Virginia Wine Board
Year-end Report – July 2017

Fungicide sensitivity and resistance; continuation of monitoring and evaluation of powdery and downy mildew and Botrytis bunch rot

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Objectives

1. Continue research on impact of quinoxyfen (Quintec) resistance in grape powdery mildew
2. Continue research on phosphite sensitivity of grape downy mildew, with particular attention to a documented control failure in 2015
3. Continue Botrytis survey as needed to keep track of emerging resistances, and conduct field trial on efficacy of polyoxin-D (Ph-D, Oso), a recently labeled different mode of action against Botrytis.
4. Respond to emerging reports and concerns about fungicide resistance in grape pathogens

Activities and Results

Powdery Mildew – Quinoxyfen (objective 1)

The results of the 2016 field trial of the anti-powdery mildew fungicide quinoxyfen (Quintec) has been fully described in the January 2017 mid-year report, and trials in two previous years have been reported in previous reports. A manuscript describing the results of three years of such trials (2014-2016) has been prepared and submitted to the professional journal Plant Disease. To summarize, a powdery mildew population with a high frequency of resistance to quinoxyfen was detected in the fall of 2013 at a commercial vineyard in western Virginia. In 2014, 65% of isolates from this population were resistant to quinoxyfen, which in untreated areas gradually dropped to 46% in 2016. Despite the presence of a Quintec-resistant population of powdery mildew, the effectiveness of Quintec for powdery mildew control was reduced only modestly. In 2014, it performed as well as a Rally/Endura rotation. In 2015, powdery mildew control on clusters was still quite good, but control of foliar mildew later in the season was clearly inferior to that by the Rally/Endura rotation. In 2016, quinoxyfen performed well again, slightly below the best treatments (Vivando and Aprovia) but comparable to the Rally/Endura rotation, and there was little difference between early- to mid-season applications and late-season application. Our overall conclusion is that the type of resistance we have studied can have some effect, but that it appears to be very modest.

To identify potential genetic markers that could be used in future studies, we sequenced expressed genes from conidia of moderately resistant and sensitive isolates germinated for 24h on quinoxyfen-treated and untreated leaves. RNA of conidia was extracted for high-throughput sequencing to generate a 'transcriptome', a reflection of the set of genes that are expressed during the early stage of infection. We chose this time point because quinoxyfen interrupts the early infection events such as host recognition and penetration. By comparing gene expression levels of quinoxyfen-resistant and sensitive isolates on treated and untreated leaves, we have identified a set of candidate genes that are responsive to quinoxyfen treatment, and genes associated with quinoxyfen resistance. These candidate genes can now be tested across multiple resistant isolates to identify a set of robust gene-based markers for identifying resistance in field populations.

We have generated 50X coverage (the number of times each nucleotide base is sequenced) which is the standard depth of coverage required in such 'RNA-seq' studies to detect important but rarely expressed variants. With this high level of coverage, the sequencing error rate drops to below 1 error in every 1 million sequenced bases, providing a high degree of confidence in the derived genetic markers. The selected markers (genes that are found to be highly responsive to quinoxyfen) will be used to increase the efficiency of screening for quinoxyfen-resistant isolates from the field. Raw reads, ranging from 15,985,594 to 27,339,714, were generated for each sample. The *Erysiphe necator* reference transcriptome was assembled from the sequencing data. A total number of 81,457 transcripts -- genes plus gene isoforms (different proteins coded for by the same gene) -- with nonzero read counts were obtained. Aligning treatment-specific sequencing reads to this reference identified 2,064 transcripts (2.5%) that were up-regulated and 1,433 transcripts (1.8%) that were down-regulated. Filtering these results with significance standards (a change in expression by a factor of 2 or more, and adjusted value of $P <= 0.01$) resulted in a much smaller dataset. In total, 119 transcripts were found responsive to quinoxyfen treatment in both resistant and sensitive isolates. Within the 119 transcripts, 39 genes were annotated by gene ontology (GO) database; of these, 28 genes were down-regulated and 11 genes were upregulated on quinoxyfen-treated leaves. Thirty-six differentially expressed genes were uniquely expressed in the resistant isolate after the quinoxyfen treatment and were considered genes important to the quinoxyfen-resistance response. Some of these genes correspond to similar biological response pathways; to characterize the corresponding biological processes, "gene ontology term enrichment" was performed. Sixteen genes were identified by this analysis, with 12 genes upregulated and 4 genes down-regulated in response to quinoxyfen treatment. To narrow down the potential markers list, these selected genes are now being analyzed with respect to their biological function and expression level.

