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

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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.

One field-plot and sentinel-vine trial was conducted in a commercial vineyard in Southwest Virginia, and a second combination at the Winchester AREC (M. Nita). In the SW Virginia trial, plots consisted of three to five vines (average of four), and plot treatments were replicated four times. Grower sprays of sulfur plus mancozeb had been applied prior to initiation of the trial during the early part of the season. Fungicides were applied with backpack mist blower, at a rate of 100 gallons per acre and 0.48 gallons per minute, starting on 7 Jun (full bloom), and repeated every 14 days until 20 Jul (which was 15 days after the preceding spray). Abound was added to the spray mix on 7 and 21 Jun, and Flint on 5 Jul to prevent black rot (powdery mildew at this site had previously been found to be QoI resistant). To prevent downy mildew (also QoI resistant), Prophyt was added on 21 Jun and 20 Jul, and Presidio on 5 Jul. Inspire Super was somewhat underdosed (14 rather than 16 fl oz per acre) because some of the plots were later measured to have a more extended trellis relative to the number of vines. Thirty clusters and 25 main-shoot leaves per plot were rated on 12 July, and 20 main-shoot leaves per plot were rated on 3 Aug**.** In addition to individual-leaf ratings, whole-plot estimates of powdery mildew severity on both foliage and clusters were taken on 3 Aug.

At the time of the first application (7 Jun, full bloom), a few powdery mildew colonies were already visible on the foliage, and disease developed quickly on untreated vines soon after initiation of the trial. Powdery mildew pressure was high. Rainfall totaled 1.16 inches in June (after 7 Jun), 2.73 inches in July, and no rain in August before the 3 Aug rating. Coverage at the second application may have been somewhat compromised because shoots had been tied up and dense leaf layers were covering clusters hindering spray penetration. At the third application, leaves had been removed from the cluster zone, and full coverage was easier to accomplish. Among the fungicides tested, Vivando provided the best powdery mildew control, Quintec and Inspire Super were in the next group in most ratings, and the remainder provided poor powdery mildew control at this site and spray interval (Table 1).

Table 1. Powdery mildew fungicide field trial in commercial vineyard in southwest Virginia.

Treatment	PM Severity 12 Jul [a]		PM Severity 3 Aug [a]	
	Leaves	Clusters	Leaves	Clusters WP [b]
Water (control)	60.7 a	85 a	71.6 a	95 a
Elite 45WP, 4 oz/A	10.1 b	53 b	24.5 ab	70 b
Vintage SC, 6 fl oz/A	8.1 b	66 b	17.3 b	83 ab
Inspire Super, 14 fl oz/A	0.4 de	16 c	0.4 d	26 c
Mettle, 5 fl oz/A	2.4 bc	52 b	4.1 c	75 b
Vivando, 15.4 fl oz	0.2 e	4 c	0.2 d	5 d
Endura, 4.5 oz/A	0.5 de	54 b	3.2 c	70 b
Quintec, 6 fl oz/A	0.7 cd	18 c	3.7 c	43 c
Microthiol Disperss, 5 lbs/A	2.7 bc	68 ab	11.0 bc	81 b

[a] Numbers in a column followed by the same letter are not significantly different according to Waller-Duncan's test (k=100, after arcsine transformation for cluster data, and log transformation for the leaf data which better stabilized variance).
[b] Whole-plot rating

Table 2. Sensitivity by bioassay of powdery mildew leaf populations from unsprayed plots to four DMI fungicides.

Sampling Date	Mean EC50 [a]			
	Teb [b]	Fen	Tet	Dif
7-Jun (*n*=8)	1.11	0.48	0.18	0.03
21-Jun (*n*=12)	1.93	0.44	0.32	0.05
12-Jul (*n*=19)	0.49	0.32	0.15	0.07
2-Aug (*n*=17)	0.71	0.37	0.21	0.05

[a] Values are EC50s (mg/L) or concentration of the active ingredient that inhibits growth by 50%
[b] DMI fungicides: Teb-tebuconazole; Fen-fenarimol; Tet-tetraconazole; Dif-difenoconazole

Isolates from control plots (Table 2) and plots treated with DMI fungicides (Table 3) were collected several times during the season and bioassayed for sensitivity to four DMI fungicides. There were significant differences in sensitivity to the individual compounds, but no consistent changes in the course of the season. The number of isolates that could be recovered from most treated plots (Table 3) was too small to allow clear conclusions.

