a so-called reporter gene that enables us to observe the fungal hyphae in plant cells. The gfp-transformed fungal hyphae will glow under a fluorescent microscope. It will help us to observe their lifecycles better, which is particularly important for ripe rot pathogens because we know so little about their infection processes. Especially when they cause latent infection where berries remain asymptomatic.
- 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:
  1) The microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organisms.
  2) The microorganism must be isolated from a diseased organism and grown in pure culture.
  3) The cultured microorganism should cause disease when introduced into a healthy organism.

4) 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. acutatum* from single spore isolation in order to establish a line of pure cultures. These isolates will be the subject for the transformation
  - For single spore isolation of *C. gloeosporioides*, we are currently investigating the best method to make the isolate sporulate on artificial medium
- Our collaborator, Dr. Khang of University of Georgia, visited Winchester AREC on 3 – 5 May 2012 to provide us demonstrations of fungal transformation (Fig. 3), and we created several candidates (please see the picture on the right).
  - We will resume transformation in this fall

Figure 1. Dr. Khang (right) and Ms. Yablonski discussing fungal transformation

Figure 2. Table grape inoculation assay: A) Unprotected natural opening at the base on the peduncle can be a point of entry; 2) With a wounding it can show symptoms within 10 days after inoculation; 3) Berries were kept dry in a chamber; and 4) After the cold shock treatment, symptom can appear within three days. Also note the effect of wax coating.



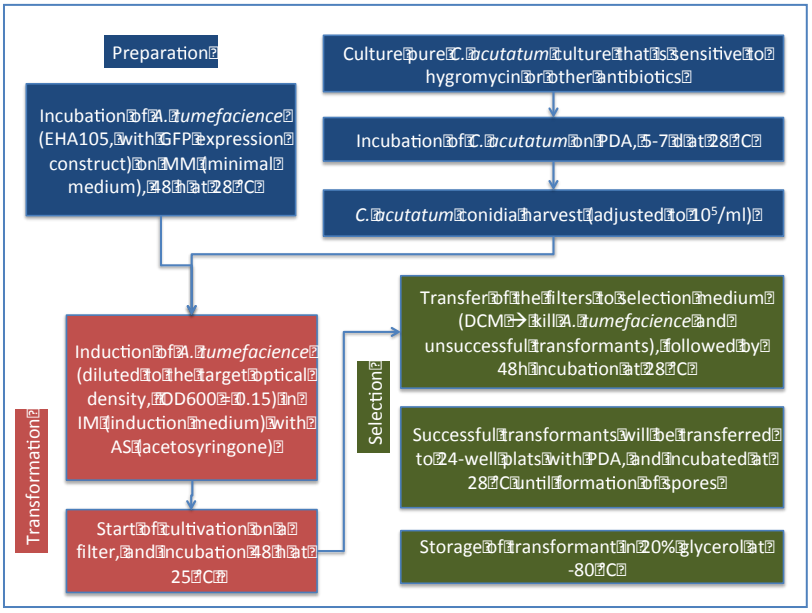
- We have been conducting an inoculation assay on a detached table grape berry. So far, we have finished several preliminary experiments, including two major experiments to determine a successful infection method.
  - Experiment 1 was conducted to determine whether inoculation could be done with isolates we have. We inoculated detached table grape berries with spore suspension (1 x 10^5 /ml, *C. acutatum*), and then leave them in a plastic bag for 7 to 21 days under room temperature (21-22C). After these incubation periods, berries are observed for disease development. If there were no symptoms, berries were placed into -80C conditions for 10 min to 1 hour (cold shock treatment).
    - After three replications, this experiment revealed that fungal isolates were able to infect berries and symptom can appear 10 days after

inoculation at the earliest. However, in the most of the time, berries remained asymptomatic.

- A cold shock treatment under -80C for 30 min helped break down of berry skin so that the fungus can develop symptoms.
- Also, the fungus was able to find natural opening at the base of a peduncle (Fig. 2, panel A), or wounds (Fig. 2, panel B). Thus, we tested several materials (pruning wound protection paste, liquid latexes, resins, wax, etc.) to seal the opening (Fig 2, panel D). We found that a double-coating with hot wax was able to efficiently prevent the infection.

- Experiment 2 was conducted to determine the length of wetness required for infection. Berries (with waxed peduncle) were inoculated with spore suspension, and then placed into a plastic bag for 6, 24, 48, and 72 hours under 25C. Then, berries are dried and placed into a chamber where berries are placed on a net that is sustained on a ½ inches base (Fig. 2, panel C). This chamber allowed air movement and prevented berries to touch each other. Berries were subjected to the cold shock treatment after 7 days.
  - The initial three experimental runs showed that 6 hours was enough to create a successful infection. (It was significantly shorter than what previous literatures reported.)
  - We will test with shorter wetness hours.

Figure 3. 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

- The experiment was initiated on mid-May. We prepared certified Chardonnay vines from the foundation planting service and also several Merlot vines from our field (where we have not observed ripe rot). Inoculation was made on 7 July 2012 by drilling a 5 mm hole into cane, place a piece of actively growing fungal culture in the

hole, and then the wound was covered with Vaseline and Parafilm (Fig. 4). These inoculated pots will be grown in a greenhouse. The assessment of disease will be done in October or November of 2012.

Fig. 4 Grapevine wood inoculation

Objective 3) Investigate on ripe rot disease cycle and determine whether modifications of the current management strategies can reduce the risk of future outbreaks.

