SRSFC Project Progress Report (2015-11)
Proposal Category: Research **Proposal Status: Previously Funded Project (second year)**

Title: Species identification and examining QoI and Captan resistance among *Colletotrichum acutatum* and *C. gloeosporioides* isolates found in VA vineyards.

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OBJECTIVES:

1. Determine whether *Colletotrichum* isolates from VA vineyards exhibits either QoI and/or caption resistance using a combination of *in vitro* and molecular approaches.
2. Identification of species among our *Colletotrichum* isolates using a targeted sequencing

JUSTIFICATION

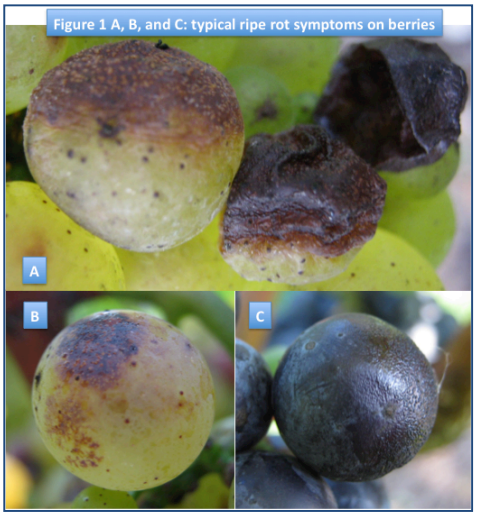


Figure 1 A, B, and C: typical ripe rot symptoms on berries

Ripe rot, which is caused by two pathogens, *Colletotrichum acutatum* and *C. gloeosporioides*, is an endemic disease for VA; in fact, there is a record of fungicide field trials in 1888 by Mr. G. Curtiss in Stafford county, VA [1]. It is often considered a relatively minor disease for wine grape production, probably because it does not cause consistent damages year after year. However, the damage can be detrimental to the production. In 2010-2014, there were several growers in VA who lost 15-30% of their potential crop due to this disease (Nita, *unpublished data*). The suffering growers all agreed that the wine quality was hugely compromised by infected berries. A study by Meunier (2009) indicated that as little as 3% contamination can results in noticeable changes in flavor and color. Thus, the actual damage can be far greater than direct reductions of yield. Although it is anecdotal, discussion with wineries revealed that it is not uncommon for them to receive ripe-rot infected berries from growers (Nita, *personal communication*). Sizable outbreaks have been observed in VA, PA, and NC in recent years (Tuner Sutton, NCSU, and Naomi Halbrendt, PSU, *personal communication*). In fact, during 2009-2013 state-wide grapevine virus survey, our group found that nearly all visited vineyards had some degree of infestation by ripe rot (Nita, *unpublished*).

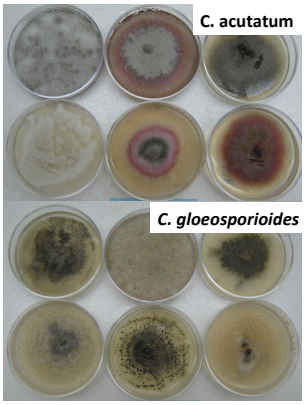
Both pathogens are able to cause infection from bloom to harvest [2]. Around the time when sugar levels increase (5-13 Brix) and/or when titratable acidity decreases in the berry, the pathogens cause necrosis; however, the actual trigger for the transition is unknown. Once the symptoms start to develop, it causes brown to tan colored, more or less circular lesion on infected berries (Fig. 1A). These lesions develop brown concentric rings in which numerous fruiting bodies (acervuli) reside. Pinkish to salmon-pink to orange masses of conidia (spores) can be observed on these fruiting bodies with a low magnification lens (10x) or even with the naked eye (Fig. 1A). These conidia can cause secondary infection on healthy berries [3]. Once a berry is covered with acervuli, it will shrink and become mummified [1].

Both *C. acutatum* and *C. gloeosporioides* are common pathogens of other crops and noncultivated plant species [4]. *C. gloeosporioides* also causes ripe rot on muscadine grapes [5], and it is one of the economically important diseases for table grape production in Asia [3]. In addition, *C. acutatum* and *C. gloeosporioides* cause bitter rot on apple and pears [4] and crown rot and anthracnose fruit rot on strawberries [6].

Although both *C. acutatum* and *C. gloeosporioides* are economically significant pathogens on many crops, the life cycle of ripe rot pathogens on grape has not been well studied [4]. Thus, our lab has been working to determine the effects of infection conditions (temperature, wetness, relative humidity, and cluster development stage), using inoculation studies as well as microscopies. Our preliminary results showed that both *C. acutatum* and *C. gloeosporioides* can cause infection from bloom to harvest, but symptom development typically happens near harvest (Nita *unpublished*).

