**Virginia Wine Board**

Project Report, July 1, 2010

# *Characteristics and Monitoring of Fungicide-Resistant Grape Powdery Mildew*


Anton Baudoin
Plant Pathology, Physiology & Weed Science
Virginia Tech, Blacksburg, VA 24061
Phone 540-231-5757
Fax (departmental) 540-231-7477
abaudoin@vt.edu

David G. Schmale III
Plant Pathology, Physiol. & Weed Science
Virginia Tech, Blacksburg, VA
Phone 540-231-6943
dschmale@vt.edu

Gill Giese
Viticulture Instructor
Surry Community College, Dobson, NC
gieseg@surry.edu

Mizuho Nita
Research/Extension Grape Pathologist
Virginia Tech, Winchester, VA
nita24@vt.edu

Lynn Rallos, Graduate Student
Plant Pathology, Physiology & Weed Science
Virginia Tech, Blacksburg, VA 24061


**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.


Project #: FY10WB09/04/07

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

Field tests for 2010 have been initiated

1. At Surry Community College, NC
2. In Franklin County, VA (commercial vineyard, high powdery mildew pressure)
3. In Rockbridge County, VA (commercial vineyard, history of high DMI resistance)
4. At the Winchester Agricultural Research and Extension Center

Each field trial is accompanied by a set of "sentinel vines", 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 are being maintained and treated at

5. Blacksburg, location as in 2008 and 2009 (relatively un-exposed powdery mildew population)

6 and 7. Two commercial vineyards in North Carolina

May and June have seen favorable conditions for powdery mildew development. Ratings are being conducted in early July.

Bioassays of powdery mildew isolates collected in 2009 are still being completed. The available data suggest that sensitivity of isolates do correlate with sentinel plant results from the same locations:

1. Sentinel vines at a commercial vineyard in Hillsboro, Loudon Co, northern VA. Plants were sprayed weekly, but downy mildew caused considerable defoliation by early August. Powdery mildew developed on control plants, and a number of colonies were also observed on plants treated with Quintec 2.5 µg/ml active ingredient, but not on plants treated with fenarimol (lowest concentration 2 µg/ml), myclobutanil (lowest concentration 5 µg/ml) and trifloxystrobin (lowest concentration 50µg/ml). Bioassay results (Table 1) indicate that the powdery mildew at this location was indeed sensitive to fenarimol, mycobutanil, and strobilurins, and that downy mildew was strobilurin-resistant. Isolates from plants treated with 2.5 µg/ml quinoxyfen were quinoxyfen-sensitive.

Table 1. Estimates of EC50 (bioassay, µg/ml) of powdery mildew isolates from sentinel vines at Hillsboro, Loudon County, VA, 2009

| Isolates from control plants: | | | | | | |
|---|---|---|---|---|---|---|
| Isolate | Fen | Myc | Teb | Qui | Bos | Azo |
| HI9-1 | <0.03 | <0.1 | <0.01 | | | <0.1 |
| HI9-2 | <0.03 | <0.1 | <0.01 | | na | <0.1 |
| HI9-3 | <0.01 | <0.3 | <0.01 | | <0.001 | <0.1 |
| HI9-4 | <0.01 | <0.3 | <0.01 | | <0.001 | <0.1 |
| HI9-5 | <0.03 | <0.1 | <1 | | na | <0.1 |
| Isolates from Quintec-treated plants: | | | | | | |
| HI-Q1 | <0.01 | <0.3 | <0.01 | <0.001 | <0.001 | <0.1 |
| HI-Q3 | <0.01 | <0.3 | <0.01 | <0.001 | <0.001 | <0.1 |
| HI-Q4 | <0.01 | <0.3 | <0.01 | <0.001 | <0.001 | <0.1 |

* Fen=fenarimol, Myc=myclobutanil, Teb=tebuconazole, Qui=quinoxyfen, Bos=boscalid, Azo=azoxystrobin

2. Isolates from Winchester were relatively tolerant to DMI fungicides (Table 2)

3. Isolates from Linden were generally sensitive to DMI fungicides, with the exception of Li9-11 (Table 2)

These bioassay data correlate with sentinel vine data (Table 3). 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.

Table 2. Preliminary estimates of EC50 (bioassay, µg/ml) of powdery mildew isolates from two sets of sentinel vines in 2009

| Winchester Isolate | Fen* | Myc | Teb | | Linden Isolate | Fen | Myc | Teb |
|---|---|---|---|---|---|---|---|---|
| Wi9-1 | >0.3 | >3 | >3 | | Li9-4 | <0.01 | <0.03 | <0.3 |
| Wi9-4 | >0.3 | >3 | >3 | | Li9-6 | <0.01 | <0.03 | <0.3 |
| Wi9-5 | >0.3 | >3 | >3 | | Li9-7 | <0.01 | <0.03 | <0.3 |
| Wi9-6 | >0.3 | >3 | >3 | | Li9-8 | <0.01 | <0.03 | <0.3 |
| Wi9-7 | >0.3 | >3 | >3 | | Li9-9 | <0.01 | <0.03 | <0.3 |
| Wi9-9 | >0.3 | >3 | >3 | | Li9-10 | <0.01 | <0.03 | <0.3 |
| Wi9-10 | >0.3 | >3 | >3 | | Li9-11 | **>0.3** | **>3** | **>3** |
| Wi9-11 | >0.3 | >3 | >3 | | | | | |

