Virginia Wine Board

Project Report
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Sentinel vines to evaluate powdery mildew sensitivity to fungicides on winegrapes

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Objectives

1. Evaluate effect of moderate ergosterol biosynthesis inhibitor (EBI) resistance of powdery mildew (PM) on effectiveness of EBI spray program, with emphasis on spray rate or frequency needed for adequate control.
   a. Determine field performance in vineyards with contrasting PM EBI sensitivities
   b. Relate field performance to EC50 values obtained in standard bioassays, and lab analysis of components of disease development (PM germ tube elongation, latent period, sporulation rate).
2. Continue to monitor fungicide resistance of grape pathogens (powdery and downy mildew), with emphasis on vineyards reporting unexpected problems, uncertainty about QoI (strobilurin) sensitivity, and vineyards with heavy use of boscalid or quinoxyfen, or metalaxyl (for downy mildew)
3. Estimate fitness of QoI-resistant PM population by initiating field experiments in commercial vineyards to determine possible decline of QoI resistance in absence of any QoI application.
4. Exploratory study to detect specific point mutations in the *CYP51* gene and promoter region of PM isolates with contrasting EBI sensitivities.

Objectives 1 and 2. Relating DMI resistance to control, and **continued resistance monitoring**

The 2010 season was more favorable than 2009 for powdery mildew (PM), which developed at all trial sites. Field tests, each accompanied by a set of "sentinel vines," in 2010 were conducted:

1. In Franklin County, VA (commercial vineyard, high PM pressure).
2. In Rockbridge County, VA (commercial vineyard, history of high DMI resistance)
3. At Surry Community College, NC, vineyard managed with a standard spray program
4. At Winchester Agricultural Research & Extension Center (standard and experimental fungicides)

Sentinel vines are potted plants that are sprayed regularly (usually weekly) with low rates of an individual fungicide to detect practical resistance in the local powdery (or downy) mildew population. Additional sets of sentinel vines were maintained and treated at:

5. Blacksburg, location as in 2008 and 2009 (relatively unexposed powdery mildew population)
6. 6 and 7. Two commercial vineyards in North Carolina

All sentinel vine rates are reported as ppm (mg/l) active ingredient. In Franklin Co. (cv. Aglianico, 3 vines per plot, 5 replications), neither Elite nor Vintage provided good control of powdery mildew on clusters; only the rotation of Endura and Quintec, both tank-mixed with sulfur, was effective (Table 1A). Control of foliar infection was significant but mediocre. On the sentinel plants, low rates of Elite and Vin-

tage provided little inhibition, but high rates suppressed powdery mildew development well (Table 1B).

Table 1A. Franklin Co. 2010 field trial, powdery mildew, percent coverage

Treatment, frequency and rate/A	PM Leaves Jul 5	WDa*	PM Clusters Jul 5	WDa	PM Clusters Aug 5	WDa
Untreated check	27.7	a	35.2	a	71	a
Elite, 7d schedule, 4 oz	1.6	de	20.0	a	46	c
Elite, 14d schedule, 4 oz	5.2	cd	28.8	a	56	abc
Vintage, 7d schedule, 4 oz	3.1	d	37.4	a	61	abc
Vintage, 14d schedule, 4 oz	12.5	b	34.5	a	67	ab
Vintage, 14d schedule, 6 oz	9.1	bc	23.7	a	50	bc
Vintage 3 oz + Elite 4 oz, 14d schedule	3.8	d	18.5	a	41	c
Endura/Quintec rotation+Sulfur, 14d	0.2	e	1.6	b	5	d

*WDa: mean separation by Waller-Duncan's test, k=100, on arcsine-transformed percentages.