Typically, our recommendations on chemical management of ripe rot are the use of mancozeb, ziram, captan, and 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 to indicate some *C. gloeosporioides* strains are not sensitive to captan [7]. In fact, the aforementioned vineyard with 30% loss relied on captan on their late season disease management; yet, remained unable to manage ripe rot. Thus, the importance of QoI fungicide is high, especially dealing with late season ripe rot outbreak.

Figure 2
"Typical" cultures from our collection



In 2012-14 seasons, our lab collected over 450 *Colletotrichum* isolates from VA vineyards. To our surprise, about 50% seems to be *C. gloeosporioides*, based on colony and spore morphologies. Since it is often assumed *C. acutatum* is a predominant species in vineyards, more than the expected number of *C. gloeosporioides* in our sample maybe the artifact of consecutive captan applications. However, our species identification methods are somewhat subjective, considering number of studies revealed that both *C. acutatum* and *C. gloeosporioides* are composed of multiple species, rather than just single species [8-13]. Our culture collections also show wide variety of colony as well as spore morphologies (Fig. 2).

In this proposal, we are requesting support to investigate 1) potential QoI resistance and 2) species identification of our collection of *Colletotrichum* species. Since ripe rot will affect grape yield and wine quality, the proposed objectives meet research priorities suggested by the VA Vineyard Association's annual survey (impact on fruit/wine flavor is listed #1 on both viticulture and enology section). The proposed research also meets research needs criteria of National Grape and Wine Initiative's Vision 2012 (the research priority 1: understanding and improving quality and 4: extension/education), as well as that of the National Road Map for Integrated Pest Management. Moreover, since both *C. acutatum* and *C. gloeosporioides* cause important diseases on strawberry and apple, our results can be applied to other regions of Southern US.

Methodologies

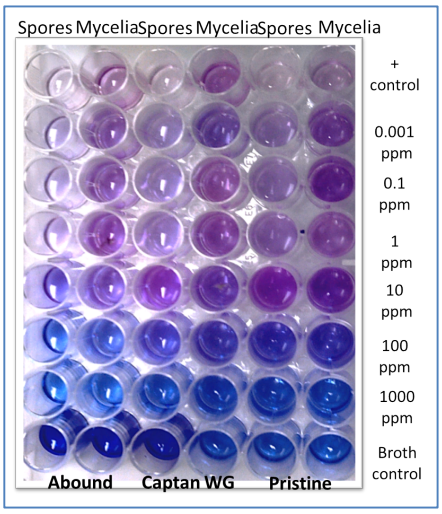
Isolate collection (completed in 2013-2014): In order to identify potential regional differences, growers were selected randomly from each of five major grape growing regions of Virginia (VA). The northern region spans from the Washington D.C./Arlington area west towards the Appalachian Mountains and 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 and 7 in 2014 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 the lab, skin from the berry was cut into approximately 5 x 5 mm piece, and the tissue was surface sterilized using 70% EtOH for 10 sec, followed by 0.53% hypochlorite (i.e. 10% bleach) solution for 1.5 min, and rinsing 1.5 min with autoclaved H_2O. Then the tissue was placed onto ¼ PDA amended with streptomycin and chloramphicol (both are 100 mg/ml). Once colony growth was observed, a hyphal tip was transferred onto a new Antibiotic ¼ PDA. Once a clean colony was established, single spore isolations were made. Five- to seven-day old culture was flooded with autoclaved H_2O, and the media surface was gently scraped with a sterilized toothpick. Then the spore suspension was filtered with two layers of Miracloth (EMD Millipore) to remove mycelium. The spore suspension was plated onto a water agar and individual spore was picked for further culturing. As of October 2014, we are estimating 450 of our collections were either *C. acutatum* or *C. gloeosporioides* based on colony morphology and spore characteristics.

Fungicide sensitivity tests: We employed Alamar blue (AB) in the fungicide amended culture plate [14], and then we also used more traditional fungicide amended media. The single-spore isolate plates were flooded by

adding 5 ml of clarified, buffered 2% Potato Dextrose Broth (PDB). Then, the suspension was filtered using two layers of Miracloth to remove mycelium. Then, 100 µl of a suspension of 10^5 conidia/ml (adjusted using a hemocytometer) or 100 µl of 2% PDB with aerial mycelium was 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, and 100.0 µg/ml). AB dye (AbD Serotec) was added as 10% of the final volume in the test wells [15]. Plates were 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 was negative control (200 µl of 2% PDB and 10% AB dye only), and positive control (100 µl of PDB, 100 µl of 10^5 conidia/ml, and 10% AB dye). A chemical control plate was also prepared to ensure that the fungicides themselves did not reduce the AB dye (100 µl of stock fungicide, 100 µl of 2% PDB, and 10% AB).

