

## SRSFC Project Report for 2017

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

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### OBJECTIVE:

1. Determine the efficacy of various SDHI fungicides, polyoxin-D, and pyriofenone, on five different species of *Colletotrichum* that were isolated from grape 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 evidence indicating certain *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 274 of our isolates with partial support from the SRSFC. Preliminary results showed that more than 25% 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 dehydrogenase 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 a 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 the control treatment. 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 a significant reduction of the number of disease-infected berries from non-treated control ( $P < 0.05$ ).



The objectives of this project is to investigate the efficacy of several SDHI fungicides, polyoxin D, and pyriofenone against a 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 progress 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. fiorinae* 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. fiorinae*, *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<sup>6</sup> 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<sup>6</sup> 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.

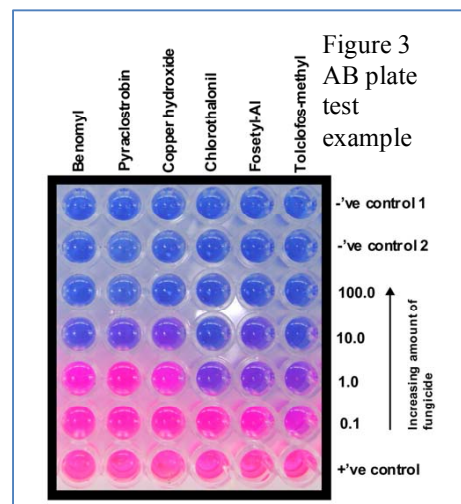
Fungicides to be tested are 1) Aprovia (soratenol/ benzovindflupyr, Syngenta), 2) Endura (boscalid, Syngenta), 3) Luna Privilege (fluopyram, Bayer), 4) Kenja (isofedamid, ISK Bioscience), 5) Oso (polyoxin D, Certis), and 6) Kabuto (pyriofenone, ISK Bioscience). The experiment will be repeated three times 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  $\frac{1}{4}$  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  $\mu\text{g/ml}$  for each fungicide. A 5 mm diameter agar block will be cut from the advancing edge of an actively growing culture on  $\frac{1}{4}$  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 ( $\%RG = [\text{mean diameter of colony} / \text{mean diameter of colony on non-amended agar}] \times 100$ ) 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  $\frac{1}{4}$  PDA) for testing of SDHI material since it is known to affect the efficacy [7].