Table 1B. Franklin Co.. 2010, sentinel vines, percent leaf coverage by powdery mildew

	Rate Jun-Jul, ppm	Aug 5 %PM	Rate after Aug5, ppm	Sep18 %PM	Oct2 %PM
Control	(water)	40	(water)	62	66
Elite	25	2	40	0	0
Elite	5	22	15	5	4
Vintage	10	2	15	0	0
Vintage	2	23	5	4	17
Quintec	2.5	25	10	17	14
Endura	5	1	5	1	4
Flint	25	27	25	42	50
Topsin	--	ND	200	80	67

In Rockbridge Co. (cv. Pinot Noir, 3 vines per treatment, 4 replications), the same rates of Elite and Vintage provided good control (Table 2A), which was reflected in better powdery mildew inhibition on sentinel plants (Table 2B). Somewhat higher sentinel rates were used, since past experience suggested greater tolerance but this did not materialize. At both locations, the Quintec 2.5 ppm rate allowed considerable mildew development and is apparently too low to serve as a discriminatory dose. The rate was raised to 10 ppm in the middle of August, but mildew development on young leaves continued.

At Surry CC (cv. Chardonnay, 3 vines per plot, 5 replications) severe mildew had developed on the field plot clusters by mid-July (Table 3A), and on the leaves by mid-August. Elite at 4 oz every 7 days and Endura plus sulfur every 14 days provided good control, but Rubigan and 4 oz Elite every 14 days provided mediocre to poor control. On sentinel plants, most treatments (except low rates of Vintage and Endura provided good inhibition of powdery mildew at Jul 21, but little inhibition on Aug 17, 2 weeks after the last application, when downy mildew had become severe on the foliage (Table 3B).

At Winchester (cv. Chardonnay, 1 vine per plot separated by unsprayed border vines, 4 replications), Rally, Quintec, Inspire Super and Vivando all provided very good powdery mildew control on leaves and clusters. Mildew was slow to develop on sentinel vines, but ratings in September showed poor control by Abound, 5 ppm Elite (but 25 ppm provided good control), 10 ppm DPX LEM-17 (penthiopyrad), 2 ppm Topguard (but 5 ppm provided partial control) and 0.5 and 2.5 ppm Quintec

In bioassays of powdery mildew isolates collected in 2010 (Table 5), EC50s for tebuconazole were 26-35 times higher than for fenarimol, and were similar at the three vineyard locations. This contrasted with the observation that there were no great differences between the efficacy of the two compounds in field and sentinel vine trials.

In 2009, sentinel vines detected clear differences in powdery mildew sensitivity at different loca-

tions. At a commercial vineyard in Hillsboro, Loudon Co, northern VA, no PM developed on plants

Table 2A. Rockbridge Co., 2010 field trial, powdery mildew, percent coverage

Treatment, frequency and rate/A	Leaves Jul 29	WDa	Clusters Jul 15	WDa
Untreated check	25.2	a	23.1	a
Elite, 7d schedule, 4 oz	0.01	c	3.1	de
Elite, 14d schedule, 4 oz	0.1	c	6.0	bc
Vintage, 7d schedule, 4 oz	0.05	c	3.5	cd
Vintage, 14d schedule, 4 oz	0.3	b	6.5	b
Vintage, 14d schedule, 6 oz	0.3	b	3.4	cd
Vintage 3 oz + Elite 4 oz, 14d schedule	0	c	2.0	de
Endura/Quintec rotation+Sulfur, 14d schedule	0	c	1.3	e

WDa: mean separation by Waller-Duncan's test, k=100, on arcsine-transformed percentages.

Table 2B. Rockbridge Co. 2010, sentinel vines, percent leaf coverage by powdery mildew

	Rate Jun-Jul, ppm	Aug 21 %PM	Rate after Aug21, ppm	Sep 11 %PM	Oct 2 %PM
Control	(water)	60	(water)		16
Elite	50	1	50		0
Elite	25	3	25		2
Elite	10	6	10		6
Vintage	25	0.4	25		0.1
Vintage	10	2	10		2
Vintage	2	2	5		2
Quintec	2.5	25	10		17
Endura	5	0	5	6	12
Flint	25	16	25		ND
Topsin			200		49