Figure 3 AB plate test example



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 [15]). A negative test result was 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) Abound (Azoxystrobin, Syngenta), 2) Flint (Trifloxystrobin, Bayer CropScience), 3) Pristine (Pyraclostrobin + Boscalid, Syngenta: will obtain Pyraclostrobin only), 4) Sovran (kresoxim-methyl, BASF), and 5) Captan 50W (Arysta LifeScience). All fungicide treatment will be evaluated with or without SHAM (salicylhydroxamic acid, (100 µg/ml) [16, 17]. Experiment was repeated six times per isolate to validate the results.

As of Dec 2015, we have been working on some of amended media assay to complete our objectives. At this point, we have decided to focus on QoI fungicide since the preliminary results indicated that the resistance is wide spread. A 5 mm diameter agar block was 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 QoI-amended PDA that is amended with 100 ppm of azoxystrobin and 1,000 ppm of SHAM. Plates were incubated in the dark at 25°C for 6 days. The growth of the mycelia was recoded at day 3 and day 6.

Validation of G143A mutation in samples (PCR-RFLP)

DNA Extraction: Mass of mycelium from each culture plate were placed into grinding bags (BIOREBA, Switzerland) containing 2 ml of a filter-sterilized grapevine extraction buffer (1.59 g/liter Na_2CO_3, 2.93 g/liter $NaHCO_3$, 2% Polyvinylpyrrolidone-40, 0.2% Bovine Serum Albumin, and 0.05% Tween 20) (Sigma-Aldrich Co. LLC, St. Louis, MO), and gently ground using a mechanical grinder (BIOREBA, Switzerland, Homex 6 [115V]). (It seems that the grinding is not necessary, but we are still tweaking the methodology.) Crude extracts were then transferred into 1.5-ml microcentrifuge tubes and stored at -80°C until the next step.

PCR: To amplify pathogen cytochrome *b* gene fragments from total DNA, the following PCR primers [18, 19] was used: GCCBF1& RSCBR2 (Ishii et al., 2001) (GCCBF1 = 5'-TTTCTTGGGTTATGTTTTACCTTA-3', and RSCBR2 = 5′ -AACAATATCTTGTCCAATTCATGG-3). PCR reaction mixtures contained total DNA, 1.5 mM MgCl2, 200 µM each dNTP, and 2.5 units of *Taq* DNA polymerase. PCR conditions were 2.5 min at 94°C, followed by 40 cycles of 0.5 min at 94°C, 1 min at 52°C, 1.5 min at 72°C, a final extension for 8.5 min at 72°C, and holding at 4°C. PCR products were separated by electrophoresis on a 1.5% agarose gel in 40 mM Tris- acetate (pH 8.0) and 1 mM EDTA (TAE) buffer and stained with GelRed (Biotium, Inc. Hayward, CA). The PCR product (~120 bp) was then treated with a restriction enzyme SatI (=Fnu4HI). The PCR products from resistant isolates carrying the mutated sequence GCN at position 143 were digested by the restriction enzyme, whereas those from sensitive isolates remained undigested. Or in the case of the heteromorphic mitochondrial DNA, there were more than two lines observed.

Species identification

Based on the observations on colony morphologies among isolates from different locations (grower/vineyard/sample site) and crops, we selected 360 samples for species ID. Using the extracted DNA (culturing of isolates and DNA extraction methods were described above.), following methods was performed to identify species. A species-specific internal transcribed spacer region 1 (ITS1) primer and the conserved universal primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') encoded in the 28S ribosomal subunit were used in pairs to identify isolates to species [20]. The ITS1 primers used were either the *C. gloeosporioides* specific ITS primer 5'-GACCCTCCCGGCCTCCCGCC-3' or the *C. acutatum* specific ITS primer 5'-GGGGAAGCCTCTCGCGG-3' [9, 21]. Isolates were assigned to the species group by which a positive amplification with a specific ITS1 primer was obtained. The PCRs were performed in a total volume of 25 µL [12.5 µl GoTaq Green Master Mix (Promega) (2X), 1 µl species-specific primer (ITS1) (10µM), 1 µl conserved universal primer (ITS4) (10µM), 2 µl DNA, and 8.5 µl H_2O]. The PCR conditions for the *C. gloeosporioides* specific/ITS4 pair were 5 min at 94 °C, then 26 cycles of 94 °C for 1 min, 60 °C for 2 min, 72 °C for 2min, and then 7 min at 72 °C. The PCR conditions for the *C. acutatum* specific/ITS4 pair were 5 min at 94 °C, then 32 cycles of 94 °C for 1 min, 60 °C for 2 min, 72 °C for 2min, and then 7 min at 72 °C. The resulting amplification products were verified by electrophoresis described above.