Table 3. Sensitivity (reported as mean EC50, mg/l) of leaf populations from DMI-sprayed plots to four DMI fungicides.

7-Jun [a]		12-Jul	2-Aug
n=8	PM from tebuconazole-plots, tested against	*n*=17	*n*=2
1.11	Teb [b]	1.14	1.95
0.48	Fen	0.65	0.64
0.18	Tet	0.27	0.28
0.03	Dif	0.07	0.02
n=8	PM from tetraconazole-plots, tested against		*n*=7
1.11	Teb		1.23
0.48	Fen		0.79
0.18	Tet		0.48
0.03	Dif		0.12
n=8	PM from difenoconazole-plots, tested against		*n*=4
1.11	Teb		0.84
0.48	Fen		0.46
0.18	Tet		0.39
0.03	Dif		0.05

[a] From control plots, before first spray
[b] DMI fungicides: Teb-tebuconazole; Fen-fenarimol; Tet-tetraconazole; Dif-difenoconazole

The second field experiment, at the Winchester AREC, included additional, not yet commercial but soon-to-be-registered fungicides. Plots consisted of three vines, and were arranged in a completely randomized design with four replications. Treatments were applied with a 4-gal backpack air sprayer with a flat fan nozzle. Treatments were started 5 d after bloom and repeated three times. The interval between applications was approximately 14 days. Treatments were tank mixed with Revus (7 fl oz/A) in order to suppress downy mildew. Prior to initiation of the trial, all vines were treated with Penncozeb (3 lb/A) and Microthiol Disperss (3 lb/A) to control various diseases. At bloom, all vines were treated with Vangard (8 oz/A) to control blossom blight by Botrytis.

Powdery mildew severity on leaves and clusters was assessed four times during the season, and results from 19 July are shown (Table 4). Bud break at Winchester was 19 April, and 50% bloom was 30 May. There were regular rain events between bud break and bloom, with total precipitation of about 3.5 inches. However, after mid-May, there was no significant rain until 8 July. Powdery mildew disease pressure was high.

With respect to leaf infection, the best treatment (Luna Experience with >96% disease control) resulted in less than 4% disease incidence while others were around 30-40% (70-60% disease control). Leaf disease severity was below 2% in all treatment except the “standard” (sulfur only), indicating that many of the colonies observed were very small. Compared to severe infection on untreated vines, all treatments presented good to excellent control on leaves. On the other hand, high disease incidence was observed on clusters with all treatments (~15% control), except with Luna Experience. As with leaves, low cluster disease severity (< 5%) indicated that many of the observed colonies were small.

Table 4. Powdery mildew fungicide trial, Winchester AREC.

Treatment[z]	Days after first application[y]	Leaves: Disease incidence[x]	Leaves: % Control[w]	Leaves: Disease severity[v]	Leaves: % Control[w]
Luna Experience 8 oz	43, 54, 69	3.8 D	96.2	0.1 D	99.8
Quintec 4fl oz	43, 54, 69	40.0 C	59.7	1.3 C	95.0
Torino 3.4 fl oz rotated with Vivando 10 oz	33, 43, 54, 69	28.8 C	71.0	1.0 C	96.2
Vivando 10 oz	43, 54, 69	36.7 C	63.0	2.1 C	92.1
Vivando 15 oz	43, 54, 69	32.1 C	67.6	1.0 C	96.4
PM check (Revus 7 oz only)	43, 54, 69	99.2 A	0.0	26.6 A	0.0
Standard (Microthiol 3 lb)	43, 54, 69	78.3 B	21.0	10.5 B	60.7

Treatment[z]	Days after first application[y]	Clusters: Disease incidence[x]	Clusters: % Control[w]	Clusters: Disease severity[v]	Clusters: % Control[w]
Luna Experience 8 oz	43, 54, 69	5.0 B	94.7	0.1 D	99.9
Quintec 4fl oz	43, 54, 69	80.0 A	14.7	2.7 C	95.5
Torino 3.4 fl oz rotated with Vivando 10 oz	33, 43, 54, 69	82.5 A	12.0	4.8 C	91.9
Vivando 10 oz	43, 54, 69	82.5 A	12.0	4.4 C	92.5
Vivando 15 oz	43, 54, 69	78.8 A	16.0	2.8 C	95.4
PM check (Revus 7 oz only)	43, 54, 69	93.8 A	0.0	59.2 A	0.0
Standard (Microthiol 3 lb)	43, 54, 69	100.0 A	-6.7	37.1 B	37.3