treated with fenarimol (lowest concentration 2 ppm), myclobutanil (lowest concentration 5 ppm) and trifloxystrobin (lowest concentration 50 ppm). Bioassay results confirmed that PM at this location was sensitive to fenarimol, mycobutanil, and strobilurins, and that downy mildew that developed on trifloxystrobin-treated plants was strobilurin-resistant. Isolates collected in 2009 at Winchester were relatively tolerant to DMI fungicides, whereas 2009 isolates from Linden (except one), were sensitive to DMI fungicides. These bioassay data correlated well with 2009 sentinel vine data. Three isolates from Linden control plants that were tested by PCR were negative for the G143A resistance mutation, whereas 2 isolates recovered from the few lesions on azoxystrobin-treated plants were positive, suggesting a presence but relatively low frequency of this mutation in the population.

Because of heavy downy mildew pressure in 2009 and to a lesser extent in 2010, and the possible need for control of insect pests such as Japanese beetles and other leaf feeders, a number of insecticides and fungicides were tested for their effect on powdery mildew. Young shoots were sprayed with a labeled rate of the formulated compound and allowed to dry. Expanding leaves were then harvested, placed in Petri dishes with water agar, and inoculated with powdery mildew. Percent coverage was evaluated after 7 days. Among fungicides, captan and mancozeb inhibited powdery mildew strongly, Prophyt had a moderate effect (56% inhibition), and Presidio, Revus 250SC and Ridomil SL had slight impact (less than 30% inhibition). Among insecticides, PM growth was strongly inhibited by Lorsban, Sevin, and Malathion (over 70% inhibition), moderately so by Danitol and Safari (40-60% inhibition), and only slightly or not at all by Avaunt and Provado (less than 10% inhibition).

Table 3A. Surry Community College 2010 field trial, powdery mildew, percent coverage.

	PM leaves		PM clusters		PM leaves	
	Jul 20	Wda	Jul 21	Wda	Aug 17	WDa
Control = water, 7 day	5.6	b	59.2	b	28.8	b
No treatment	50.6	a	95.5	a	53.9	a
Elite 4oz, 7 day	0.6	c	4.9	e	4.1	ef
Elite 4oz, 14 day	0.6	c	29.3	cd	16.6	bcd
Elite 8oz, 14 day	0.4	c	13.5	de	8.8	de
Rubigan 3oz, 7 day	0.1	c	29.7	cd	10.3	cde
Rubigan 4oz, 14 day	1.5	bc	43.7	bc	23.6	b
Rubigan 6oz, 14 day	1.4	c	60.2	b	18.4	bcd
Elite 4oz plus Rubigan 3oz, 14d	0	c	22.2	cd	23.3	bc
Endura 4.5oz + Sulfur 3lb, 14d	0	c	8.1	e	0.9	f

WDa: mean separation by Waller-Duncan's test, k=100, on arcsine-transformed percentages.

Table 3B. North Carolina 2010 sentinel vines, powdery mildew, percent coverage

Treatment, rate in mg ai/l		SCC 7/21	SCC 8/17	CV1[1] 7/21	CV1[1] 8/17	CV2[1] 7/21	CV2[1] 8/17
Control		13.5	10.9	29.0	10.6	12.5	22.2
Elite	25	0	6.3	0.7	14.5	0	11.7
Elite	10	0	13.0				
Elite	5	0	5.4	19.2	29.3	8.6	23.0
Quintec	5	0	5.6				
Quintec	2.5	0	16.4	6.2	9.1	2.5	14.0
Quintec	1	0	4.9				
Quintec	0.5	0	10.8				
Flint	50	0	13.7	3.8	5.9	1.2	12.0
Mettle	5	0	11.5				
LEM-17[2]	5	0	11.2				
Rally	25	0	7.0				
Rally	5	0	13.3				
Topguard	5	0	11.4				
Endura	10	0.5	9.5				
Endura	5	0.5	9.0	7.8	18.8	0.1	10.0
Endura	2	9.5	3.8				
Endura	1	10.0	13.3				
Vintage	10	0.5	2.6	0.0	22.4	0.0	8.3
Vintage	4	0.1	2.8				
Vintage	2	6.0	11.0	6.0	17.5	3.5	22.3
Vintage	1	4.4	8.8				
Inspire	1	1.5	18.4				

[1] Commercial vineyard

[2] Not currently expected to be commercialized for grape

Table 4. Sentinel vine 2010 trial, Blacksburg and Winchester, powdery mildew severity (Blacksburg) or incidence (Winchester) on leaves. Plants were sprayed every 7 days or 14 days at Blacksburg as indicated in the table, and every 7 days at Winchester.