After initial screening using ITS sequences, we employed other genes to further specify species. These are CAL (Calmodulin), GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase), and TUB (beta-tubulin 2).

CAL_CL1C_Foward:	GAA TTC AAG GAG GCC TTC TC (Weir et al., 2012)
CAL_CL2C_Reverse:	CTT CTG CAT CAT GAG CTG GAC (Weir et al., 2012)
GD_Forward:	GCC GTC AAC GAC CCC TTC ATT GA (Templeton et al. 1992)
GD_Reverse:	GGG TGG AGT CGT ACT TGA GCA TGT (Templeton et al. 1992)
TUB2_T1_ Foward:	AAC ATG CGT GAG ATT GTA AGT (O'Donnell & Cigelnik, 1997)
TUB2_Bt2b_ Reverse:	ACC CTC AGT GTA GTG ACC CTT GGC (Glass & Donaldson, 1995)

Once sequencings were completed, sequences were trimmed and aligned using Lasergene 9.1 software. Each consensus sequence were queried against GenBank and Qbank databases. In addition, phylogenetic analyses leveraging maximum likelihood and Bayesian methods were conducted to identify species of *Colletotrichum* in our collections. Colony morphology of the each culture on ¼ PDA, as well as spore size and characteristics were also recorded to aid our species identification.

Results

Fungicide sensitivity test with AB dye

Table 1 shows the results from our preliminary experiments. EC50 (Effective concentration with 50% inhibition) was determined by estimating the intercept and slope using a generalized linear model in SAS (PROC GENMOD, ver. 9.4, SAS institute, Cary, NC) where logit was used as a link function. Then, effective concentration with 50% (EC50) and 80% (EC80) of inhibition (i.e., no change in color) were then estimated using a nonlinear mixed model (PROC NLMIXED in SAS), using the estimated intercept and slope from the generalized linear model. Based on the assay, we did not find significant difference ($P < 0.05$) between two isolates (*C. acutatum* and *C. gloeosporioides*), and many of fungicide resulted in less than field rate EC50. However, one of fungicides that showed an excellent efficacy with our table grape assay (*data not shown*), Mettle, did not work with AB assay. We used a buffer (sodium bicarbonate) to increase the pH of Mettle treated well; however, based on the concentration, it should not affect the efficacy of the fungicide (Table 1). As with any other *in vitro* tests, tests using a live plant tissue should be conducted to validate true efficacy. The poor performance by Endura was also shown with our table grape assay, thus, SDHI group might not have efficacy against *Colletotrichum acutatum* and *C. gloeosporioides*.

Table 1. Estimated effective concentrations (EC50 and EC80) of ten commercial fungicides and three assay additives for control of mycelial growth and conidial germination for the pooled data of C. gloeosporioides (CTCH1A11A) and C. acutatum (ACAP1A11A) determined in alamarBlue® in vitro assay.

