**SRSFC Project Report for 2016**

**Proposal title: Examining effects of three potential mode of action group fungicides among *Colletotrichum acutatum* and *C. gloeosporioides* isolates found in VA vineyards.**

**Principal Investigators:**

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**OBJECTIVE:**

1. Determine efficacy of various SDHI fungicides, polyoxin-D, and pyriofenone, on five different species of *Colletotrichum* that were isolated from grape berries.

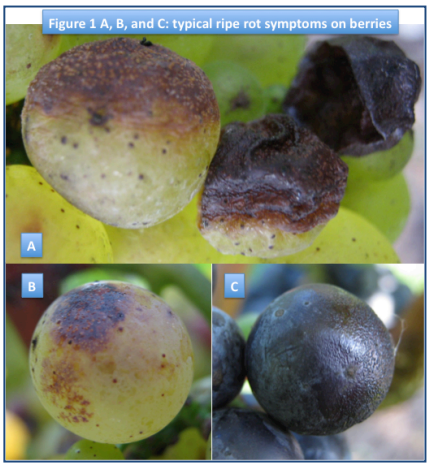

Figure 1 A, B, and C: typical ripe rot symptoms on berries

**Brief overview of the project background**

Ripe rot, which is caused by two pathogen complexes, *Colletotrichum acutatum* and *C. gloeosporioides*, is an endemic disease for VA [1]. Typically, our recommendations for chemical management of ripe rot are the use of mancozeb, ziram, captan, and quinone outside inhibitors (QoI) fungicides. However, mancozeb and ziram have a 66-day and 21-day PHI, respectively, for grape; thus, we cannot use them in the late season. In addition, there is some evidenceindicating some *C. gloeosporioides* complex strains are not sensitive to captan [2]. In fact, the aforementioned vineyard with 30% loss relied on captan for their late season disease management; yet, remained unable to manage ripe rot. Thus, the importance of QoI fungicide is high, especially in dealing with late season ripe rot outbreak.

In 2012-15 seasons, our lab collected over 1,200 *Colletotrichum* isolates from VA vineyards. In 2013-15, we investigated the efficacy of QoI and captan among 300 of our isolates with a partial support from the SRSFC. Preliminary results showed that more than 20% of our isolates were insensitive to QoI at a discriminatory dose of 100 ppm, and about 15% of our isolates contains G143A mutation that is common among QoI-resistant fungal species [3, 4]. These results showed that we have very limited options for chemical management for ripe rot of grapes.

In 2013-15, we have begun screening of modes of action using two of our isolates using AB assay (described below), fungicide amended culture media, detached fruit assay, and field trials, and found that there are several that produced promising results. These were: DMI (Sterol demethylation inhibitor), SDHI (succinate de-hydrogenase inhibiting), copper, phosphorous acid, polyoxin D, and pyriofenone.

Boscalid and other SDHI materials were often used against late season rot (especially Botrytis bunch rot) management, which are applied about the same time ripe rot starts to appear; therefore, if they work against ripe rot it would be ideal; however, our tests with boscalid amended media results were not consistent among our isolates, indicating that there may be varying sensitivity among *Colletotrichum* isolates. In a recent field test on a newer SDHI material (solatenol from Syngenta) showed significant reduction in ripe rot severity; however, the data is only from one year, and further examination is required. In addition, in a field trial conducted in 2015, polyoxin D products (Oso from Certis and Ph-D from Arysta, FRAC 19) resulted in significant reduction in disease severity ($P < 0.05$) compared with a control. As with SDHI group, polyoxin D products are labeled for Botrytis control. Pyriofenone is a new mode of action (FRAC U8) introduced from ISK bioscience as a powdery mildew material. When we examined the material using a table grape inoculation assay, the results indicated significant reduction of the number of disease-infected berries from non-treated control ($P < 0.05$).

The objectives for this project is to investigate efficacy of several SDHI fungicides, polyoxin D, and pyriofenone against collection of *Colletotrichum* species from VA vineyards and orchards. Also, if there is an evidence of SDHI insensitivity among our isolates, we will to investigate for known SDH gene patterns, which is the target for the SDHI fungicides.

**Experimental procedures and progresses made**

***Isolate collection (conducted in 2013-2016)*:** 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 Viticultural 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, and 300 of them were sequenced for identification.

***Progresses made:*** In 2016, 200 samples from 70 random locations in three vineyards were also collected. An additional 200 isolates collected in 2014 were sequenced for general species complex identification with 44 isolates identified to in species within the complex using multilocus sequencing (MLST).

Of 500 isolates we sequenced, we have identified at least two *Colletotrichum acutatum* species (*C. fioriniae* and *C. nymphaeae*) and five *C. gloeosporioides* species (*C. aenigma, C. fructicola, C. siamense, C. kahawae,* and *C. gloeosporioides*). We selected five isolates from *C. fioriniae*, *C. nymphaeae, C. aenigma, C. fructicola,* and *C. alienum* from different geographic locations per species to conduct experiment. One of species (*C. nymphaeae*) was found only from one location, thus we are expecting to examine a total of five species. We are currently in the process of preparing mycelium plugs for following experiments.

