Virginia Wine Board
Project #: FY12WB11/30/03

Project Progress Report
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Deployment of Sentinel Vines, Further Studies on Powdery Mildew Fungicide Resistance, and Botrytis Survey

Report prepared by Lynn Rallos and Anton Baudoin

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Objective 1. Continue development and implementation of a method to monitor powdery mildew sensitivity to fungicides in individual vineyards through the use of sentinel vines treated with discriminatory dosages of individual fungicides, and relate data to fungicide field performance and bioassay results.

Sentinel plant experiments completed in 2011 have been reported in the previous semi-annual report. In 2012, one additional experiment has been initiated, to monitor for possible boscalid and quinoxyfen resistance in a northern Virginia location. No results are available as of yet.

Several additional experiments to determine discriminatory dosages for DOWNY mildew fungicides have been conducted. Updated estimates of minimum inhibitory rates for several fungicides are shown in Appendix Table A1 (Appendix, updated version of Table 9 of the previous report).

Objective 2. Determine fitness costs associated with QoI-resistance of powdery mildew

Laboratory-scale experiments and a 4-year survey of QoI resistance of powdery mildew populations in one vineyard have indicated that QoI resistance persists even without QoI selection pressure. To confirm this phenomenon under field conditions, we conducted an inoculation experiment in 2011 at the Glade Road Research Center, Virginia Tech. This involved infecting fungicide-free potted grape plants with a mixed spore suspension consisting of resistant and sensitive powdery mildew isolates. Results for the 2011 field test were reported previously: the resistance proportion dropped quickly, possibly due to background infection by sensitive populations coming from the infected plants or from nearby gardens. In order to confirm or refute these results, we initiated a repeat of the field trial with several modifications in **June 2012**. Modifications included using a 50 resistant:50 sensitive inoculum and closely monitoring the first appearance and severity of disease on non-inoculated and inoculated plants. Four plants per treatment (mixed, resistant only, sensitive only, non-inoculated control) were used, and new plants were added two

weeks after inoculation to provide new host tissue for colonization. Leaves were sampled from all plants on June 15 and June 28. DNA samples will be subjected to real time PCR for quantitation of the G143A mutation that is associated with resistance.

Objective 3. Continue development of molecular assays: characterize the cytochrome b gene in E. necator strains of moderate resistance that do not have the G143A mutation, and continue search for EBI resistance mutations in our powdery mildew collection and correlate with resistance phenotype to different EBI fungicides.

QoI resistance mechanisms. A high percentage (89%) of our isolates collected in 2005-2008 had a high level (>95%) of the G143A mutation. However, the remaining 11% had none. We are attempting to test three hypotheses to explain the resistance not associated with the G143A mutation: (1) other point mutations in the *cytb* gene; or (2) the presence of the mutation below detectable levels, and (3) the existence of an alternative oxidase (AOX), which has been associated with a lower level of resistance in other pathogens such as *Botrytis cinerea, Venturia inaequalis* and *Blumeria graminis* f.sp. *tritici*, and which has also been recently reported for trifloxystrobin-resistance in *E. necator* isolates from Michigan (Miles et al. 2012).

To test the first hypothesis, we have been attempting to amplify the entire cytochrome b gene from *E. necator*, but have found that the region of interest is difficult to amplify in regular PCR amplification. This suggests the presence of a large intervening sequence within that region or a secondary structure (e.g. hairpins) in the DNA. The addition of PCR enhancing agents such as dimethyl sulfoxide (DMSO) or betaine will be attempted to improve amplification.

To test the second hypothesis, we have grown three QoI-resistant isolates on strobilurin-treated (3 mg/L) and strobilurin-free host tissue in parallel. After three transfers, the mutation was measured by real-time PCR to determine if exposure to the fungicide had allowed for quick selection of cells carrying the mutation in their mitochondria, but there was no change in the genotype of these isolates. We extended the parallel transfers to 28-29 cycles on grape leaves, and are in the process of completing the sampling for DNA extraction.

The third mechanism has been thought to have low importance in the field, but this is unconfirmed. We attempted to determine the presence of an AOX using AOX inhibitors salicylic acid hydroxamate/SHAM and propyl gallate. However, both had a very strong negative effect on spore germination, so that no clear conclusions on the contribution of AOX in QoI resistance could be drawn.

DMI resistance mechanisms. Three mechanisms for DMI resistance are known so far for plant pathogens: (1) point mutations in the CYP51 target, (2) over-expression of the target protein, and (3) increased efflux pump activity. We have explored these mechanisms in our isolates.