Treatment	Rate, ppm ai	Freq[1]	Aug 1, % severity	Oct 4-10, % severity	Rate, ppm ai	Sep 1, % incidence	Sep 21, % incidence
Water	na	7	31.0	53.6	(water)	13.3	11.7
Abound					150	12.5	45
cyflufenamid[2]					5	0	5
Elite	10	14	0.2	0			
Elite	2.5	7	0	0	25	0	0
Elite	2	14	1.5	0.1			
Elite	0.5	7	0	0	5	5	12.5
Endura	50	14	0	4.8			
Endura	20	14	0.3	5.5			
Endura	10	7	0	2.0	5	0	5
Endura	4	7	0	2.6	1	0	2.5
Flint	25	7	0	0.1			
fluopyram[3]	2.5	7	0	0.6			
fluopyram	0.5	7	0.2	12.8			
Inspire	2.5	7	0	0	5	10	12.5
Inspire	0.5	7	0	0	2	0	0
LEM-17[4]	25	7	0.03	7.2			
LEM-17	10	7	1.4	40.3	10	10	10
metrafenone[5]	2.5	7	0.7	0.2	5	0	0
metrafenone	0.5	7	1.9	6.5			
Mettle	2.5	7	0	0	5	0	0
Mettle	0.5	7	0	0	2	0	0
Quintec	25	14	0	1.6			
Quintec	10	14	3.5	2.6			
Quintec	2.5	7	1.7	2.1	2.5	0	5
Quintec	0.5	7	0.8	8.0	0.5	2.5	20
Rally	2.5	7	0	0	25	0	0
Rally	0.5	7	0	0	5	0	0
Topguard	2.5	7	0	0	5	10	5
Topguard	0.5	7	0	0	2	0	17.5
Vintage	4	14	0	0			
Vintage	1	14	0	0			
Vintage	2	7	0	0	5	0	0
Vintage	0.4	7	0.03	0	2	0	0
Topsin M	100	7	0	0			

[1] Freq = every 7days or every 14 days. Severity ratings (Blacksburg) are based on 10 leaves on each of 2 or 3 plants (control 5 plants), incidence ratings (Winchester) on 20 leaves from each of 2 plants per treatment (control 3 plants).

[2] Proposed trade name Torino

[3] Proposed trade name Luna (in mixtures with other compounds)

[4] Penthiopyrad, not currently expected to be commercialized on grapes

[5] Proposed trade name Vivando

Table 5. Bioassays of isolates collected at three locations with field trial and sentinel vines.

	Mean EC50 tebuconazole	n	teb SD	teb range	Mean EC50 fenarimol	n	fen SD	fen range
Rockbridge	5.2	18	6.6	1.3-23.0	0.18	18	0.30	0.001-1.20
Franklin	5.7	17	3.5	0.3-12.8	0.22	16	0.19	0.05-0.70
Surry	7.0	15	5.3	0.3-19.3	0.20	15	0.20	0.02-0.70

Objective 3: Determine fitness costs associated with QoI-resistance

In order to predict whether QoI resistance carries a fitness penalty when QoI fungicide use is withdrawn, a competition assay was conducted wherein different spore ratios of QoI-resistant and -sensitive strains were mixed and inoculated on healthy susceptible plants. Two inoculations were done. The first two experiments (Set A) consisted of resistant isolates with EC50≥10; the second set of experiments (Set B) consisted of resistant isolates with EC50 =1-10. All isolates were verified to possess the G143A mutation by real time PCR. The changes in population composition were determined based on the proportion of the mutant allele A143 that is responsible for a high-level strobilurin resistance. Individual resistant strains show very high %G143A (>90%), whereas sensitive strains show very small values (<1%). The fitness of resistant strains can be gleaned by monitoring the changes in %G143A in mixed populations on fungicide-free plants.