[z] Rates were calculated based on an application volume of 100 gal/A, PM Check received mancozeb (Penncozeb 3 lb/A) during the experiment to prevent downy mildew and black rot.

[y] The first application was made on 25 April. Before initiation of the trial on day 43, sulfur at 3 lb per acre was applied to all vines on days 1, 10, 17, 23, and 33. The treatment applied for day 54 contained Ridomil Gold MZ for downy mildew control

[x] Disease incidence = percentage of diseased leaves: Numbers presented are the least square mean of percentage. The same letter within a column indicates there was no significant difference between treatments (Tukey-Kramer adjustment method, the overall error rate = 0.05)

[v] Disease severity = diseased percentage of surface area of leaves or bunches. The same letter within a column indicates there was no significant difference between treatments (Tukey-Kramer adjustment method, overall error rate = 0.05)

[w] %Control = the percentage of disease controlled, compared with the check

Sentinel vines were deployed at Winchester and Blacksburg, as well as two commercial vineyards in Virginia and one on northern North Carolina. Disease pressure at Blacksburg was moderate (Table 5); Winchester, light (Table 6); commercial vineyard in SW Virginia, heavy (Table 7); and commercial vineyard in northern Virginia, moderate (Table 8).

Table 5. Sentinel experiment Blacksburg 2011. Powdery mildew population was fungicide-sensitive at this location.

		Estimated minimum inhibitory concentrations (mg/L) allowing less than 10% disease severity relative to the check	
Common name	Trade name	Treatment every 7 days	Treatment every 14 days
Tebuconazole	Elite	0.5 - 1	Variable
Quinoxyfen	Quintec	7.5-10	25-50
Boscalid	Endura	<5	20
Vintage	Vintage	0.5	>1
Flint	Flint	10	25
Difenoconazole	Inspire	0.5	NT
Tetraconazole	Mettle	0.5	NT
Myclobutanil	Rally	0.5	2 - variable
Fluopyram	Luna solo	2 - 5	<10
Metrafenone	Vivando	2 - 5	10 - 25
Flutriafol	Topguard	0.5	NT
Cyflufenamid	Torino	2.5	NT
Thiophanate methyl	Topsin M	<100	NT

Table 6. Sentinel experiment Winchester AREC, 2011. Values represent disease severity on Sep 6.

7-day schedule	Rate	Plant A	Plant B	Plant C	Mean
Control	NA	19.0	6.0	5.2	10.1
Vivando	5	3.0	6.0	3.6	4.2
Vivando	2.5	0.2	1.8	1.2	1.1
Luna	5	0	0	0	0
Luna	2	0	0	0	0
Inspire	5	0.2	0	0	0.1
Inspire	2	0.2	0.2	0.4	0.3
Torino	5	0	0	0	0
Torino	2	0	0	0	0
Quintec	7.5	0	0.2	0	0.1
Quintec	5	1.0	4.4	0.4	1.9
Elite	5	0	0	0	0
Elite	2	0	0	0	0

Table 7. Sentinel experiment in commercial vineyard in SW Virginia, 2011, estimated minimum inhibitory concentrations (disease less than 10% of control), weekly applications.

Product	Minimum inhibitory concentrations, mg/L
Cabrio	12.5
Elite	20
Endura	5 - 10
Inspire	< 5
Merivon	< 5
Mettle	5
Quintec	7.5-10
Vintage	10
Vivando	5-10

Table 8. Sentinel experiment in commercial vineyard in northern Virginia, disease severity

Treatment	Rate	Mean PM severity of infected leaves
Control (water)		8
Rally	10	16
Rally	5	23
Rally	2	23
Endura	5	0
Quintec	7.5	0
Flint	50	4

An additional set of sentinel plants was situated at a commercial vineyard in northern N. Carolina. However at this location, none of the treatments provided significant powdery or downy mildew control compared to the check. It seems likely that the treatments were not properly timed or applied at this location.