In our CYP51 **sequencing** project we found only a single amino acid change in codon 136 (Y136F), which was present only in tebuconazole-resistant isolates (Colcol, Rallos, Baudoin 2012). We have found two additional nucleotide differences (SNPs, Appendix Table A2) but these did not change an amino acid. Only Y136F was clearly associated with DMI-resistance.

A few of the tebuconazole-resistant isolates, which were also fenarimol-resistant, lacked the Y136F mutation, indicating another mechanism for DMI resistance. Furthermore, we found isolates that possessed both wildtype and mutant codons, TAT as well as TTT and denoted as TWT in Table A2, suggesting duplicate or multiple copies of the gene. Having more than one copy of the *cyp51* gene or its homologue is common in other fungal species (Da Silva Ferreira et

al. 2005). How this might relate to fungicide resistance in *E. necator* is still not clear; however, gene amplification is known to have conferred chemical resistance to some insect species (Devonshire and Field 1991). Having two variants of the gene might allow for differential expression in different environments (e.g. fungicide or no fungicide), or these variants may produce the same function but have different cell localization, or different substrate affinities. Alternatively, duplicate copies can simply increase the mRNA levels (over-expression) for more protein production to counteract the effect of the fungicide.

To confirm the genotypes from the sequencing data, we adopted a **SNP genotyping protocol** based on the Taqman chemistry. As summarized in Table A2, the SNP genotyping supported and even clarified ambiguities in the sequencing data, except for one isolate (VAHP6), which was reported 'wildtype' in the 136th codon by sequencing but 'undetermined' in the SNP genotyping method.

We also designed a protocol to determine if the CYP51 protein is **differentially expressed** in *E. necator*. The preliminary assay was done for one sensitive isolate (BLP4) and two resistant isolates with the TWT genotype. We found that CYP51 expression was increased more than 10-fold (RQ=13 and 17) in the resistant isolates (Fig. 1, Table A3). We have collected RNA samples from other isolates to expand on this preliminary finding.

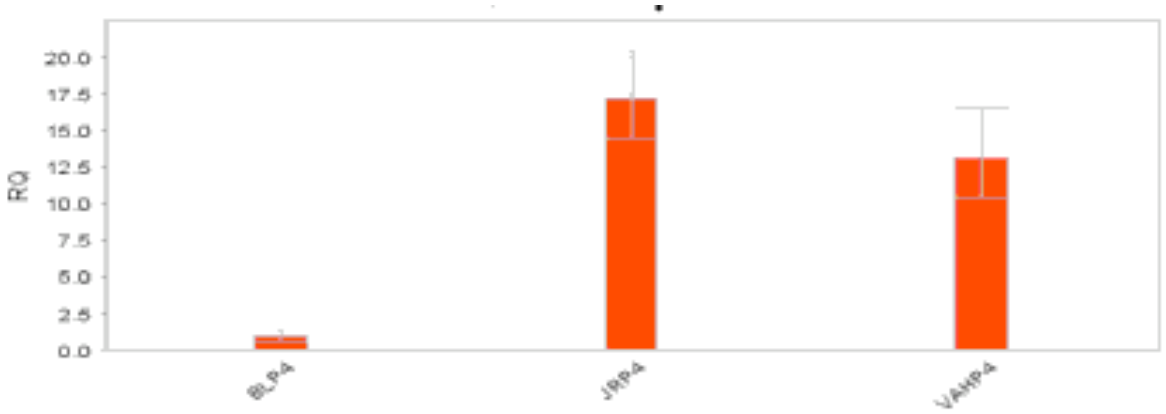

Fig. 1. Gene expression plot for CYP51 in sensitive (BLP4, left) and resistant isolates (JRP4, VAHP4, middle and right) grown on fungicide-free host tissue.

The **involvement of efflux pumps** in DMI resistance is also being investigated. We use the efflux pump inhibitors verapamil and CCCP (carbonyl cyanide-3-chlorophenyl-hydrazine). Efflux pumps in eukaryotes are known to expel fungicides and other foreign chemicals from the cell. In the presence of pump inhibitors, the cell loses the ability to do so, and exhibits greater sensitivity to the chemical. Experiments are underway to determine the effect of efflux pump inhibitors on DMI resistance of *E. necator*.