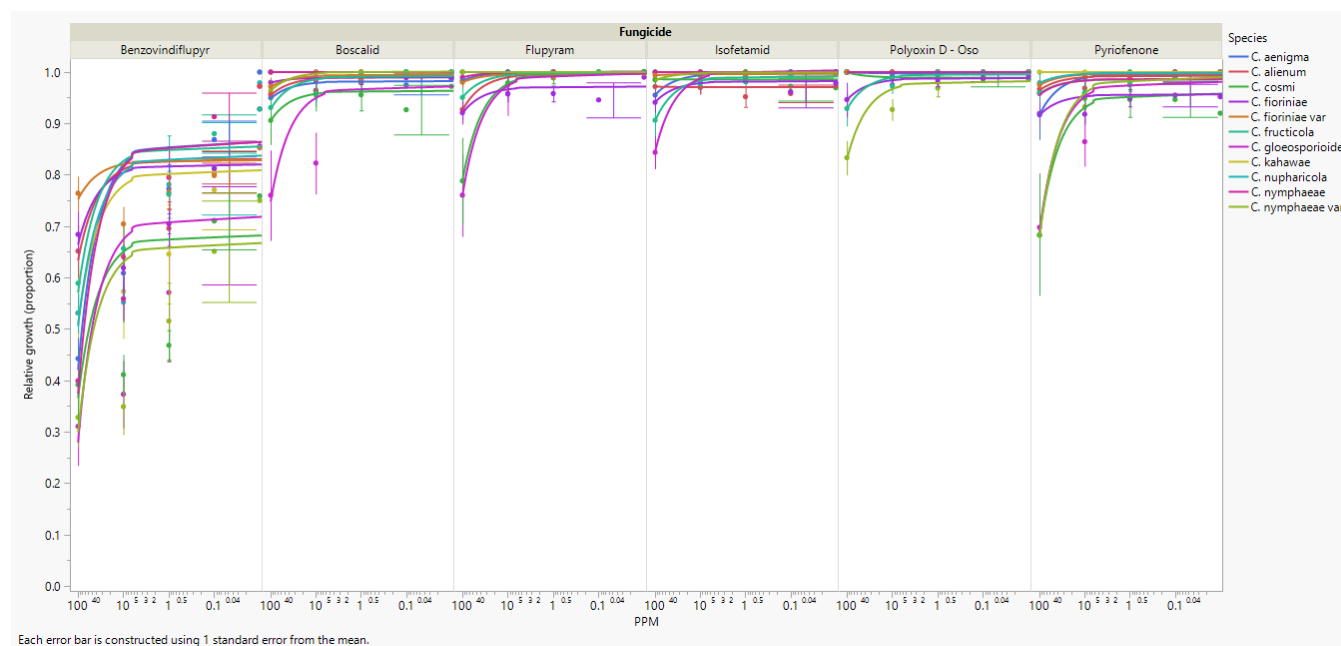
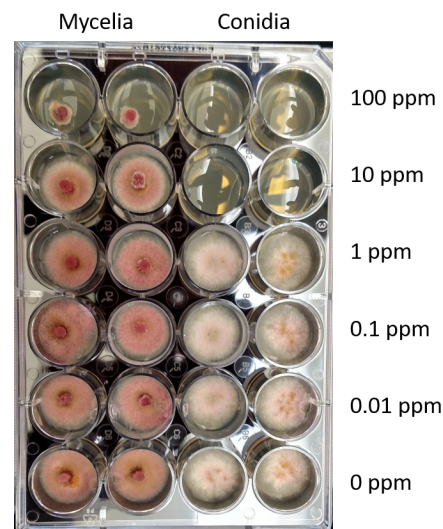
**Germination rate:** Four 5  $\mu\text{l}$  of spore suspension with  $1 \times 10^6$  spore/ml will be placed on to aforementioned amended (and non-amended) PDA or MM. Then spore germination rate (and formation of appressorium) will be determined using a microscope (40x and 100x objectives, Nikon Eclipse 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 the sample) and  $EC_{90}$ .

**Progress 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 was 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 were then added to the incubated wells after 24 hrs.

We initiated set of experimental runs in 2016; however, we noticed many cases of false positives with certain fungicides. This was due to the dye's requirement of a 7.5 pH to work correctly and some highly acidic fungicide formulations. A significant time was spent to investigate the effects of broth additives, but we found that although an adjustment with sodium bicarbonate worked, several *Colletotrichum* species we tested were sensitive to the material at high concentration. Moreover, even with less acidic fungicides, we observed inconsistent results, probably due to the variable acidity of water we use. After extensive troubleshoots in 2016, we developed a protocol that we could use (which uses a relatively low level of sodium bicarbonate to adjust pH and use newly opened store-bought purified water each time we conduct assay), but at the same time, we were never able to trust the assay 100%. Thus, we decided to use a different assay method to achieve our objectives.



We employed the traditional mycelium growth and spore germination tests using a 24-well culture plate. Plates of PDA were amended with stock fungicide solutions to give final concentrations of 0.0, 0.01, 0.1, 1.0, 10.0, and 100.0  $\mu\text{g/ml}$  for each fungicide. Conidial suspensions and mycelial plugs were tested in separate wells of the same 24-well plate. Conidial suspensions were prepared as described above to  $2.5 \times 10^5$  conidia/ml in sterile distilled water and 5  $\mu\text{l}$  was applied to the center of the test wells. A 3 mm diameter agar block was excised from the advancing edge of an actively growing culture on WA and placed in the center of the dish, mycelia-side down. Plates were incubated with a diurnal light cycle at 25°C for 3 days. The radial diameter (perpendicular measurements in millimeters) was recorded for each colony. The percent relative growth inhibition was calculated as described above for both conidial and mycelial wells (Fig. right). For each experiment, one plate with two sets of test wells per propagule was used and the experiment was conducted twice. The data collection for this assay was completed on Nov. 27, 2017. The Fig. below shows a quick look at the data where the x-axis is the fungicide concentration in a log scale, and the y-axis is a relative growth in proportion. Each of nine species and two “unknown” species is represented in different color. Each dot is the mean value at the concentration, and the error bar represents a standard error. The line is a polynomial line that JMP Pro (ver. 13) places as a default. A low value in the figure indicates a larger level of inhibition by the material. Both propagule (mycelia and spore) are combined for this figure.