In Set A, a spore suspension consisting of either 8 resistant (R) or 8 sensitive (S) cultures was prepared. The R and S suspensions were mixed in different proportions based on spore count. The %G143A of mixed spore suspensions was calculated from real-time PCR data to determine the initial composition. A total of 6 mixtures were generated : Trial 1- 10%R:90%S, 20%R:80%S and 60%R:40%S and Trial 2- 5%R:95%S, 30%R:70%S and 40%R:60%S. Each spore suspension was sprayed on two young, healthy grape plants until large droplets formed on the leaves. After air drying, individual plants were placed in closed plastic chambers connected to an air pump. The plants were incubated in a 12-hr light period, with relative humidity of 75-81%. Every 14 days, the spores were harvested and re-inoculated onto healthy plants. A total of three cycles or harvests were done. The %G143A of each spore harvest was determined by real time PCR. The same procedures were employed for Set B except that only one cycle (14 days) was considered, and fewer isolates were used (2 or 3 R or S).

In Set A, the %G143A in the population either remained unchanged or tended to increase (Fig. 1), indicating that there is no fitness cost associated with QoI-resistance under these conditions. However, in Set B employing isolates with lower EC50s, the %G143A generally declined or remained constant from Day 0 to Day 14 (Fig 2). These initial results led us to ask further if fitness differs between “highly” and “moderately” QoI-resistant PM.

A new powdery mildew sample was obtained in 2010 from a commercial vineyard where we have obtained samples for several years, and where QoI fungicides have not been used since 2007. The G143A mutation continues to be present at considerable frequency (1.4-100% of individual samples).

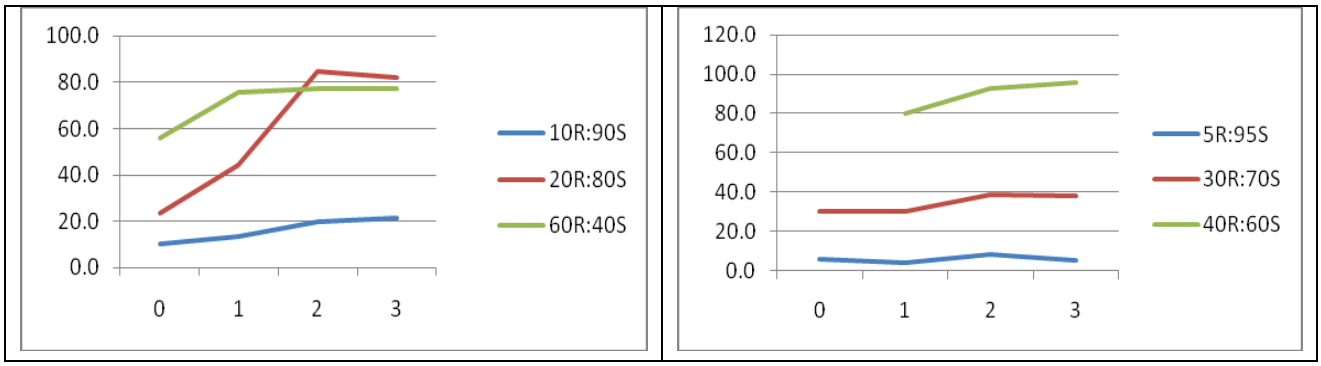

Fig. 1. Changes in the G143A frequency in mixed populations of azoxystrobin-resistant and sensitive powdery mildew isolates cycled every 14-days on grape plants. Plants individually grown in plastic columns under a 12-hr light cycle.