Our interest in the efflux pump mechanism stemmed primarily from observations of decline in resistance among isolates maintained in the laboratory for several years. We have noted an appreciable reduction for tebuconazole and slight reduction for fenarimol resistance (Table A4). The same isolates are being assayed at present to test for a decline in myclobutanil. Our results indicate that DMI resistance could be lost through several generations on DMI-free host tissue. This has obvious implications for the use of DMI fungicides for disease control and fungicide resistance management.

To verify these observations, we collected fresh field isolates in 2010 and 2011. Most of the isolates exhibited a declining degree of resistance to tebuconazole (Tables 1 and A5). In 2012, we treated field plots with Elite (tebuconazole), Rally (myclobutanil) and Inspire Super (difenoconazole) to induce resistant populations to develop. From these populations, we are screening for highly resistant powdery mildew isolates to determine how rapid this resistance decline occurs, and if it occurs for all three DMIs tested.

Table 1. Sensitivity shifts (mean resistance factor=RF=concentration needed to inhibit isolate, divided by concentration needed to inhibit sensitive reference isolates) in isolates from sentinel plants, collected 2010.

2010 Isolates	*Initial RF_{teb}	*Final RF_{teb}
SC10-15	115	73
SC10-17	41	30
SC10-19	63	7
SC10-20	196	37
SC10-21	148	63
AF10-22	493	56
AF10-23	70	30
RO10-25	185	4
RO10-34	156	4
RO10-21	148	<37

*Initial RF observed after 4-6 transfers on DMI-free leaves from field collection. Final RF after an additional 4-6 transfers following the initial bioassay.

Objective 4. Initiate a summer/fall 2011 survey of Botrytis to determine sensitivity status to cyprodinil/pirimethanil, boscalid, fludioxonil, and iprodione, and continue to monitor fungicide resistance of other high-risk grape fungal pathogens with emphasis on vineyards reporting unexpected problems.

Botrytis samples from 26 vineyards, mostly in Virginia but including a few in the Yadkin Valley of North Carolina were collected or received in the fall of 2011. Table A6 lists results for anti-Botrytis fungicides registered for use in Virginia grapes that are thought to be subject to resistance development due to their single-site mode of action.

Not previously documented in Virginia grapes, but Flint (QoI or strobilurin, FRAC group 11) resistance in Botrytis was VERY common (84%, present in 25 of 26 vineyards). This follows the widespread strobilurin resistance of grape powdery and downy mildew in our area. Although only Flint was tested, Abound, Sovran, and the QoI component of Pristine are expected to be affected equally based on results from other areas.

Boscalid resistance was also common: 61%, present in 23 of 26 vineyards. Boscalid is marketed as Endura, and in combination with QoI, as Pristine, and in locations where both QoI and boscalid resistance are found, both Endura and Pristine are likely to suffer control failure. 71% of all isolates was resistant to both components of Pristine, and this combintion was detected in 23 of 26 vineyards.

With respect to the Vangard and Scala mode of action group (FRAC group 9), only Vangard has been tested so far. Results are more difficult to classify than for other groups for the following reasons: (1) a number of different types of resistance have been described in the literature, spanning the range from high, to moderate to low, making it difficult to distinguish distinct groups in the test results; (2) two different nutrient media have been used in our tests, and results often depend on the medium used. Some isolates grow poorly on one or the other medium or both, even without fungicide, making it difficult to interpret equally poor or slightly poorer growth in the presence of the fungicide. Nevertheless, as many as 30-40% of isolates tested did not have normal sensitivity and exhibited various degrees of resistance, and these were collected in 19 of the 26 vineyards. Additional work is needed with this group.

With respect to the fludioxonil component of Switch (a mix of fludioxonil and cyprodinil, the latter being the same active ingredient found in Vangard), only a slightly reduced level of sensitivity (very low degree of resistance) was found in 8 or 129 isolates tested. Hight levels of resistance have also not been reported elsewhere in the world. Low levels of fludioxonil resistance have been shown to be due to a "multi-drug resistance" mechanism elsewhere, but this has not yet been demonstrated in our isolates.

No fenhexamid (Elevate) resistance was found in the fall 2011 grape samples tested. However, one Botrytis isolate resistant to fenhexamid was obtained from a pelargonium (geranium) plant purchased from a garden center in Christiansburg, VA, Elevate resistance has been found in a greenhouse in Pennsylvania, in strawberry fields in Florida, California, and the Carolinas, in grapes in Germany and in other countries at considerable frequencies, so it would not be at all surprising if we were to find it in Virginia.