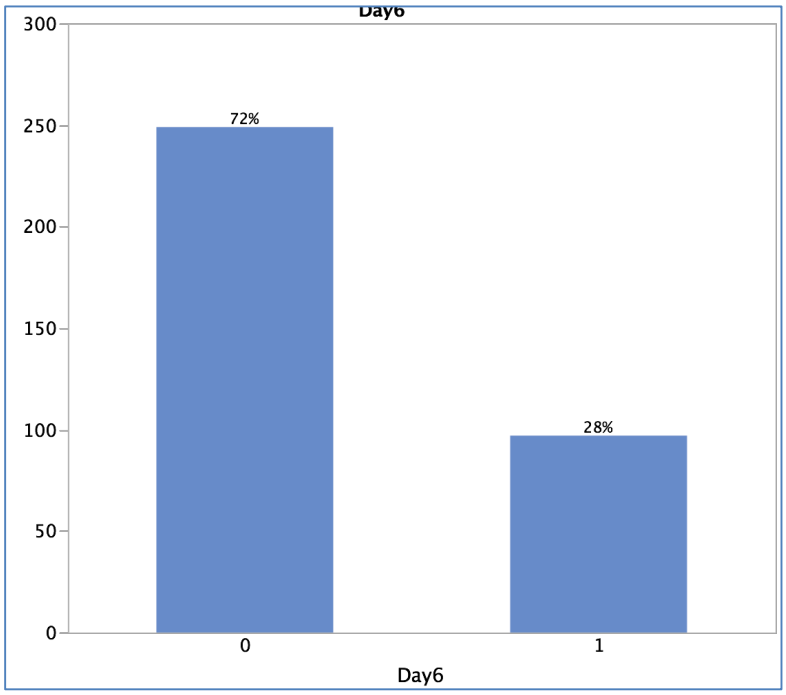
Propagule	Fungicide	Intercept[Z]	Slope[Z]	EC50[Y] (ppm)		EC80 (ppm)	Rate per Acre	PPM[X] in field
Mycelia	Abound®	2.50	-1.00	608.63	+	9260.60	355 mL	215.3
	Captan Gold™ 80 WDG	3.64	-2.16	47.99		129.31	907 g	1922.2
	Champ® Dry Prill	7.57	-3.88	88.05		152.31	1361 g	2775.2
	Cueva®	273.77	-137.49	97.01		98.53	18927 mL	2000.0
	Dithane® 75DF Rainshiel	4.18	-3.09	21.37		43.24	1361 g	1333.5
	Endura®	19.21	-6.33	1087.66	+	1518.61	237 mL	420.5
	Property®	14.98	-5.13	829.05	+	1251.05	148 mL	70.3
	Mettle® 125 ME	7.67	-4.63	44.60	+	70.95	118 mL	36.0
	ProPhyt®	8.14	-3.55	194.91		353.80	2366 mL	3382.9
	Topsin® M 70 WDG	9.90	-3.68	490.55		871.20	454 g	841
Conidia	Abound®	7.01	-2.75	355.64	+	767.97	355 mL	215.3
	Captan Gold™ 80 WDG	3.73	-3.41	11.48		22.19	907 g	1922.2
	Champ® Dry Prill	5.66	-3.67	33.97		61.18	1361 g	2775.2
	Cueva®	5.33	-3.32	39.37		75.29	18927 mL	2000.0
	Dithane® 75DF Rainshield	4.45	-5.08	6.53		10.40	1361 g	1333.5
	Endura®	11.68	-3.31	3357.09	+	6348.51	237 mL	420.5
	Property®	3.95	-0.82	63275.00	+	822967.00	148 mL	70.3
	Mettle® 125 ME	5.32	-3.71	26.11		46.84	118 mL	36.0
	ProPhyt®	12.53	-6.85	66.36		90.64	2366 mL	3382.9
	Topsin® M 70 WDG	8.8	-3.6	255.83		456.05	454 g	841
Supplemental chemicals used in the alamarBlue® assay								
Mycelia	Ethanol	256.59	-85.15	1030.18		1056.05		
	SHAM	258.68	-86.22	1000.00		1024.80		
	Sodium bicarbonate	11.86	-5.21	188.89		283.78		
Conidia	Ethanol	36.98	0.09	-1.00	o	-1.00	o	
	SHAM	37.38	0.14	-1.00	o	-1.00	o	
	Sodium bicarbonate	7.49	-4.05	69.52		117.67		

[Z] Intercept and slope were calculated using PROC GENMOD of SAS (ver. 9.4).
[Y] Concentration that effectively suppresses 50% or 80% of growth of mycelia or germination of conidia (EC50 and EC80) was calculated using PROC NLMIXED of SAS. A '+' denotes an EC that is higher than the labeled concentration for field applications. A 'o' denotes an EC could not be calculated for the chemical
[X] PPM = parts per million (μg/mL) of active ingredient applied recommended by the label in a field application in Virginia vineyards based on application volume of 100 gallons per acre (or 935 liters per ha)