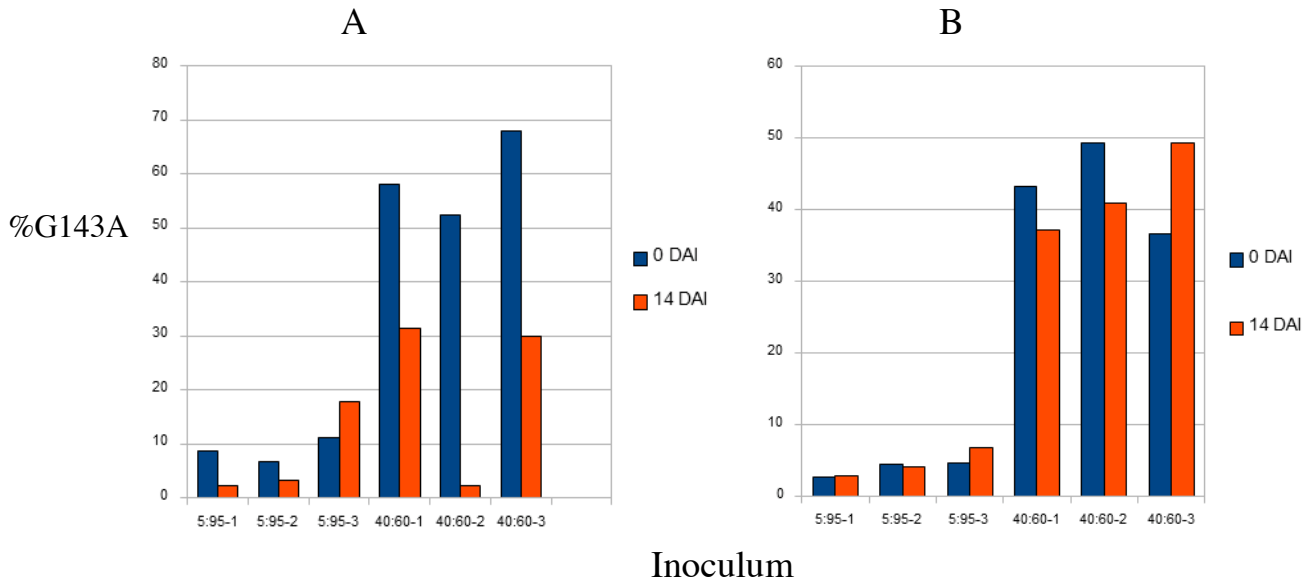

Fig.2. %G143A at 0 and 14 days after inoculation. A and B are data from two independent experiments employing resistant isolates with EC50s=1-10 mg/L.

Objective 4. Exploratory study to detect mutations in the *CYP51* gene.

It is generally expected that a pathogen will have cross-resistance to fungicides having the same mode of action. A group of our isolates having high resistance factors to tebuconazole and myclobutanil (triazoles), were shown to have various levels of resistance to triadimefon, which is the applied parent form of triadimenol (both also triazoles), but low resistance to fenarimol (pyrimidine-DMI). This can be due to the inherent activity of the fungicides but may also be conferred by group-specific mechanisms. Mutations gleaned from the *CYP51* sequences of isolates coming from different resistance phenotypes may provide an explanation for this phenomenon. The grape powdery mildew *CYP51* gene has been completely sequenced and characterized previously (Delye et al. 1997b, Pestic Sci 51:309-314). A point mutation conferring the Y136F change has been detected in European isolates resistant to triadimenol (Delye et al. 1997a Appl Environ Microbiol 63:2966-2970). This report provides results of our initial sequencing

effort for *E. necator CYP51* gene to determine whether this same mutation or other mutations occur in DMI-resistant isolates from the USA.

Based on their EC50s for fenarimol, myclobutanil, tebuconazole, triadimefon, and triflumizole, *E. necator* isolates were categorized into the following groups: sensitive (EC50 <<1), moderately resistant (EC50s 1-9 for most of the fungicides), highly resistant (EC50>10 for most of the fungicides). Other isolates that did not fall into these categories (e.g. highly resistant for tebuconazole but moderately resistant to others) were assigned their own category.