***Fungicide sensitivity tests**:* We are employing two methods to compare results; use of alamarBlue® (AB) in a fungicide amended culture plate [5], and a traditional fungicide amended media for potentially resistant isolates. Single-spore isolate plates will be flooded with 3 ml of a clarified, buffered 2% Potato Dextrose Broth (PDB) or minimal medium (MM). The suspension will be filtered using two layers of Miracloth (EMD Millipore) to remove mycelium. Then, 100 µl of a suspension of $10^6$ conidia/ml (adjusted using a hemocytometer) will be added to test wells of 48-well cell culture plate (Corning Costar), and stock fungicide solutions will be 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) will be added as 10% of the final volume in the test wells [6]. Plates will be 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 will be 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 will be 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 was recorded as a color change from blue to pink, which indicated that the dye had been reduced due to the presence of viable conidia (Fig 3 from [6]). A negative test result will be recorded as no color change or the dye remained blue, i.e., the dye was not reduced due to the absence of viable conidia/fungal growth. One mean inhibitory concentration (MIC) endpoint will be 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.

Fungicides to be tested are 1) Aprovia (soratenol, Syngenta), 2) Endura (boscalid, Syngenta), 3) Luna Privilege (fluopyram, Bayer), 4) Kenja (isofedamid, ISK Bioscience), 5) Oso (polyoxin D, Certis), and 6) Kabuto (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 will be conducted to confirm their lack of sensitivity to fungicides. Plates of ¼ PDA or MM will be 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 will be incubated in the dark at 25°C for 4-6 days. The radial diameter (perpendicular measurements in millimeters) will be recorded for each colony. The corrected diameter (mean radial diameter minus the length of the agar block) will be 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 will be used per isolate, the experiment will be conducted twice, and the mean corrected colony diameters will be used in all calculations. As with AB assay, we will 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 [7].

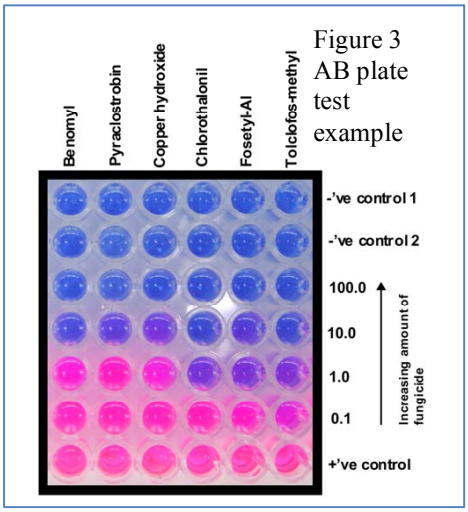

Figure 3 AB plate test example

Germination rate: Four 5 µl of spore suspension with 1 x $10^6$ spore/ml will be placed on to aforementioned amended (and non-amended) PDA or MM. Then spore germination rate (and formation of appressorium) will be 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 will be examined per drop (i.e., 100 spores will be examined per isolate per run). Percent germination and relative germination inhibition rate will be determined. The assays will be conducted three times. Data from mycelium growth and spore germination rate will be analyzed using linear (PROC REG) or non-linear (PROC NLIN) or other methods such as beta model [8] regression to determine $EC_{50}$ (Effective Concentration to inhibit 50% of sample) and $EC_{90}$.

***Progresses made:*** In 2016, we have begun the testing of various minimal nutrient broths for compatibility with the AB dye, commercial fungicides, and *Colletotrichum* species. Additionally, method modifications to produce consistent mycelial quantities have been tested. The new method for assembling mycelial wells is to distribute a $10^5$ conidial suspension (process described above) into test wells and seal the plates. Sealed plates will be incubated with a diurnal light cycle (12 light/ 12 dark) on an orbital shaker. Fungicides are then added to the incubated wells after 24 hrs. The timing of dye addition has also been reevaluated to account for the time required for fungicide uptake into cells. The optimum time frame for dye addition is currently under evaluation. Due to the dye's requirement of a 7.5 pH, additional time has been taken to investigate the effects of broth additives for fungal growth inhibition after observing inhibition from sodium bicarbonate.

We are also testing several growth media (MM, PDA, and ¼ PDA) for the fungicide amended-plate testing in 2016. Once we set up both AB and fungicide amended media, we will commence with two experiment simultaneously. We are planning to finish all the groundworks in December 2016-January 2017, and commence with the experiments in February 2017.