QoI resistance

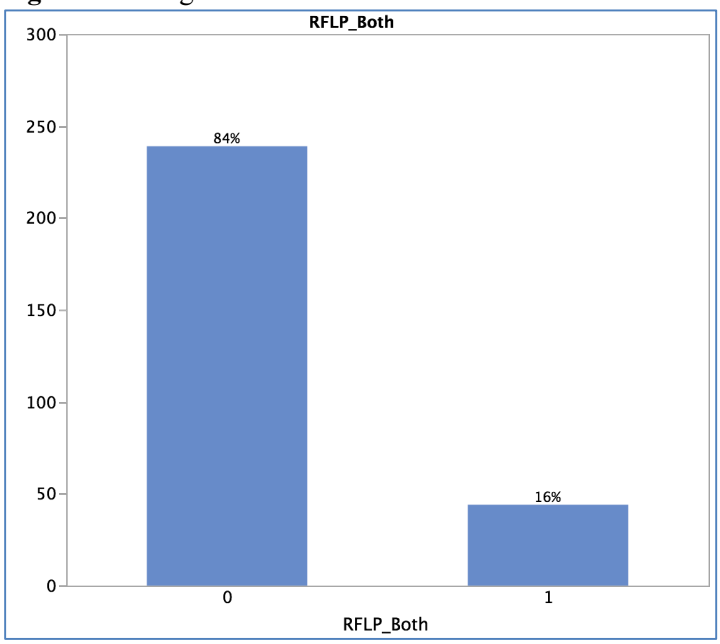
Fig. 4 shows that mycelial growth of day 6 on the azoxystrobin (+ SHAM) amended media, which indicate 28% of our 346 samples were able to grow at 100ppm.

Fig. 4 Percentage of isolate that is able to grow on a medium amended with 100 ppm azoxystrobin and 1,000 SHAM (noted as '1' in the figure), measured at day 6 of incubation.



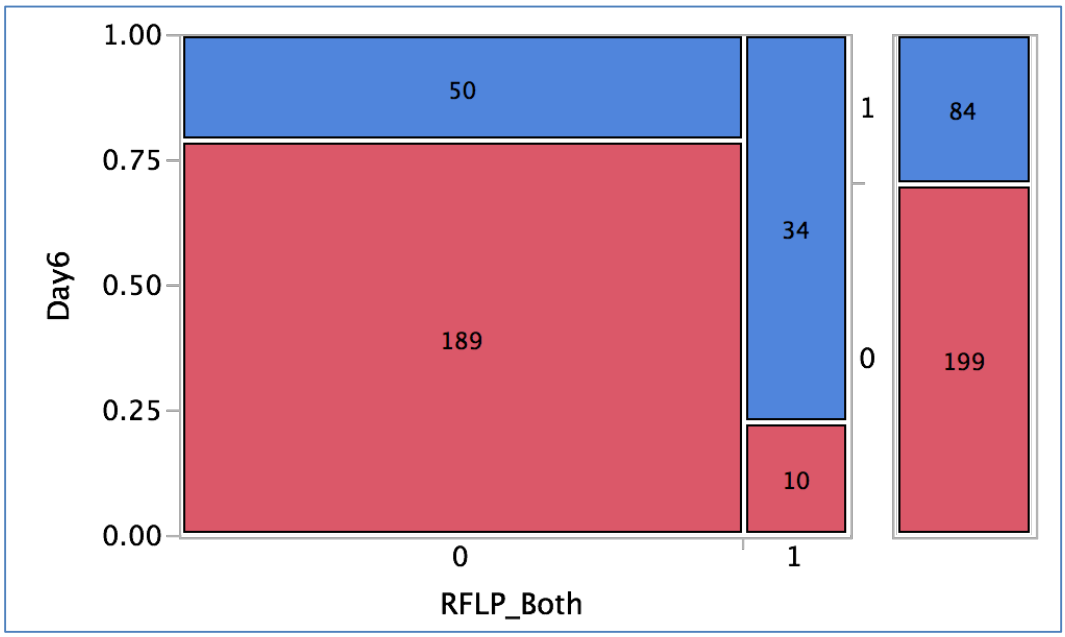
In addition, the validation of G143A mutation indicated that 17% of our sample contained G143A mutation in some degree (Fig. 5). Since *C. acutatum* and *C. gloeosporioides* contain heteromorphic mitochondrial DNA, some isolates contained mixture of mitochondria with and without G143A mutation.

