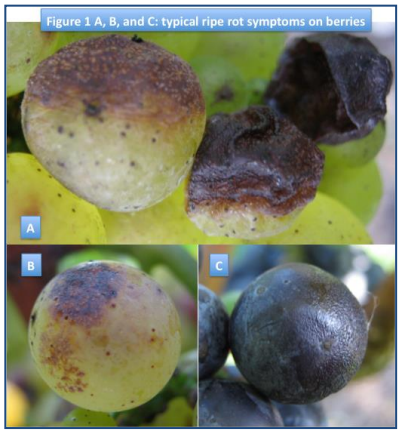
Final report, Virginia Wine Board FY 2016

"Investigations on chemical management option against ripe rot of grape"

Mizuho Nita and Charlotte Oliver

7 August 2017



Objectives: In this proposal, we requested support to

1) Investigate efficacy of several SDHI fungicides, polyoxin D, and pyriofenone against collection of *Colletotrichum* species from VA vineyards using *in vitro* assays and field trials. In order to have much better understanding of options we have against existing ripe rot pathogens.
2) If there is an evidence of SDHI insensitivity among our isolates, we would like to investigate for known SDH gene patterns, which is the target for the SDHI fungicides.
3) Combine knowledge we obtained from the previous round of our research by applying fungicidal treatments on leaves and flowers to see if that can prevent development of further infection by the pathogen or formation of secondary conidia on leaf tissues, which could be a missing link to understand the mechanism of ripe rot outbreak.

Summary of procedures

Isolate collection (conducted in 2013-2015): In order to identify potential regional differences, growers were selected randomly from each of five major grape growing regions of Virginia (VA). The northern region spans from the Washington D.C./Arlington area west towards the Appalachian Mountains and south to the edge of the Monticello American Viticulural Area, which is the start of the central region that spans as far east as Richmond, VA. The western region of VA contains the Appalachian and Blue Ridge Mountain areas while the eastern region of VA spans the coastline from Virginia Beach up to the Chesapeake Bay area. The southern region of VA is the southern piedmont of Appalachia. Our lab visited 35 growers in 2013, another 8 locations in 2014 and 2015 to collect symptomatic ripe rot grape berries. Since some growers had more than one vineyard, we collected from 90 unique locations, and within the same location we were able to collect some isolates. In addition, in 2015, we collected 60 samples from 20 random locations in two vineyards. Thus, overall, we processed more than 1,200 samples. Majority of our collections were either *C. acutatum* or *C. gloeosporioides* based on colony morphology and spore characteristics, and targeted DNA sequences of more than 300 of them have been submitted. We are expecting to send out 200 more isolates, which will be selected based on location and year, for sequencing in upcoming year to finalize our isolation collection and identification.