Experiments have been initiated to determine discriminatory dosages for DOWNY mildew fungicides; these will be continued in Spring and summer of 2012. Because of the spottier nature of infection conditions, the protocol for downy mildew is different from the approach used with powdery mildew. Instead of placing plants at vineyard locations and keeping them sprayed, potted plants will be deployed only when downy mildew makes an appearance. Previously untreated plants are then sprayed with discriminatory dosages of fungicides (the lowest rate that would be expected to give near-complete control of disease development by sensitive downy mildew). One day later, they are inoculated with a field isolate, and disease is evaluated after 10 days. Preliminary estimates for several fungicides are shown in Table 9.

Table 9. 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		
Abound	Azoxystrobin	11		
Ranman	Cyazofamid	21		
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	100-200	0.4-0.8
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

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

QoI resistance in powdery mildew is now widespread in Virginia and nearby states (Baudoin et al. 2008). We wanted to address the question whether withdrawal of the fungicide might be followed by a decreasing frequency of resistant isolates due to a possible fitness cost of the resistance. If this were to be the case, the QoI group might then still be useful for occasional use against powdery mildew. Reports of fitness costs in other plant pathogens have conflicted: some report some fitness costs, and others none; this may vary among pathogens.

In our investigation, the fitness cost associated with QoI resistance in *E. necator* was determined by competition assays employing mixed populations of resistant and sensitive isolates maintained in the laboratory as single-spored cultures. The competitiveness of resistant isolates in mixed populations was determined by measuring through a time series experiment the fraction of the mutation (G143A) correlated with QoI resistance. The %G143A is expected to decline through time if a fitness cost is associated with QoI resistance. G143A quantification was done using real-time PCR (Baudoin et al. 2008).

In competition assays under laboratory conditions, the %G143A generally did not decline in most inoculation experiments (data reported previously), and even had a tendency to increase.

The %G143A was also monitored through several years (2007-2011) in one commercial vineyard that has stopped using QoI fungicides. QoI resistance was found to persist at fairly high frequencies (Table 10, only partial results for 2011), with no convincing evidence of a decline, and persistence of resistance at levels that would preclude efficacious use.

Table 10. Resistance profiling of *Erysiphe necator* population in one vineyard that has not used QoI fungicides for several years.

	2007[a]	2008	2009	2010	2011
# resistant isolates (%G143A>95%) / # tested:	5/5	4/4	2/2	-	-
%G143A profile of leaf populations[b]:	-	-	99	100	100
			95	100	100
			55	100	59
			42	100	65
			21	100	43
			5	99	43
			5	90	22
			0.1	43	16
				27	5
				5	0.02
				1	0.01
					0.005
					0.002
Mean			40	70	35

[a] Reported by J. Colcol (2008), a graduate student in the Baudoin lab
[b] One leaf population=all powdery mildew growth on one leaf

In an attempt to collect a separate set of data to confirm the field experience described in the previous paragraph, we started a competition experiment under field conditions at the Virginia Tech Blacksburg Glade Road Research Center in June 13, 2011. Clusters of potted plants (Fig 1) with different powdery mildew isolate mixes were separated by distances of about 100 m, and, as much as possible, by bushes, trees, and buildings. Powdery mildew developed quickly on the inoculated plants, and samples of infected leaves were collected in two periods, July 4-8 and September 19-27. Powdery mildew growth was rubbed onto clean grape leaves with or without detaching from the plant. Rubbed leaves were incubated until profuse sporulation was obtained. DNA extracted from the spores from a single leaf was considered a sample. DNA from each sample was subjected to realtime PCR for G143A quantification.

In contrast to the other two experiments, the %G143A in this experiment quickly dropped to a sensitive range (mean=0.1) in the course of the summer. The population on plants inoculated with QoI-sensitive PM remained sensitive. Migration of spores could have been a major contributor to the establishment of the sensitive strains. There are no known vineyards nearby, and few known grapevines, but they are likely to exist in residential neighborhoods several 100 m to a km away. Control plants (not inoculated) did develop powdery mildew somewhat later than inoculated plants, but disease development was still vigorous by mid-summer. Mere mixing of inoculum among treatments would have been expected to result in approximately 30% G143A while the actual percentage in July-September was clearly lower than 30%. It appears plausible that the decline in the resistant population was due to background infection from outside sources.