Fig. 5 Percentage of isolate with G143A mutation based on PCR-RFLP



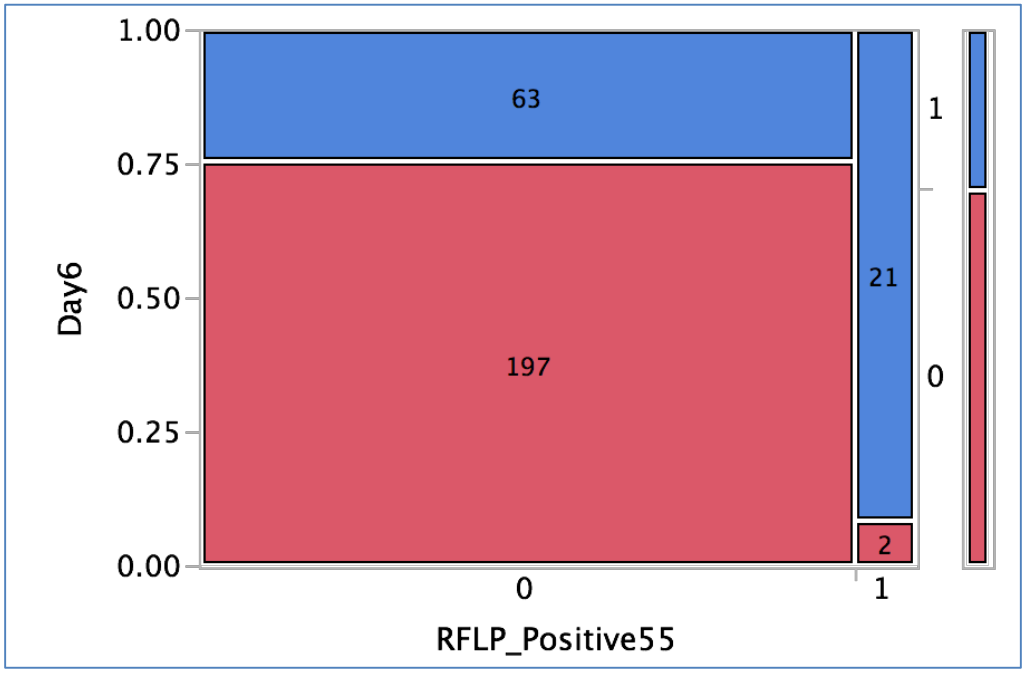
Then the relationship between the PCR-RFLP and fungicide amended media results are compared. Fig 6. Shows the number of isolate that contained G143A mutation (X-axis) and number of isolate that were able to grow on the QoI-amended media (Y-axis). Fifty of isolate that does not contain G143A mutation were able to grow on the QoI-amended media, indicating that there might be other resistance mechanism (e.g., F129L and Y275N) involved. In addition, ten isolates were not able to grow on the QoI-amended media even though they contain G143A mutation.

Fig. 6. Contingency table to describe number of isolate with G143A mutation and isolates with mycelial growth at day 4 on the QoI-amended media



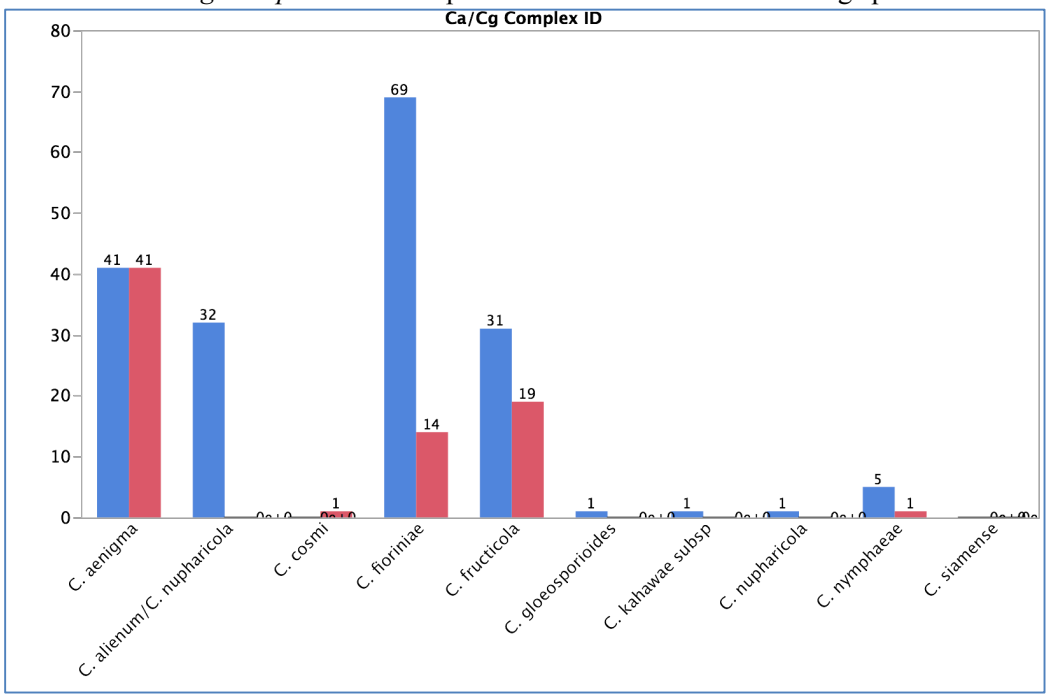
However, we remove the isolates shows evidence of heteromorphic mitochondrial DNA (i.e., three lines present after the restriction enzyme treatment), and the analysis was done with isolate that did not show mixture of mitochondrial DNA (Fig. 7). Then there were only two isolates that were not able to grow with the possession of G143A mutation. These two isolates were from the same vineyard (= most likely be processed at the same time), and will be reviewed in 2016.