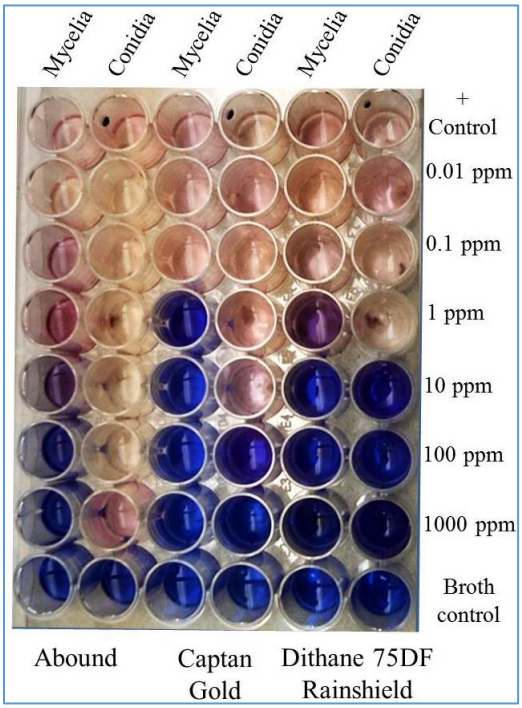
Fungicide sensitivity tests

We are employing two methods to compare results in the lab. The first is a use of Alamar blue (AB) in the fungicide amended culture plate [1], and then we use more traditional fungicide amended media with potentially resistant isolates. The single-spore isolate plates are flooded by adding 5 ml of clarified, buffered 2% potato dextrose broth (PDB) or minimal medium (MM). (Note: For the assay with SDHI, we use several different types of medium since it is known to affect the efficacy of the SDHI group [2]. Several media (MM, PDB, ¼ PDB, Complete Medium [for different fungal species], etc. [3] prior to the experiments.) are tested for their compatibility to the assay. Then, the suspension are filtered using four layers of cheesecloth to remove mycelium. Then, 100 µl of a suspension of 10^6 conidia/ml (adjusted using a hemocytometer) are added to test wells of 48-well cell culture plate (Corning Costar), and stock fungicide solutions are added to give final concentrations of each fungicide (0.0, 0.01, 0.1, 1.0, 10.0, 25.0, 50.0 and 100.0 µg/ml (Note: the concentration is subject to be adjusted based on the fungicide)). AB dye

(AbD Serotec) is added as 10% of the final volume in the test wells [4]. Plates are 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 are negative control (200 µl of 2% PDB and 10% AB dye only), and positive control (100 µl of PDB (or MM), 100 µl of 10^6 conidia/ml, and 10% AB dye). A chemical control plate are also prepared to ensure that the fungicides themselves did not reduce the AB dye (100 µl of stock fungicide, 100 µl of 2% PDB (or MM), and 10% AB).

A positive test result is recorded as a color change from blue to pink, which indicated that the dye has been reduced due to the presence of viable conidia (Figure on the right). A negative test result is recorded as no color change or the dye remained blue, i.e., the dye is not reduced due to the absence of viable conidia/fungal growth. One mean inhibitory concentration (MIC) endpoint is 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.

Of 300 isolates we sequenced, we have identified at least two *Colletotrichum acutatum* subspecies (*C. fioriniae* and *C. nymphaeae*) and four *C. gloeosporioides* subspecies (*C. aenigma, C. alienum, C. fructicola,* and *C. kahawae* subsp.). We will select five isolates from different geographic locations per subspecies to conduct experiment. Two of subspecies were found only in one location, thus we are expecting to examine a total of 5 species.



Fungicides to be tested are 1) Aprovia (solatenol, Syngenta), 2) Endura (boscalid, Syngenta), 3) Luna Privilege (fluopyram, Bayer), 4) Isofedamid (isofedamid, ISK Bioscience), 5) Oso (polyoxin D, Certis), and 6) property (pyriofenone, ISK Bioscience). Experiment will be repeated three times per isolate to validate the results. The results will be analyzed using a generalized linear mixed model (PROC GLIMMIX, SAS, ver. 9.4, Cary, NC) to determine the effect of fungicide and its concentration. Fungicide type, and concentration will be considered as fixed effects and experimental repetitions will be considered as a random factor.

In addition, traditional mycelium growth and spore germination tests are conducted to confirm their lack of sensitivity to fungicides. Plates of ¼ PDA or MM are amended with stock fungicide solution to give final concentrations of 0.0, 0.01, 0.1, 1.0, 10.0, 25.0, 50.0 and 100.0 µg/ml for each fungicide. A 5 mm diameter agar block will be cut from the advancing edge of an actively growing culture on ¼ PDA (using a 60mm Petri dish) and placed in the center of the dish, mycelia-side down, on the surface of the amended PDA. Plates are incubated in the dark at 25°C for 4-6 days. The radial diameter (perpendicular measurements in millimeters) are recorded for each colony. The corrected diameter (mean radial diameter minus the length of the agar block) are used to calculate percent relative growth (%RG = [mean diameter of colony/mean diameter of colony on non-amended agar] × 100) and percent relative growth inhibition (%RGI = 100 – %RG) compared with the non-amended controls. At each experiment, three plates are used per isolate, the experiment is conducted twice, and the mean corrected colony diameters is used in all calculations. As with AB assay, we select several different types of medium (e.g., minimal, and ¼ PDA) for testing of SDHI material since it is known to affect the efficacy [2].