Downy Mildew – Phosphite (objective 2)

In recent years, several growers and consultants in Virginia and neighboring states have raised suspicions that phosphorous acid/phosphite products (e.g. Prophyt, Phostrol, Fosphite, Rampart, Aliette) may not be as effective as they used to be. In 2012, a downy mildew sample was collected in Virginia that was able to develop a considerable amount of sporulation on plants that were treated with 0.2% Prophyt (0.5% being the maximum field rate) while very little disease developed on plants treated with 0.4% Prophyt. The downy mildew was maintained through

several transfers on similarly treated plants, with the amount of downy mildew gradually decreased. Despite Prophyt being applied every 14 days, the trial rows experienced a serious downy mildew outbreak after four applications; although 14 days is a relatively long interval under high disease pressure, our expectation was that it should have performed better. Those experiences suggested a hypothesis that fungicide resistance to phosphonates can develop, but perhaps an unstable type of resistance, that may be maintained only under selection pressure. In 2016, a field trial was set up to examine the efficacy of Prophyt in the same location in comparison with other downy mildew fungicides. Prophyt was applied every 14 days at 0.5% (the highest label rate). Downy mildew control was poor again (details presented in the January 2017 semi-annual report), confirming the 2015 experience. Isolates collected from trial vines were bioassayed along with a "standard" (reference) isolate collected before phosphites became commonly used.

Potted-plant experiment

One bulk sample taken from a non-phosphite-treated field trial plot (Y4) and one bulk sample from a Prophyt treated plot (RS33) were cultured on potted Catawba plants either treated with 0.2% Prophyt (treated) or distilled water (nontreated). Isolates were bioassayed every five generations. The EC50 values (concentration estimated to provide 50% disease inhibition) along with their 95% confidence intervals are shown in Table 1, and the degree of reproducibility of the assay is illustrated in Table 2. The initial EC50 of Y4 (0.033) was similar to that of RS33 from Prophyt treated plot (0.027) (Table 1). The EC50 value of RS33 slightly increased in the period of ten transfers, however, EC50 values of Y4 fluctuated without showing an increase. There appeared to be no consistent loss of sensitivity of either Y4 and RS33 on either the Prophyt-treated or the nontreated plants

Isolates collected from vineyards throughout Virginia were bioassayed to screen for possible differences in sensitivity to phosphite fungicides. Isolate SuD1, which was collected in 2006, was included for comparison since it was collected before use of phosphite or phosphonate fungicides became common. The EC50 values along with their 95% confidence intervals are shown in Table 3. None of the tested isolates showed a notable lower of sensitivity. Isolates from the 2016 field trial plots were bioassayed along with the reference isolate; some isolates were not as sensitive as the reference isolate, but the reduction in sensitivity was small.

Table 1. EC50 values of downy mildew isolates "trained" by regular exposure to 0.2% Prophyt

	Start of pot trial			5th generation		10th generation	
Isolate	EC50 (%)	95% Confidence interval	Potted plant treatment	EC50 (%)	95% Confidence interval	EC50 (%)	95% Confidence interval
Y4, from nontreated plots	**0.033**	0.026-0.041	Nontreated	**0.041**	0.023-0.059	**0.020**	0.013-0.027
			Treated	**0.044**	0.020-0.068	**0.022**	0.015-0.029
RS33, from treated plots	**0.027**	0.011-0.043	Nontreated	**0.042**	0.028-0.056	**0.020**	0.005-0.036
			Treated	**0.024**	0.011-0.038	**0.036**	0.014-0.059

EC50 = concentration estimated to cause 50% inhibition; smaller numbers mean greater sensitivity

Table 2. Reproducibility of the biosassays of *Plasmopara viticola* (n=3) against Prophyt.