Fig. 6. Contingency table to describe number of isolate with G143A mutation (without sign of heteromorphic mitochondrial DNA) and isolates with mycelial growth at day 4 on the QoI-amended media



Species identification: We are still in a progress of species ID. We had some difficulty in the sequence quality, which made us very difficult to obtain enough length of DNA to be used. However, based on the preliminary analysis, we are certain that our isolates were classified into two species of *C. acutatum* complex (*C. fioriniae and C. cosmi*) and seven or eight species of *C. gloeosporioides* complex (*C. aenigma, C. alienum/C. nupharicola, C. fructicola, C. gloeosporioides, C. kahawae, C. nupharicola, C. nymphaeae, and C. siamense*). *C. fioriniae, C. aenigma, C. alienum/C. nupharicola,* and *C. fructicola* compose majority of our sample (Fig. 7).

Proportion of isolates that were able to grow on the QoI-amended media was also examined (Fig. 7). Many of commonly found species, with an exception of *C. alienum/C. nupharicola*, contained considerable number of isolates that can grown on the QoI-amended media, confirming our concern on the selection of QoI-resistant isolate in the field. Our results also indicated that the susceptibility to a chemical among species in *C. acutatum* and *C. gloeosporioides* complex seemed to be different among species.



Conclusions

We have confirmed that quick screening method using the AB dye works with *Colletotrichum acutatum* and *C. gloeosporioides*. It still needs some modifications (e.g., timing of dye placement, especially with mycelial samples) to increase the quality of the test, and we are working on it. Our results from the fungicide screening showed that some products (cupper, DMI, and phosphonate) were promising candidates for ripe rot management. We also revealed that nearly 30% of our samples were insensitive to the QoI, and the degree of it varied among ten *Colletotrichum* species we identified from wine grape samples. It seemed that some of prevalent species contained considerable percentage of resistant isolate, indicating that the use of QoI might be a part of species selection pressure.

Impact

Since we have very limited number of options for chemical management of ripe rot of grape, our findings have a significant impact on the management of this disease. Moreover, since our results suggested the variation of fungicide sensitivity among *Colletotrichum* species, further study is warranted to determine the best materials for each species. In addition, the development of relatively rapid fungicide screening method can benefit wide array of researchers as well as growers.

Also, it is difficult to estimate the impact of the project; however, it is possible to estimate the impact of the disease. The worst case we have observed is about 30% loss of crop as fruit rot across different cultivars (i.e., both reds and whites). The average-sized vineyard/winery in VA produced about 5,000 cases of wine, and each wine was priced around $20, thus, in theory, the winery lost about $360,000 (5,000 case x 12 bottles/case x

$20/bottle x 30%) per year. Moreover, if they can sell the rest of flavor-compromised wine, it can damage VA wine's reputations. Overall quality of VA wines has been improved from less than ideal reputations from early 80's, to the point that some wineries produce $80-$100 bottle wines. We do not want to hurt our standings. Also, based on the communication with several wineries and wine makers, they have recognized the issues with ripe rot for a several seasons now. They have been reducing the risk by hand sorting the harvested berries, which cost both time and money to wineries.

Citations

My MS student, Ms. Charlotte Oliver is currently working on her MS thesis and publications, which will be ready in the Spring 2016 semester.

Related presentations

Oliver, C. and Nita, M. "Effect of cultivar and cluster maturity on ripe rot of grape caused by *Colletotrichum acutatum* and *Colletotrichum gloeosporioides*" at the American Phytopathological Society Annual Meeting, Pasadena, CA, 1-5 August 2015.

Oliver, C. and Nita, M. "Characterizing the Infection conditions and potential control methods of Ripe rot of grape, *Colletotrichum acutatum* and *C. gloeosporioides*" at the Virginia Vineyard Association Annual meeting, Charlottesville, VA, 7 February 2015

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