Germination rate: Four 5 µl of spore suspension with 1 x 10^6 spore/ml are placed on to aforementioned amended (and non-amended) PDA or MM. Then spore germination rate (and formation of appressorium) are determined using microscope (40x and 100x objectives, Nikon Eclipse Ci, Nikon, Inc.). The observation will be made at 6 hours after inoculation, and 25 spores are examined per drop (i.e., 100 spores will be examined per isolate per run). Percent germination and relative germination

inhibition rate are determined. The assays are conducted three times. Data from mycelium growth and spore germination rate are analyzed using linear (PROC REG) or non-linear (PROC NLIN) or other methods such as beta model [5] regression to determine EC_{50} (Effective Concentration to inhibit 50% of sample).

Field trials: We will also examine the efficacy of these materials in the field. A block of Chardonnay vineyard at AHS AREC, which has been experiencing consistent occurrences of ripe rot since 2013, will be used for the field trial. The vines are trained in vertically shoot positioning system with two cordons. The vine spacing is 5 ft within a row and 10 ft between rows. A vine will be an experimental unit, and there will be six blocks (i.e., 6 vines will be used per treatment). The same 6 fungicides (+ one non-sprayed control) will be examined. Fungicide application will be made from bloom in two-week interval until harvest to determine the efficacy of the product. The timing of application will be examined as 1) at bloom alone, 2) from veraison (2 sprays), and 3) from bloom to harvest. Thus, the effect of 6 fungicides applied in 3 different timings will be examined. Other fungal diseases will be managed using fungicide that does not affect ripe rot development. Visual estimation of disease incidence (yes/no) and severity (% of area infected) of 10 clusters per vine (= 120 clusters total per treatment) will be conducted at harvest, and then taken into the lab to incubate for 7-14 days. This incubation allows to show latent infection that may not resulted in symptom development at the time of harvest. The data will be analyzed with a generalized linear mixed model to determine the effect of treatment on the development of ripe rot symptoms.

Progresses made

AB and fungicide amended media assays: During 2016, broth and juice were examined for their compatibility to the AB assay. Currently, we are testing the suitability of several grape juices and synthetic broths (PDB and MM) for supporting fungal growth and limiting false positives. Once we finalize the medium to be used, both assays to test 500+ isolates will start. We are expecting the starting date to be mid-February 2017, and finish date to be mid-April 2017.

In 2017, the broth pH for the AB assay was proving to be problematic. Do to the sensitivity of the AB dye, fungicide additions were causing the pH to shift and provide false positives. Instead, our lab shifted to a 24-well fungicide amended solid media format. This method allows for a range of fungicide concentrations (0.0, 0.01, 0.1, 1, 10, 100 µg/mL) and the testing of both spores and mycelia on the same plate. Currently, our lab started screening isolates in Apr. 2017 and are on track to finish in Nov. 2017.

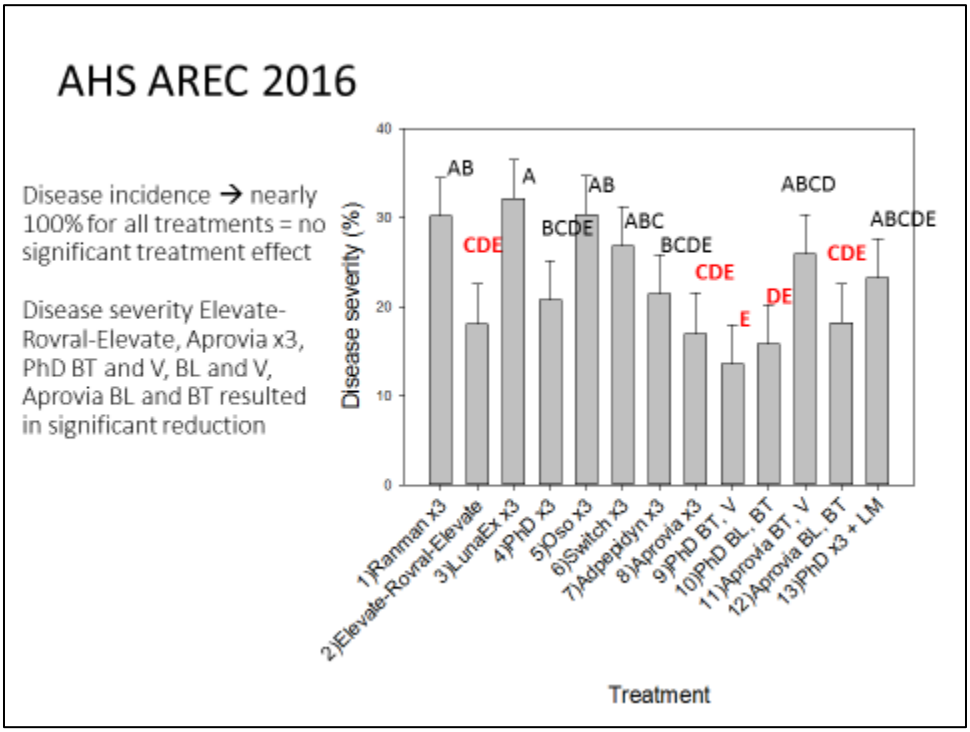
Field trials: Trial at the AHS AREC showed that although disease incidence on cluster was nearly 100% for all treatments, there was significant treatment effect on disease severity (infected % area of cluster). Rotation of Elevate and Roval, Aprovia applied three times, Aprovia applied at bloom and berry touch, Ph-D applied at bloom and berry touch, Ph-D applied at berry touch and veraison resulted in significantly lower disease severity than the check. (please refer to figures below)