**Analysis of DNA sequence of the SDH subunits***:* Once we identify SDHI insensitive isolates, a subset of isolates will be selected for sequencing to identify characteristics of the SDH complex. Three to five day old mycelia culture on PDA will be used for the extraction of DNA using the Bioline Isolate II Plant extraction kit (Bioline, Taunton, MA) [9], then a set of primers will be used to amplify the gene codes for the SDHB, SDHC, and SDHD subunits (Table 1). Polymerase chain reactions (PCRs) will be carried out using HotStart Taq plus polymerase kit (Qiagen). Amplification conditions will be initial preheat for 5 min at 95C, followed by 40 cycles of denaturation at 94C for 40 sec, annealing at 51C for 50 sec, and extension at 72C for 1 min, and a final DNA extension at 72C for 10 min [9]. PCR products will be purified using the ExoI/SAP method [10]. Both directions of the PCR products will be sequenced at Virginia Tech's Bioinformatics facility or equivalent lab. Multiple DNA sequence alignments and translation to amino acids will be performed with DNAStar program (ver. 11).

Table 1. List of primers for detecting known three mutations in SDH subunit (Table 2 from [9])

| SDH subunit (gene)[x] | Primer | Primer sequence (5′–3′) | Reference | Tm (°C)[y] | Amplified DNA fragment[z] |
|---|---|---|---|---|---|
| Iron-sulfur protein (*sdhB*) | SDHB-F1 | TAC GAG CTC GAC CTC AAC AAG AC | 8 | 58.4 | 341 to 1,049 |
| | SDHB-R1 | CTC GGC AAC GCG GGG TTC AGT C | 8 | 65.0 | … |
| | SDHB-F2 | GTG GCG TCG AAG GGC CGA AGA AGC CG | 8 | 68.9 | –33 to 349 |
| | SDHB-R2 | CAG CAT CAT GGG TCC GGT CTT GTT GA | 8 | 62.7 | … |
| | SDHB-F4 | CGA CGG ACT CTA CGA ATG C | 8 | 55.4 | 801 to 1,020 |
| | SDHB-R4 | GCA TGT CCT TGA GCA GTT GAG | 8 | 56.3 | … |
| CybL protein (*sdhC*) | SDHC-F2 | ATG GCT TCT CAG CGG GTA TTT CAG | 5 | 59.1 | 1 to 623 |
| | SDHC-R3 | TCA TCC GAG GAA GGT GTA GTA AAG GCT G | 5 | 60.3 | … |
| | SDHC-F4 | CCA GCG GAG GTA TGT CAT AAT AG | This study | 54.6 | 84 to 570 |
| | SDHC-R4 | TCC ATC CAG TGC GGA TAA CC | This study | 56.9 | … |
| CybS protein (*sdhD*) | SDHD-F1 | ATG GCC TCC GTC ATG CGT | This study | 59.9 | 1 to 636 |
| | SDHD-SeqR2 | TAT CTA TGC GTG CCA CAA CC | 5 | 55.1 | … |
| | SDHD-F2 | CTG CGA CAT CGA CCA TGA A | This study | 55.2 | 77 to 596 |
| | SDHD-R2 | CCA ACA TCG TTT GTC TCG AAA G | This study | 54.3 | … |

[x] SDH = succinate dehydrogenase.
[y] Primer melting temperature.
[z] Fragment numbering corresponds to nucleotide positions in the SDH subunits B, C, and D of *A. alternata*, with the first nucleotide of the start codon at position 1.

**Summary of progresses and bottlenecks**

The major bottleneck we encountered was the selection of samples due to difficulty of correctly identify species. We spent the most of 2016 for sequencing and analyzing DNA sequence data. The majority of species identification went relatively smooth, but there are two groups of variant isolates that are different enough to require closer look, and we are still working on these. Since it may take more time, we have decided to commence with fungicide assays with known species.

So far, we are finalizing the AB assay's protocol, and start working on a protocol for the fungicide amended media assay. Once both protocols are set, we will start testing sample isolates. As noted above, our plan is to commence assays in February 2017. Our estimated time to finish the first round assay is around April 2017. Since it involves quite a bit of work, we have submitted the request for second year (note: when requested this project, we indicated that it will be two-year project).

Also, I should note other achievements from the SRSFC funded projects:

1) Ms. Charlotte Oliver has graduated with her MS degree in Dec 2015. We are in the process of preparing two manuscripts based on her work. She continued on her PhD, and now working on this project.
2) An oral and a poster presentation was made based on the previous funded project:
    a. Mizuho Nita and Amanda Bly (2015) "Screening for QoI resistance among several *Colletotrichum* species associated with ripe rot of grape found in VA vineyards" 91st Annual Cumberland-Shenandoah Fruit Workers Conference, Winchester, VA, 3 and 4 Dec. 2015
    b. Mizuho Nita and Amanda Bly (2016) "Screening for QoI resistance among *Colletotrichum* species associated with ripe rot of grape in Virginia vineyards" American Phytopathological Society Annual Meeting 2016, Tampa, FL, 30 Jul. - 3Aug. 2016

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