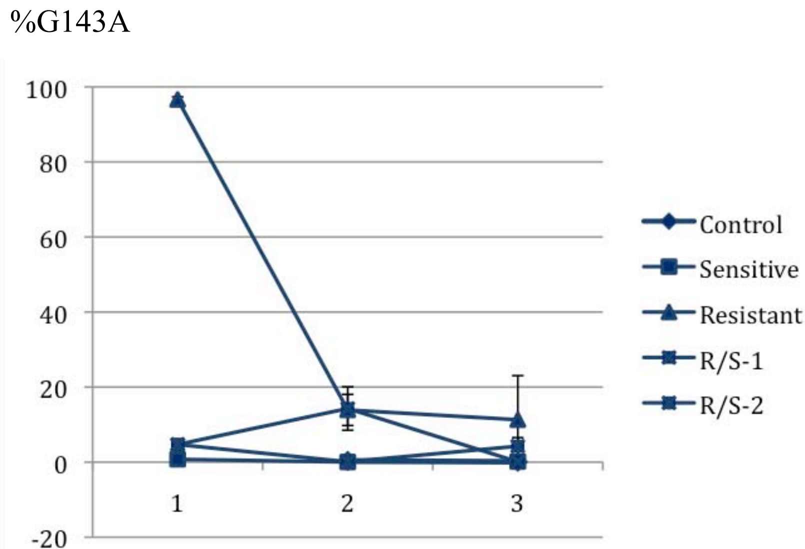

Fig. 1. Changes in %G143A in powdery mildew inoculated with either a mixture of resistant and sensitive isolates (1R:9S spore ratio) or a single-type inoculum (R or S) on small groups of potted grape plants grown under field conditions. 1=Jun, 2=Jul 4-8, 3= Sep 19-27.

Alternating or tank-mixing fungicides with different modes of action is a recommendation to delay resistance development. Only very few reports are available to show this phenomenon. Results from these studies revealed that the delay effect may vary with the kind of fungicide used (Genet et al. 2006; Ma and Uddin 2009). We attempted to test the hypothesis that resistance development is delayed when the pathogen population is exposed to mixed fungicides (QoI and a different chemistry). Our preliminary experiments, using detached grape leaves soaked in mixed fungicides produced very variable and insignificant results. We concluded that determining the delaying effect of a partner fungicide would require testing several partner products, which poses a huge limitation to the conduct of this study.

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.

A. Mechanism of QoI resistance beside G143A mutation

Although 89% of our QoI resistant isolates collected in 2005-2008 had a high level of the G143A mutation, about 11% did not. We are exploring two hypotheses to explain the resistance in these 11%: (1) other point mutations such as the F129L in the Cytb gene; or (2) the G143A mutation IS actually present and responsible for the resistance, but is present below detectable levels.

To search for other point mutations in the Cytb gene, a draft sequence provided by Dr. H. Sierotzki of Syngenta was used to design primers spanning the hot-spot (amino acids 129-143) and downstream regions of *cytb*. In preliminary runs, we failed to amplify the hotspot region using our primers. However, we obtained amplicons for the region downstream of the 129-143 section. The products were sent to the University of Chicago DNA Sequencing facility (UCDSF).

Sequences were analyzed using the Lasergene 8.1.3(DNAStar Inc.). Alignment with the draft sequence provided by Dr. Sierotzski matched perfectly. The next step will be to detect nucleotide polymorphisms by sequencing from DNA of sensitive, "moderately" resistant (EC_{50}>1, G143A<1%) and resistant isolates.

To generate the 129-153 region, the use of degenerate primers (BgtF1/BgtR1) designed by Sierotzki et al. (2000) for amplifying a portion of the *cytb* of *Blumeria graminis* f.p. *tritici* was attempted on *Encytb*. After PCR amplification and clean-up of the product from a G143A resistant isolate (VaHP4), we sent the sample to the UCDSF for sequencing. The predicted amino acid sequence was obtained using Geneious Pro 5.5.5 (Biomatters Ltd.) then aligned with a section of *cytb* from the QoI-resistant *B. graminis* f.sp. *tritici* (AAK26622.1) and from *E.necator* isolated from Italian vineyards (Hajjeh et al.2010). This region includes the 129-143 amino acids (Fig. 2). The next step is to design primers specific to our *Encytb*, optimize the PCR protocol, then amplify this section from the isolates of interest (high EC50, low %G143A) to check the presence of the F129L mutation.