- Initially, our aim was to test several candidate fungicides in the field; however, I have realized that we are missing the critical information to be addressed.
    - Simply, we do not have a clear understanding of when and how these pathogens can infect grape berries, thus we need to track their lifecycle.
    - Literature search revealed that although both pathogens were extensively studied with other crops, there were not many researches done with grape
- *Thus, I took a step back on the research objective,* and have been conducting inoculation assays a) in a greenhouse with potted vines and b) in the field.
    a) Four or five potted vines with a total of 10 clusters were inoculated with 5 x 10^5 /ml of *C. gloeosporioides* spore suspension
        a. The concentration was adjusted using a hemocytometer
        b. Spore suspension was mixed with a drop of Tween 40 (a surfactant) and sprayed with a hand atomizer
        c. Clusters were bagged with a ziplock bag (Fig. 5), and then the pots were placed in a growth chamber where the average temperature was maintained at 26C and constant light was provided
        d. Incubation time was either 24 hours or 72 hours
            i. Note: all previous inoculation study on grape was done with 72-hour incubation time; however, that kind of a long wetness period rarely happens in the nature.
        e. In 2011, berries were inoculated at fruit set, BB size, Pea size, bunch closure, Veraison, and 15-degree Brix with *C. gloeosporioides* spores.
        f. In 2012, berries are inoculated at bloom, fruit set, BB size, Pea size, bunch closure, and Veraizon with *C. acutatum* spores.
        g. Potted vines are placed in a greenhouse where a shade cloth was installed in order to keep temperature low.

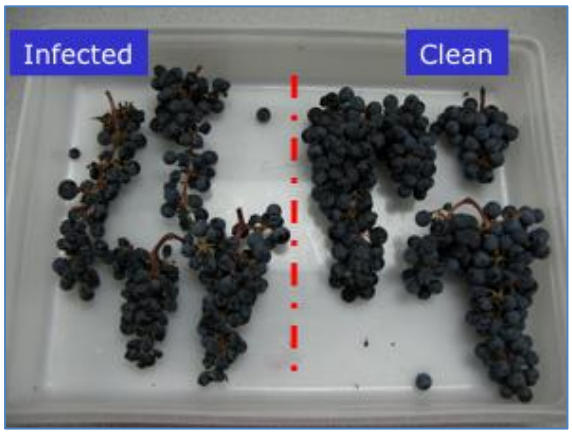
Figure 5. Field inoculation of Merlot vine

    b) In addition, we inoculated field-grown Cabernet Franc and Merlot berries at fruit set, BB size, Pea size, bunch closure and veraison during 2012 season.
        a. After inoculation, a plastic bag was placed on to the inoculated cluster and kept for 24 hours to ensure infection (Fig. 5). Inoculated vines have been evaluated for development of disease over the course of the season
- The results showed *C. gloeosporioides* successfully infected grape berries regardless of the developmental stage of the grape berries.

- Development of symptoms varied and it can be developed within 10 days after inoculation. However, it seems that almost all treatments, regardless of the timing of infection produced symptoms at the same time, indicating the role of grape maturity to the development of ripe rot symptoms.
- The trend has been similar in the field. A few berries showed symptom within 14 days of inoculation; however, others are remain asymptomatic, as we saw in a greenhouse assays.
- Latter inoculation seems to develop severe symptoms; however, the sample size is too small to determine conclusively.
- There is no "critical timing" as we discuss with cluster infection of downy mildew and other major grape diseases

- The severely infected clusters showed shrinking of berries without obvious discoloration on the rachis, indicating that the rot starts at the berry (Fig. 6). In some cases, we observed discoloration on peduncles (cluster stems), thus, the fungi may be able to move toward rachis.
- The results of 2012 inoculation assay will be reported in the next report, since data will be collected during later part of the growing season.

Figure 6. Comparison of severely infected clusters and clean clusters: infected clusters are showing shrinking of berries (i.e., berry shriveling). The picture on the right is a close-up of infected berry, which shows spore production (pinkish dots) on the surface of the berry.



Ripe rot on Cabernet Sauvignon berry

Summary and future objectives

We have successfully demonstrated that the pathogens of ripe rot can cause infection regardless of the growth stages of grape berry. The results also showed that it could cause not only brown lesions on the skin of grape berries, but also shriveling of berries. In addition, a series of lab experiment showed that this pathogen found natural opening of grape (or a wound) and cause infection through it. At the same time, even without the presence of the natural opening or wounds, they can cause infection, which indicates that these fungi have a structure called appresorium where they build a pressure to puncture grape tissues at the time of infection.

We have been working on the transformation of these pathogens so that we will be able to track their movement under the fluorescent microscope. This will add more insight on how the fungi survive (or thrive) during latent infection period. Based on studies on other crops,

they can either form a superficial infection and produce spores, or cause latent infection where it stays dormant to wait for environmental cues for them to become pathogenic. Once we observe what is happening during early part of the season, it will help us to determine when and how these pathogens establish themselves in grape tissues to cause ripe rot later in the season.

Currently, we are focusing to develop inoculation techniques so that we can develop a reliable inoculation method. In addition, we repeated 2011 experiment in a greenhouse and also in a field to confirm that the results. Next step will be an addition of a fungicide application to determine if any of products can stop infection effectively. We are planning to use our field plot for that purpose. We will also infect grape flowers to see if it can cause any latent infection as discussed with *Botrytis* spores.

This project has been an educational opportunity as well. Ms. Kathleen Yablonski, an Ag technician, has been hired since the winter of 2012, and she has been involved in many experiments in the lab and field. Also, Ms. Charlotte Oliver will join my lab in the fall of 2012 to start her masters. The preliminary results have been delivered to stakeholders at various meetings, including the VVA meeting and IPM workshops.