Since we just completed the experiment a few days before the report due, what we can report here is very limited. What we found so far is that the only benzovindiflupyr showed a promising result (i.e., showed low relative growths with higher doses). With other SDHI materials, polyoxin-D, and pyriofenone, only a few strains showed any sign of reaction to the material. Considering low concentrations (less than 10 ppm with many chemicals we have tested) applied in the field, even if we see some reductions at 100 ppm, it is less likely that the product is effective in the field. We will spend more time with data analysis to determine benzovindiflupyr is a viable option. (note: we did observe a consistent reduction of ripe rot with benzovindiflupyr in our field trials, which is not covered by this grant.) We also noticed that there are differences in the sensitivity to benzovindiflupyr among *Colletotrichum* isolates we have tested.



**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) <sup>x</sup>	Primer	Primer sequence (5'-3')	Reference	T <sub>m</sub> (°C) <sup>y</sup>	Amplified DNA fragment <sup>z</sup>
Iron-sulfur protein ( <i>sdhB</i> )	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 ( <i>sdhC</i> )	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 ( <i>sdhD</i> )	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	...

<sup>x</sup> SDH = succinate dehydrogenase.

<sup>y</sup> Primer melting temperature.

<sup>z</sup> 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.

**Progress made:** Since the outcome of the media assay suggests that all of the isolates are resistant to at least one SDHI fungicide, amplification of all three *sdh* fragments for all tested isolates began in Oct. 2017. Currently, we are finalizing the amplification conditions for the PCR reactions and fragments will be submitted for sequencing by the end of January 2018.

### Summary of progress and bottlenecks

We were able to finish data collections for the fungicide-amended media test with four SDHI products, polyoxin-D, and pyriofenone for nine *Colletotrichum* species. We will analyze data more closely, and at the same time, prepare to submit PCR products of *sdh* fragments in the next few months. Unfortunately, a suppression of mycelial growth (whether using spores or mycelium as propagule) was observed only with benzovindflupyr. (Note: it is not covered by this grant, thus, not shown in this report, but we did test other modes of action. Materials that are known to provide a certain level of efficacy in the field, such as mancozeb and captan, resulted in clear dose-response curves. Thus, we believe the assay is working as we intended.) There is some evidence of difference among species to fungicides, which we will investigate more.

The major bottleneck we encountered was the selection of samples due to the 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 a 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.

We were not able to address issues with pH with the AB assay. The dye is very sensitive to low pH, and several fungicide formulations are highly acidic. Unfortunately, pH adjustments affected the growth of the *Colletotrichum* isolates. Therefore, we shifted our efforts to a fungicide amended media in 2017.

We encountered issues with one of *sdh* fragments amplifications that delayed the production of PCR products for the sequencing. We think the current fix will solve the issue, and planning to submit PCR products to the sequencing by the end of January.

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

- 1) A Ph.D. student, Ms. Charlotte Oliver has been working this project as a part of her Ph.D. project. We expect her to graduate in Fall 2018
- 2) Oral and poster presentations made based on this and the previously funded (and related) 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" 91<sup>st</sup> 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. - 3 Aug. 2016
  - c) Charlotte Oliver and Mizuho Nita (2017) "Survey and identification of wine grape ripe rot (*Colletotrichum gloeosporioides* & *C. acutatum* complexes) in VA vineyards" American Phytopathological Society – Potomac Meeting Annual Meeting 24-26 Mar. 2017,
  - d) Charlotte Oliver and Mizuho Nita (2017) "Identification, histological investigation, and evaluation of potential fungicide controls of grape ripe rot *Colletotrichum* species" Plant Pathology, Physiology and Weed Science Dept. seminar, 19 Apr. 2017
  - e) Diana McHenry and Mizuho Nita (2017) "QoI resistant among *Colletotrichum* species found in vineyards" the American Phytopathological Society Potomac Division Meeting, Morgantown, WV, 22-23 March 2017.
  - f) Charlotte Oliver and Mizuho Nita (2017) "*In vitro* fungicide screening for *Colletotrichum* species isolated from Virginia vineyards" 93<sup>rd</sup> Annual Cumberland-Shenandoah Fruit Workers Conference, Winchester, VA, 30 Nov. and 1 Dec. 2017

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