With respect to the older fungicides that have seen less use in recent years, Topsin M resistance was common (69%, present in 25 or 26 vineyards). Low-level Rovral resistance was found in 34% of isolates, and 18 of 26 vineyards, in all regions. This reduced Rovral sensitivity is likely to hamper its performance but may not produce outright control failure. High-level Rovral resistance was not found in this study.

Presentations 2012

Baudoin, A., L. Rallos, M. Nita, and G. Giese. 2012. Sentinel vines to monitor fungicide resistance in grape powdery mildew. Annual meeting of the Potomac Division of the American Phytopathological Society, Winchester, VA. March 16, 2012.

Publications 2012

Baudoin, A. 2012. Botrytis fungicide resistance in Virginia: preliminary report. Viticulture Notes, Vol. 27 No. 1, April 2012, pages 3-4.

Baudoin, A and L.E. Rallos 2012. Evaluation of fungicides for control of grape powdery mildew, 2011. Plant Disease Management Reports 6: SMF019.

Detailed appendix data may be obtained by contacting: Kay Thompson, Virginia Vineyards Association, 1122 Roses Mill Road, Amherst VA 24521, Phone: (434) 277-9463, Email: vvaresearch@gmail.com.

Appendix to July 2012 Report on
Project #: FY12WB11/30/03. Deployment of Sentinel Vines, Further Studies on Powdery Mildew Fungicide Resistance, and Botrytis Survey
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Impact statement

Newly documented cases of widespread resistance to fungicide used against *Botrytis cinerea*, the cause of Botrytis bunch rot of grapes, will help growers used effective fungicides rather than those that no longer work.

Additional Tables and References Cited

Table A1. Anti-downy mildew fungicides, their mode-of-action (FRAC) grouping, and preliminary estimate of maximum dilution that would still give near-complete disease control.

Trade name	Common name	FRAC Group	MDR [a]	MIC [b]
Ridomil (used solo for this test)	Mefenoxam	4	200	
Abound	Azoxystrobin	11		
Ranman	Cyazofamid	21	100-200	0.4-0.9
Tanos (incl. famoxadone)	Cymoxanil	27 (+ 11)	5 [c]	30
Aliette	Fosetyl Al	33		
Prophyt, Fosphite	Phosponate	33	4 [d]	250
Forum	Dimethomorph	40	50-100	2.3-4.7
Revus 250	Mandipropamid	40	200	0.4
Presidio	Fluopicolide	43	100-200	0.75-1.5

[a] Maximum dilution range that still gives near-complete disease control, expressed relative to labeled full field rate calculated on basis of 100-gallon application volume
[b] MIC= minimum inhibitory concentrations (ppm or mg/liter active ingredient)
[c] Use of a strobilurin (Group 11)-resistant isolate allows determination of maximum dilution range for the cymoxanil in this combination product
[d] Gradual loss of disease control in this dilution range

Table A2. SNP characterization in *EnCYP51* gene of resistant and sensitive isolates.

Code	136th codon from sequencing[a]	SNP Genotyping[b]	SNP 2 nt[c]	SNP 3 nt[d]
BLP1	TAT	wildtype	A	G
BLP4	TAT	wildtype	A	G
MVP1	TAT	wildtype	A	A
MVP5	TAT	Wildtype	A	A
MVP9	TAT	Wildtype	A	A
PBP1	TAT	Wildtype	A	A
SCCP4	TAT	Wildtype	A	A
SNP1-A	TAT	Wildtype		
SNP1-B	TAT	Wildtype		
SNP3-1	TAT	Wildtype		
SNP3-2	TAT	Wildtype		
BLP11	-	wildtype		
FH9-1	TAT	-		
VAHP6	TAT	Mix or WT?	C	G
BXP1A	TTT	Pure mutant	A	A
GRP15	TTT	Pure mutant	A	A
GRP18	TTT	Pure mutant	A	A
IVP3	TTT	Pure mutant	A	A
IVP11	TTT	Pure mutant	A	A
MDMRP5	TTT	Pure mutant	A	A
MDMRP7	TTT	Pure mutant	A	A
PRP7	TTT	Pure mutant	A	A
ROP14	TTT	Pure mutant	A	A
SUP13-2	TTT	Pure mutant	A	A
VAHP1	TTT	Pure mutant	A	A
MDMRP3	TTT	Pure mutant	A	A
AMP1	TWT	Mix	C	R
VAHP4	TWT	Mix	C	R
IVP4	TWT	Mix	C	R
JRP1	TWT	Mix	C	R
JRP3	TWT	Mix	C	R
JRP4	TWT	Mix	C	R
JWP1	TWT	Mix	C	R

[a] Based on sequencing results; corresponding to nucleotide 467 from translation start site
[b] Based on Taqman chemistry SNP genotyping
[c] Nucleotide 1086 from translation start site; synonymous mutation (Gly=GGG/A)
[d] Nucleotide 1170 from translation start site; synonymous mutation (Ala-GCC/A)

Table A3. CYP51 expression in *E. necator*.