To test our second hypothesis, three isolates with resistant phenotype based on the standard leaf disc bioassay but with low %G143A were tested. The isolates were obtained from Virginia vineyards and have been maintained as single spore cultures on fungicide-free grape leaves for over three years. These have been tested to grow well on >1 mg/L azoxystrobin. Parallel transfers for each isolate on fungicide-free and aAbound-treated leaves (3 mg/L active ingredient, azoxystrobin) were started in December 2010. Spores were collected after 3 parallel transfers, subjected to DNA extraction then real-time PCR to quantify the %G143A. The transfers were continued for all of 2011 (total of 12-15 transfers). In the case of one culture, the experiment was terminated after five transfers because of failure of growth on fungicide-treated leaves. We started the parallel transfer anew for this isolate from the culture that we maintain in our collection. After the first three parallel transfers, the isolates produced well-sporulating growth on both treated and untreated leaves, but the %G143A remained low (Table 11). Spores are currently being collected again to determine any later change in the %G143A.

Table 11. %G143A in three *E.necator* isolates that have been inoculated in parallel on fungicide-free and azoxystrobin-treated (3mg/L) grape leaves.

Isolates [a]	Initial G143A [b]	After 3 parallel transfers fungicide-free	After 3 parallel transfers azoxystrobin
GRP15	0.03	≤0.02	≤0.002
GRP18	0.01	≤0.11	≤0.002
SUP13	0.17	≤0.15	≤0.001

[a] First isolated in 2005-2007
[b] Colcol 2008

B. EBI – CYP51 mutations

It is generally expected that a pathogen will have cross-resistance to fungicides having the same mode of action. A group of isolates in our collection having high resistance factors to tebuconazole and myclobutanil (triazoles) were shown to have various levels of resistance to triadimefon, which is the applied form of triadimenol (also a triazole), but low resistance to fenarimol. Mutations gleaned from the *CYP51* sequences of isolates coming from different "resistance groups" may provide an explanation for this phenomenon. The grape powdery mildew *Erysiphe necator CYP51* gene has been sequenced completely and characterized previously (Delye et al. 1997b). A point mutation conferring the Y136F change has also been detected in European isolates resistant to triadimenol (Delye et al. 1997a). The Y136F mutation is a change in the codon TAT (tyrosine) to TTT (phenylalanine). Whether this same mutation or other mutations occur in DMI-resistant isolates from the United States can be determined from gene sequence analysis of DNA from isolates with different sensitivities to tebuconazole and fenarimol.

Isolates (n=28) with a wide range of tolerance to DMIs were used in sequencing the C-14-α-demethylase gene (*cyp51*). PCR amplification using three primer sets that generated overlapping sequences were designed from the Delye et al. (1997) sequence. PCR products were sent to the UCSF for sequencing. The sequences were analyzed using the Lasergene® Seqman Pro (v.8.1.3) Alignment of the sequences with *Erysiphe necator CYP51* sequence in NCBI (EF649777; EF649776) revealed a high degree of homology (99%) indicating that the sequences belong to *E. necator cyp51*. Results for each isolate are summarized in Table 12. To determine the presence of the Y136F mutation, each sequence was inspected for a single change at nucleotide position 418 (from A to T) from the transcription start site. This translates to an amino change from tyrosine (TAT) to phenylalanine (TTT). The Y136F was found in 12 isolates with a resistance factor $RF \geq 50$ for tebuconazole. All seven sensitive strains ($RF_{teb} < 3$) possessed the wild-type codon. The remaining isolates had high resistance to tebuconazole but possessed a wild-type codon in the sequence. Further inspection of the sequence chromatographs for these six isolates revealed that on the 136th codon the sequence may be TAT or TTT. This double peak phenomenon was also found in 3 of the 12 resistant isolates previously described. These findings show, despite the unresolved peaks in some isolates that the Y136F is correlated with tebuconazole resistance because of its absence in all sensitive isolates.