Isolate	Mean EC50 (%)	Coefficient of Variation (%)	95% Confidence interval, lower level	95% Confidence interval, upper level
C4Y	**0.028**	54.3	0.011	0.046
GRS28	**0.022**	53.2	0.009	0.035

Table 3. EC50 values of downy mildew isolates against Prophyt

Isolate	Location	Collection date	EC50 (%)	Confidence interval (95%)
C4Y	Raphine, VA	August, 2015	**0.022**	0.016-0.029
C6R	Raphine, VA	August, 2015	**0.018**	0.016-0.021
E4Y	Raphine, VA	August, 2015	**0.045**	0.038-0.052
F13Y	Raphine, VA	August, 2015	**0.023**	0.017-0.029
F4Y-2	Raphine, VA	August, 2015	**0.032**	0.030-0.033
GRS28	Raphine, VA	August, 2015	**0.009**	0.008-0.011
GRS33-2	Raphine, VA	August, 2015	**0.018**	0.009-0.027
FLB	Florida	August, 2013	**0.005**	0 -0.010
FLC-1	Florida	August, 2013	**0.015**	0.013-0.017
FLC-2	Florida	August, 2013	**0.002**	0.001-0.004
GHCh-2	Free Union, VA	October, 2013	**0.007**	0.006-0.007
GHV-1	Free Union, VA	October, 2013	**0.006**	0.005-0.007
GHV-2	Free Union, VA	October, 2013	**0.005**	0.005-0.006
Mar	Warrenton, VA	October, 2014	**0.006**	0.005-0.007
Medcel	Warrenton, VA	October, 2014	**0.007**	0.003-0.010
MJCab	Crozet, VA	October, 2013	**0.025**	0.023-0.028
MJCh	Crozet, VA	October, 2013	**0.003**	0.002-0.004
MJCh-2	Crozet, VA	October, 2013	**0.005**	0.004-0.005
MJTa	Crozet, VA	October, 2013	**0.020**	0.017-0.022
Mu	Brownsburg, VA	July, 2014	**0.003**	0.001-0.004
RoPin	Raphine, VA	August, 2015	**0.010**	0.008-0.012
SuD1	Greene Co, VA	2006	**0.004**	0 -0.008
SuD2	Greene Co, VA	2006	**0.004**	0 -0.008
SuD3	Greene Co, VA	2006	**0.006**	0 -0.014
Wil	Lynchburg, VA	July, 2015	**0.020**	0010-0.029
WinCh	Winchester, VA	August, 2014	**0.018**	0.013-0.022

Downy Mildew sequencing

The downy mildew research focuses on generating genetic markers for screening population diversity of *Plasmopara viticola* across Virginia and other nearby states, as well as identifying specific markers associated with fungicide resistance. As a first step, we have generated whole

genome sequences for two geographically and genetically distinct isolates, one from Virginia and one from Florida. Using these two genomes we have derived genetic markers for population identification from field-collected samples.

We have collected sufficient high-quality DNA and sequenced samples to 200X coverage which is sufficient for a *de novo* assembly (assembling the sequenced fragments without the aid of a reference genome). These *de novo* assemblies will be important for identifying novel structural variants (large physical repositioning in the genome), an important consideration as recently these sorts of genetic variants have been associated with fungicide resistances among crop oomycetes (the group to which downy mildews belong). Using these assemblies in conjunction with a recently published genome of a European isolate we have identified over 30,000 isolate-specific genetic markers. These genomic resources enable the identification of population-specific markers. Thus, isolates that are identified as resistant can be screened with this panel of markers and we can quickly identify genetic markers for screening field populations for resistant genotypes.