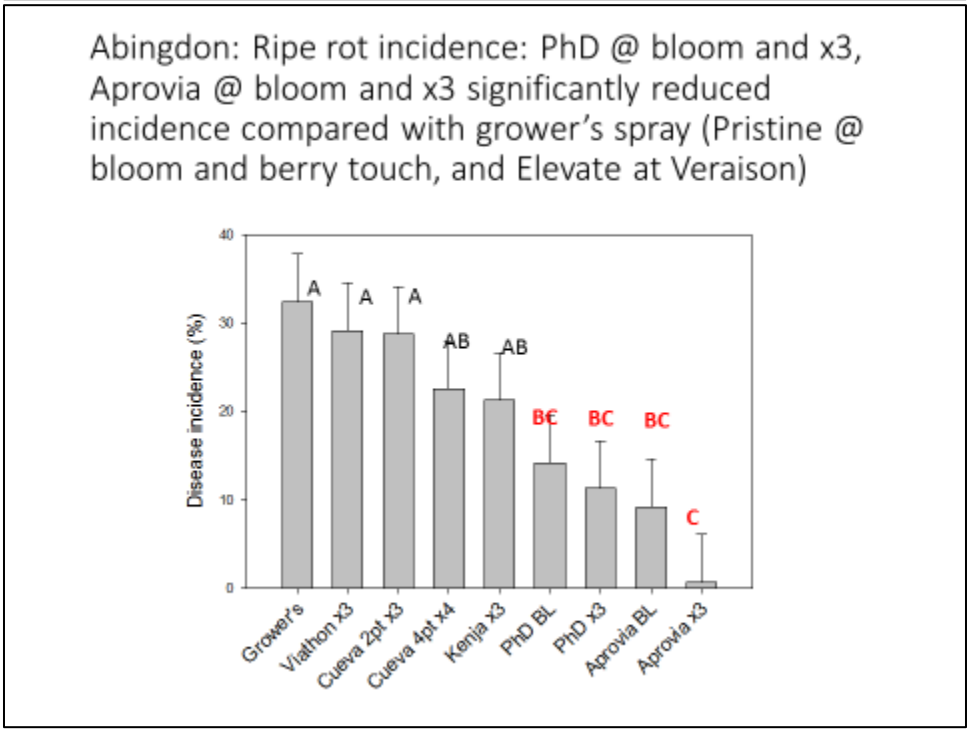
Trial at Abingdon showed that ripe rot disease incidence of Ph-D applied at bloom, Ph-D applied three times, Aprovia applied at bloom, and Aprovia applied three times significantly reduced incidence compared with grower's spray, which consisted of Pristine at bloom and berry touch, and Elevate at veraison. When ripe rot severity level was compared, Viathon, Cueva (4qt), Kenja (isofedamid), PhD x3, and Aprovia (both at-bloom application and standard three-application) resulted in significantly lower severity than the grower's spray.