The presence of two *CYP51*-related genes has been described and explained for the opportunistic human pathogen *Aspergillus fumigatus* and other *Aspergillus* species (Mellado et al. 2001). We hypothesize therefore that for resistant isolates ($RF_{teb} > 50$), the presence of double peaks in the sequence chromatographs indicate two copies of the gene. We wish to test this hypothesis by cloning the gene and sequencing. Screening clones with two variants of the gene will involve denaturing gel electrophoresis which is separation based on sequence differences, including single nucleotide polymorphisms. Clones will then be sequenced to determine whether two types of the gene (wildtype or mutant at position 136) are present.

The Y136F mutation was also found in isolates with $RF_{fen} > 5$. These isolates were the same ones that had high RF to tebuconazole. However, two tebuconazole-resistant isolates (RF>700 and RF=81, respectively) were sensitive to fenarimol (RF=0.1) but still possessed the mutation, suggesting that there could be other independent mechanisms responsible for fenarimol resistance. All tebuconazole-sensitive isolates also had low RF for fenarimol (RF<2).

We have also found another SNP at a nucleotide upstream from the 136 position at the 1119 nucleotide position (Table 12). The nucleotide change from A to C was present in only some tebuconazole-resistant isolates but none of the sensitive isolates had this mutation. This SNP was confirmed by repeat sequencing but does not confer an amino acid change, so is not expected to be relevant to DMI resistance.

Table 12. Sequence results on *E. necator cyp51*.

Isolate	DMI	136th codon sequence[a]	No. of align-ments	Remarks[b]	SNP nt 1119	
AMP1	R	TAT	3	all double peaks	C	
IVP4	R	TAT	3	all double peaks	C	
JRP3	R	TAT	4	all double peaks	C	Group1
JWP1-2	R	TAT	2	all double peaks	C	(resistant [c])
VAHP6	R	TAT	3	2 double; 1 weak	C	
JRP1	R	TWT	3	double peak	C	
JRP4	R	TWT	3	double peak	C	Group 2
VAHP4-1	R	TWT	2	double peak	C	(resistant [c])
BXP1A	R	TTT	2	clean peak	A	
GRP15	R	TTT	2	clean peak	A	
GRP18	R	TTT	2	clean peak	A	
IVP3	R	TTT	2	clean peak	A	
IVP11	R	TTT	2	clean peak	A	
MDMRP3	R	TTT	3	clean peak	A	
MDMRP5	R	TTT	2	clean peak	A	
MDMRP7	R	TTT	2	clean peak	A	Group 3
PRP7	R	TTT	2	clean peak	A	(resistant [c])
ROP14	R	TTT	2	clean peak	C	
SUP13-2	R	TTT	2	clean peak	A	
VAHP1	R	TTT	2	clean peak	A	
SCCP4	S	TAT	2	clean peak	A	
BLP4	S	TAT	2	clean peak	A	
MVP1	S	TAT	2	clean peak	A	
MVP9	S	TAT	1	clean peak	A	Group 4
MVP5	S	TAT	2	clean peak	A	(sensitive)
BLP1	S	TAT	2	clean peak	A	
PBP1	S	TAT	2	clean peak	A	

[a] Sequence reported by the sequencing program
[b] Double peaks = peaks for A and T appear on the chromatograph
[c] Resistant: EC50 > 1 ppm

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 28 vineyards were collected in the fall of 2011, and 171 isolates are in storage awaiting testing. Several methods of collecting and shipping/processing samples were tested, including immediate plating to culture dishes in the field, and collecting samples on cotton swabs and shipping those (Figure x). It turned out that the latter method was very simple and successful: swabs that had been touched to Botrytis sporulation in a vineyard could be mailed by regular mail, and stored dry at room temperature. Botrytis was easily recoverable several weeks and even months after collection.

Figure 2. Top left: Botrytis collection kit as used by several cooperators. Top right: sterile cotton swabs in paper sleeves and mailed in a regular envelope were just as effective in allowing Botrytis recovery. Bottom: culture plates with fungal growth from clusters from several vineyards. Five of six plates shown yielded Botrytis (plus other fungi).

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