* Fen=fenarimol, Myc=myclobutanil, Teb=tebuconazole

Table 3. Sentinel vine powdery mildew ratings (selected fungicides) at Linden and Winchester, VA, Sep 1, 2009.

| Fungicide (selected) | Concentration (active ingredient, µg/ml) | Powdery mildew rating, % of leaf surface | |
|---|---|---|---|
| | | Winchester | Linden |
| Control | na | 8.7 | 7.6 |
| Abound (azoxystrobin) | 300 | 2.7 | 0.1 |
| Elite (tebuconazole) | 25 | 0 | 0 |
| Elite | 5 | 3.3 | 0 |
| Rally (myclobutanil) | 25 | 0.2 | 0 |
| Rally | 5 | 4.7 | 0 |
| Rubigan (fenarimol) | 10 | 0 | 0.1 |
| Rubigan | 2 | 0.4 | 0.1 |

Two plants per treatment, 10 leaves per plant

Because of heavy downy mildew pressure in 2009, and the possible need for control of insect pests such as Japanese beetles and other leaf feeders, and number of insecticides and fungicides were tested for their effect on powdery mildew. Young shoots were sprayed with a labeled rate of the compound and allowed to dry. Expanding leaves were then harvested, placed in petri dishes with agar, and inoculated with powdery mildew. Percent coverage was evaluated after 7 days (Table 4).

Table 4. Effects on nontarget fungicides and insecticides on powdery mildew (PM) development.

| Treatment | PM Growth as % of Control | Degree of PM inhibition |
|---|---|---|
| **Fungicides** | | |
| Captan | 7 | Strong |
| Mancozeb | 0 | Strong |
| Presidio | 71 | Little |
| Prophyt | 44 | Moderate |
| Revus 250 SC | 99 | Little |
| Ridomil SL | 83 | Little |
| **Insecticides** | | |
| Avaunt, indoxacarb | 94 | Little |
| Capture, bifenthrin | 52 | Results variable |
| Danitol, fenpropathrin | 41 | Moderate |
| Lorsban, chlorpyrifos | 0 | Strong |
| malathion | 29 | Moderate |
| Provado, imidacloprid | 114 | Little |
| Safari, dinotefuran | 43 | Moderate |
| Sevin WP or XLR, carbaryl | 10 | Moderate to strong |

**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. The changes in population composition were determined based on the proportion of the mutant allele G143A that is responsible for high-level strobilurin resistance. Individual resistant strains show very high %G143A (>90% G143A), 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.

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 for two trials: 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 %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.

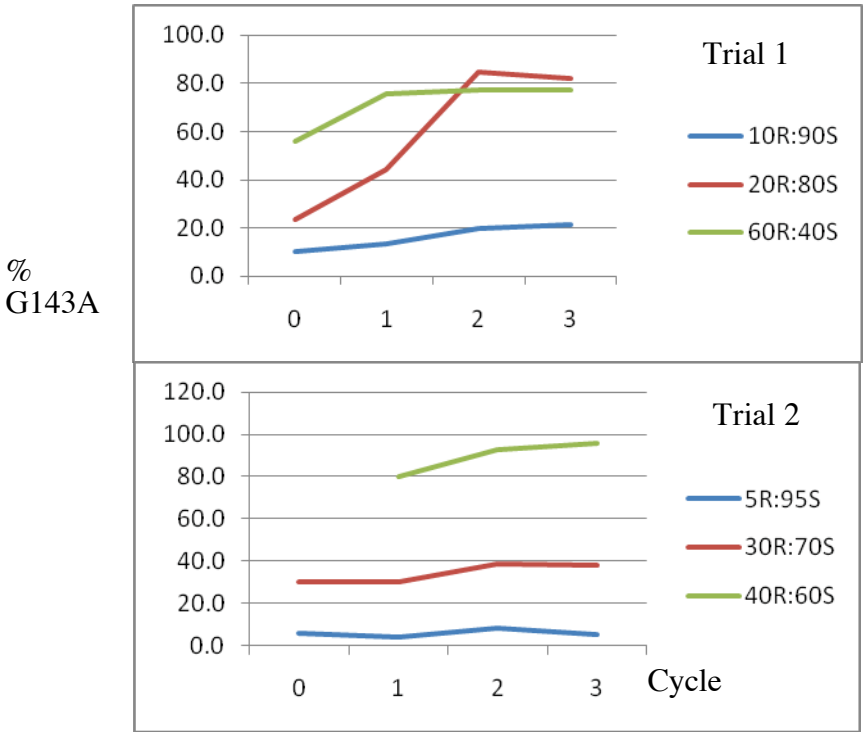


Fig. 1. Changes in the G143A frequency in mixed populations of azoxystrobin-resistant and sensitive powdery mildew isolates cycled every 14-days on grape plants. Plants individually grown in plastic columns under a 12-hr light cycle and a RH=75-81%.

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

It is generally known 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 groups" may provide an explanation for this phenomenon. The grape powdery mildew *Erysiphe necator 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). can be determined from gene sequence analysis of DNA from different strains. 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. We then designed our own primers 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

A change from A to T was found in only resistant isolates, corresponding to a change in nucleotide 664 of the reference sequence (GenBank accession # U72657.2). This change described the Y136F mutation associated with DMI resistance. Since our sequences were less than 1000kb, fungicide resistance-related mutations in other sections of the gene were not obtained. 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 contig.

This research is being continued as 2010-2011 project "*Sentinel vines to evaluate powdery mildew sensitivity to fungicides on wine grapes*"