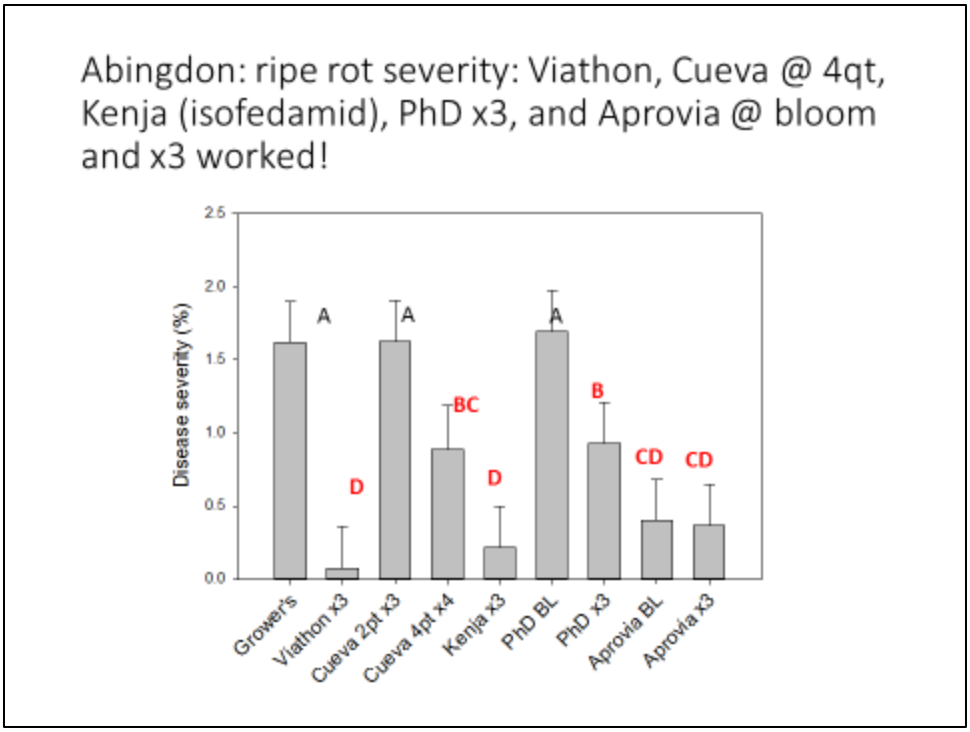
These results indicate the efficacy of Ph-D, Aprovia (a SDHI fungicide), and Kenja (another SDHI), and the importance of fungicide application at bloom. Interestingly, the other SDHI material (Luna) was not as effective as Aprovia. We would like to repeat the same experiments in 2017 to confirm our results.

In 2017, we have repeated the field experiments conducted at the AHS AREC and Abingdon vineyards using the same treatments. In addition, another field plot was added at Boordy, MD. The final application at Boordy occurred at berry touch. Final veraison applications have been made at the AHS

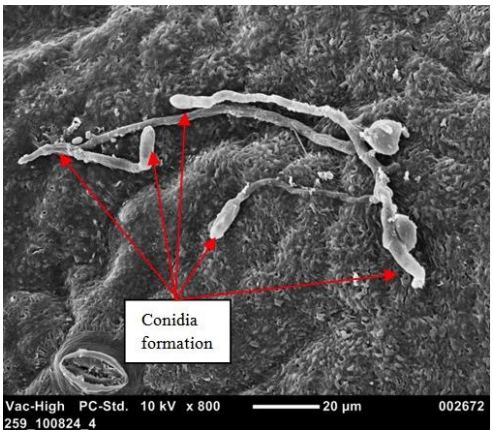
AREC and Abingdon. Ratings of disease incidence and severity will begin during late September, when fruit is ripe for harvest.







Observation of fungal activities on leaf and flowers: As noted earlier, we have identified the evidence of fungal activity on seemingly non-symptomatic leaf and flower tissues (Figure below). What we have observed is production of spores on the leaf surface, that probably become a bridge phase between their over-winter phase and infection on fruit that happen much later in the season. We would like to examine the effect of fungicide application to this bridge phase of fungal life. If the fungicide application is effective, it would probably lead to a lower risk of ripe rot infection at early part of the season hence, a lower risk at latter part of the season.