Cultures were grown on young grape leaves and spores were collected. DNA was obtained from spores using the Biosprint 15 DNA Plant Kit (Qiagen®). Initial amplification was accomplished using Delye et al.'s (1997b) primers. A ~1.7 kb band of amplicon was generated, but succeeding amplifications generated two bands that were difficult to separate. New primers were designed using *Primer 3* software (http://frodo.wi.mit.edu/primer3/) based on Delye et al.'s (1997b) published sequence (GenBank accession number U72657). The primer sequences are:

Forward Primer – 5'GTA TTG AGG CGG GTA AAT CG-3'

Reverse Primer – 5'TCA TCT CTT TTC CCA GCC TAT C-3'

A product size of 1.7kb was obtained for the following cultures:

AMP1	highly resistant to triflumizole, moderately resistant to others
GRP10	moderately resistant
IVP17	no DMI data
PBP1	sensitive

PCR products were cleaned and submitted to the University of Chicago Sequencing Facility. Sequences were edited then aligned using DNAStar Lasergene v.8.1.2. Single nucleotide polymorphisms (SNPs) were determined by comparison with published sequences of *E. necator CYP51* in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The following nucleotide accessions were used for alignment: *U. necator*, U83840.2; *E. necator* triadimenol-sensitive, EF649776.1; *E. necator* triadimenol-resistant, EF649777.1; *U. necator,* U72657.2 (Delye et al 1997b, reference sequence); *U. necator*, AF042067.1
The Y136F mutation associated with DMI resistance was found in only DMI-resistant isolates. Since our sequences were less than 1000kb, fungicide resistance-related mutations in other sections of the gene may exist but not have been detected. Our next goal is to obtain a complete section of the transcribed region for isolates with different sensitivities. This will be done by designing primer sets that will generate shorter amplicons with overlapping sequences that can be concatenated to produce a contiguous sequence.

Summary and Conclusions

The logistics of the sentinel vine method have been worked out. Four brief narrated Powerpoint files with explanations were developed and have been posted (still under password protection) at https://filebox.vt.edu/users/abaudoin/animations/. (Login name: filebox.ed. Password: vce).
The approach works well for QoI fungicides and is expected to work well for boscalid if resistance develops (has not yet been observed). Differences in DMI resistance between unexposed, sensitive and heavily exposed PM populations were detected clearly, but it is still uncertain whether smaller differences among exposed commercial vineyards can be detected and to what extent such smaller differences are of practical significance. Relative field performance of Elite compared to Rubigan/Vintage correlated better with sentinel vine data than with bioassay results. Discriminatory rates (weekly sprays) have been established for commonly used powdery mildew fungicides as well as for several recently or soon to be registered compounds. Compounds that can be used to prevent downy mildew and insect leaf feeders without interfering with powdery mildew development have been identified. Time requirements for implementation of a sentinel vine test depend to a large extent on the number of fungicides one wishes to include in the treatments, and the availability of a convenient location where it's easy to maintain the plants and keep them watered. Some support for growers willing to implement this method may continue to be needed

(provision of premeasured small amounts of fungicide, evaluation of leaves with uncertain powdery mildew infection). Either researchers or agricultural consultants may play a role in this.

Publications 2010

Baudoin, A. 2010. Evaluation of Revus Top for control of grape downy mildew, 2009. Plant Disease Management Reports 4: SMF017

Baudoin, A.B. 2010. Current status of benzimidazole resistance of *Erysiphe necator* in Virginia. Phytopathology 100: S12 (Abstr.)

Nita, M and Miller, K. 2010. Fungicide performance trial for powdery mildew of grape, 2009. Plant Disease Management Reports 4: SMF030

Presentations 2010

Nita, M. Fungicide performance trials for powdery mildew and downy mildew, Winchester, VA 2009. Annual Meeting of Virginia Vineyards Association. Charlottesville, VA. March 5 and 6, 2010.