Sample	Ct Mean	ΔCt Mean	ΔCt SD	ΔΔCt	RQ
BLP4	33.751	-0.0186	0.2087	0.0	**1.0**
JRP4	30.546	-4.1241	0.0896	-4.1055	**17.2**
VAHP4	31.923	-3.7418	0.1037	-3.7232	**13.2**

RQ - calculated relative level of gene expression for the replicate group that is associated with the test sample; calculated using the Ct values for CYP51 and the beta-tubulin gene as reference gene.

Table A4. Sensitivity shifts in tebuconazole for isolates transferred for more than 50 times on fungicide-free grape leaves.

	Tebuconazole			**Fenarimol**	
Isolate	RF-2008*	RF-2010	RF-2012	RF 2008*	RF 2011
AMP1	40	66	34	78	108
BXPIA	515	6	7	11	12
GRP15	69	34	12	44	33
GRP18	49	15	18	78	22
IVP3	82		2	11	0.1
IVP4	>714	35	23	33	56
IVP11	47	29			
JRP1	8	10	33	>11	13
JRP3	35	63	30	133	78
JRP4	248	58	24	33	50
JWP1	248	112		144	167
MDMRP5	39	18	22	22	11
MDMRP7	99	32	26	22	44
PRP7	>714	51	47	33	33
SUP13	246	5	6	33	9
VAHP1	33	9	10	11	9
VAHP4	233	66	15	189	56
VAHP6	>714	33	15	44	33
Ave. resistant	**>217.5**	**31.4**	**20.3**	**58**	**43**
BLP1	0.2	1	1	0	1
BLP4	0.3	1	1	3	1
BLP6	0.3	0.1		1	1
MVP9	0.04	0.4	0.6		1
MVP1	0.07				
MVP5	0.07			0.9	
SCCP4	0.2		2	0.2	
Ave. sensitive	**0.18**	**0.85**	**1.2**	**2**	**1**

*Data by JF Colcol (Colcol 2008; Colcol, Rallos and Baudoin, 2012)

Table A5. Sensitivity shifts (mean resistance factor=RF) in isolates from sentinel plants, collected 2011.

Isolates	Initial on Teb[a] RF	STW[b] RF	STW[c] RF
MR11-B3	104	28	20
MR11-B10	37	37	-
MR11-B2	100	33	-
MR11-B1	30	22	-
AF11-2	28	36	20
AF11-3	32	44	56
AF11-4	24	40	65

[a] Initial reading obtained after one single-spore transfer for AF11s and MR11-B1, and two single-spore transfers for all other MRs on teb 3 ug/ml-treated leaves

[b] Mean EC_{50} after one transfer from initial EC50

[c] Mean EC_{50} after additional transfers from b, for MR isolates = 9 transfers, for AF isolates =7 transfers

Table A6. Results of a resistance survey of *Botrytis cinerea* from Virginia grapes to single-site anti-Botrytis fungicides.

FRAC code	Fungicide	Trade names	Year[a]	2011 Resistance survey Vineyards	% [b]	Level
1	Thiophanate methyl	Topsin M	1973	25	69%	High
2	Iprodione	Rovral, Meteor	1979	18	36%	Low
7	Boscalid	Endura, component of Pristine	2003	23	61%	Mod-High
9	Cyprodinil	Vangard, component of Inspire Super, Switch	1998	19	NE	Various, moderate
9	Pyrimethanil	Scala	2005	--	--	
11	Trifloxystrobin	Flint, component of Adament	2000	25	84%	High
11	Azoxystrobin	Abound	1997	--	--	
11	Kresoxim methyl	Sovran	2000	--	--	
11	Pyraclostrobin	Component of Pristine	2003	--	--	
12	Fludioxonil	Component of Switch	2000[c]	8	6%	Very low
17	Fenhexamid	Elevate	1999	0	0	--

[a] Year: approximate year of introduction.

[b] Resistance frequency: number of isolates with resistance / number of isolates tested

[c] On other crops; registered for grape only in 2010

[d] --: not tested, but performance can be estimated based on compounds in the same FRAC (mode of action) group

References cited

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