Artificial inoculation will be conducted on pot-grown grapevine cv. Chardonnay. Younger leaves (2wk old from emergence) will be selected for inoculation. A fungicide treatment at field rate will be applied 24 hours prior to the inoculation. (Note: The selection of fungicide to be tested will be based on the results from the objective 1, but I am expecting to test at least three different mode of action groups.) At 48 and 72 h after inoculation, samples will be obtained and processed for scanning electron microscopy (SEM) observation. Infected tissues will be prepared for SEM as in Leandro et al., 2001 [6]. The SEM work will be performed at the lab of Dr. Jon Eisenback at Department of Plant Pathology, Physiology, and Weed Science of Virginia Tech.

In addition, light microscopy will be performed on the similarly inoculated tissues. Tissues will be prepared as described in Marques [7] where plant and fungal tissues will be stained in two different colors. The sample will be embedded in a plastic resin, sliced with a microtome in to 5-7 micro meter section for examination under microscope. Sample preparation, sectioning, and initial screening of samples will be conducted at the Molecular and Cellular Imaging Center at the Ohio State University.

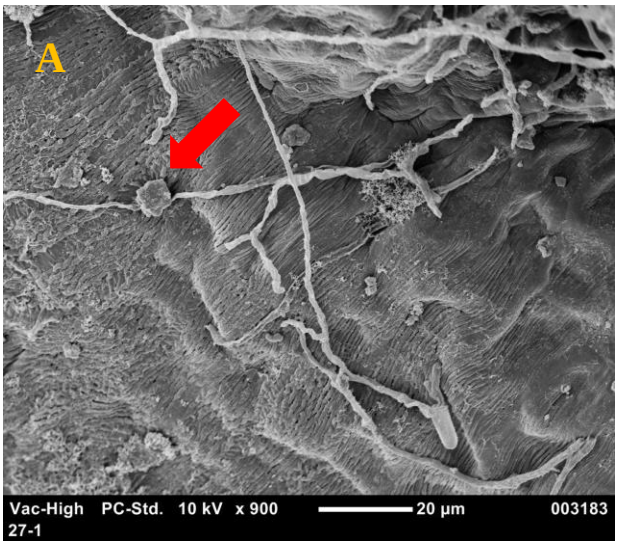
Progresses made

To explore the infection process of *Colletotrichum* species in 2016, berries were inoculated at BB/pea-size, berry touch and veraison with species from both complexes; *C. aengima, C. alienum,* and *C. fructicola* from the *C. gloeosporioides* complex and *C. fioriniae* and *C. nymphaeae* from the *C. acutatum* complex. Berries were harvested after 24 hrs, 72 hrs, 1 week and 2 weeks after inoculation ($5x10^5$ spores/mL).

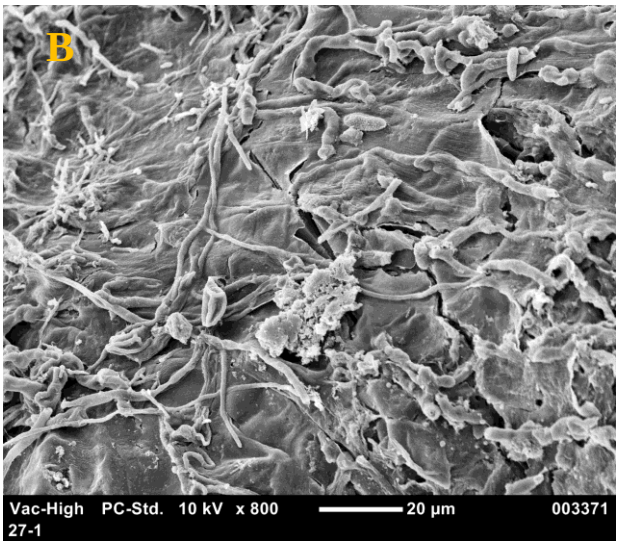
All samples collected during the growing season were stained with an osmium tetroxide and uranyl acetate (1%) solution, dehydrated and stored in 100% ethanol for SEM or embedded in Embed 812 resin for light microscopy and transmission electron microscopy (TEM). Samples will be sectioned at AHS AREC and imaged at the OSU Molecular and Cellular Imaging Center (MCIC) in Wooster, Ohio during the winter of 2017.

Additionally, leaf inoculation experiments will be completed during the winter of 2017 using new incubation intervals: 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.

A portion of samples that were prepared from SEM imaging were observed in April 2017, on campus in Dr. Eisenback's lab. Differences in growth patterns were observed between different phenological berry ages (See images below). These differences prompted our lab to repeat this experiment to confirm our potential results and fill in gaps in our sample collections.



Sample inoculated at BB/Pea-size and harvested within 24hrs. The red arrow indicates the presence of appressoria, an infection structure used for tissue penetration.



Sample inoculated at veraison and harvested within 24hrs.

In 2017, the berry inoculation was repeated using the same 5 species at bloom, BB/pea-size and veraison, with the addition of fungicide applications. Berries were inoculated ($5x10^5$ spores/mL) before or after applications of Aprovia, Captan Gold (captan, Adama), Cueva (copper octanoate, Certis), and Topsin M WSB (thiophanate methyl, United Phosphorous, Inc). Samples were harvested after 24, and 72 hrs. Tissues were prepared for SEM in the same method as 2016, without the osmium tetroxide and uranyl acetate stain. Only tissues that Aprovia and Topsin M were applied to were prepared for TEM (systemic fungicides). SEM samples were stored at 4°C until observation on campus.

Leaf preliminary studies are underway. Currently, our lab is solidifying our protocol for leaf tissue processing before staining and imaging. This process should be completed before the end of Sept. 2017. Data collection should be completed during the winter of 2017.

Other achievement

Teaching component has been the strength of the project as well. A master's level student, Ms. Charlotte Oliver who has been working on this project since 2013, has been making an excellent progress with her study (GPA 3.8). She won a student competition at a regional scientific conference in 2014, and 2017 based on her research on ripe rot. She defended her MS thesis in May 2015, and stayed for her PhD, working on this project. Coursework for her PhD was completed during Spring 2017 term with her final departmental seminar course. Currently, she has completed 39 hrs of coursework with a 3.8 GPA.

Presentations provided by our group in 2016 & 2017

Oliver, C. and Nita, M. "Identification, histological investigation, and evaluation of potential fungicide controls of grape ripe rot *Colletotrichum* species" Plant Pathology, Phyisology and Weed Science Departmental seminar, 19, Apr. 2017

Oliver, C. and Nita, M. "Survey and identification of wine grape ripe rot (*Colletotrichum gloeosporioides* & *C. acutatum* complexes) in VA vineyards" American Phytopathological Society – Potomac Division Meeting, 23, Mar. 2017.

Oliver, C. and Nita, M. "Investigations on infection Timing of Ripe Rot of grapes caused by *Colletotrichum* species complexes, & their sensitivity to the QoI fungicides" Southeast Regional Fruit and Vegetable Conference, 7, Jan. 2017.

Oliver, C. and Nita, M. "Methods for identifying *Colletotrichum* species causing ripe rot of grape in Virginia", Cumberland-Shenandoah Fruit Worker's Conference, 1 December, 2016.

Nita, M. and Bly, A. "Screening for QoI resistance among *Colletotrichum* species associated with ripe rot of grape in Virginia vineyards", American Phytopathological Society National Meeting, 1 Aug. 2016.

Personnel

- Mizuho Nita, PhD (AHS AREC, PPWS, Virginia Tech): PI, oversee the project, advise Charlotte Oliver, communicate among collaborators, design the experiment, analyze data.
- Charlotte Oliver (Graduate Research Associate, AHS AREC, PPWS, Virginia Tech): Conduct research and experiments
- Vannette Trumm (Project Associate, AHS AREC, Virginia Tech): provide assistance to Charlotte Oliver, especially on maintenance of fungal culture and in vitro fungicide assays.

Selected references

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6. Leandro, L.F.S., et al., *Germination and Sporulation of Colletotrichum acutatum on Symptomless Strawberry Leaves.* Phytopathology, 2001. **91**(7): p. 659-664.
7. Marques, J., et al., *Histopathology of postbloom fruit drop caused by Colletotrichum acutatum in citrus flowers.* European Journal of Plant Pathology, 2013. **135**(4): p. 783-790.