Botrytis and powdery mildew field trial (objective 3)

Results of a field trial of several relatively new fungicides (PhD, Aprovia, Kenja) against Botrytis bunch rot and powdery mildew were reported in the January 2017 semi-annual report. Aprovia was the most effective anti-powdery mildew treatment in this trial followed by Kenja, and the Switch or Elevate plus sulfur combination, whereas PhD had at best a small effect on powdery mildew. None of the treatments were effective in reducing cluster rot. *Botrytis cinerea* resistance to the first Group 7 fungicide, boscalid (Endura, component of Pristine), is common in Virginia, but boscalid resistance has generally not entailed resistance to another group 7 fungicide fluopyram (Luna, from Bayer). Aprovia (benzovindiflupyr, from Syngenta) and Kenja (isofetamid, from SummitAgro) are fungicides that have become recently registered for use on grapes, and have FRAC group 7 mode of action (SDHI or succinate dehydrogenase inhibition). Among 9 boscalid-resistant isolates, 7 were sensitive to the two other fungicides, and 2 had uncertain reactions, and need to be repeated.

Downy Mildew – Revus (mandipropamid) resistance (objective 4)

A sample of downy mildew-diseased leaves yielding one *P. viticola* isolate was received in July of 2016, and additional samples were collected in October 2016 from one vineyard in west-central Virginia, where Revus (23.4% SC mandipropamid) or Revus Top was reported to have failed to control downy mildew. Revus had been applied three times to this vineyard in 2016. In our bioassays, the EC50 values of all 8 isolates from the commercial vineyards were >240 µg/ml for mandipropamid, which was above the field rate (8 fl oz/acre, if applied in 70 gallons of water/acre would be 223 µg/ml active ingredient) showing their insensitivity to mandipropamid, whereas the EC50 value of the reference *P. viticola* isolate was <0.2 µg/ml.

A recent paper from Italy by Nanni *et al.* (2015. Differences in the efficacy of carboxylic acid amide fungicides against less sensitive strains of *Plasmopara viticola*. Pest Management Science 72: 1537–1539) indicated that Italian isolates with a resistance mutation (G1105S) were no

longer controlled by curative applications (one day after infection) but were still well controlled by preventative applications (one day before infection). Two experiments with potted plants were conducted, with the results (Table 4) indicating that treatment both before and after infection were ineffective in controlling the Virginia resistant isolates.

Table 4. Downy mildew disease severity of five most-infected leaves

Revus treatment	Trial 1 Severity 10d (%)	Trial 2 Severity 12d (%)
None	38	88
One day before infection	34	58
One day after infection	28	70

A sample of our mandipropamid-resistant downy mildew has been provided to Syngenta for DNA extraction and mutation characterization.

In order to determine how widespread this resistance may be, potted "sentinel" vines were distributed to four vineyards in the general area around the vineyard where the mandipropamid resistance was detected. Some of these will be regularly sprayed with mandipropamid while other will be left untreated. If/when downy mildew shows up on these vines, samples will be collected for further characterization.

An article in the April 2017 Viticulture Notes was used to alert Virginia growers to both the status of our quinoxyfen research and the detection of mandipropamid resistance.

Publications in FY 2016-2017

Rallos, L.E.E and A. B. Baudoin. 2016. Co-occurrence of two allelic variants of CYP51 in *Erysiphe necator* and their correlation with over-expression for DMI resistance. PLoS ONE 11: e0148025. doi:10.1371/journal.pone.0148025.

Baudoin, A. 2017. Fungicide resistance to Quintec or Revus in Virginia: good news versus bad news. Viticulture Notes Vol 32 No. 2 (April 2017), Virginia Cooperative Extension

Feng, X. and Baudoin, A. B. 2017 (submitted). Evaluation of quinoxyfen resistance of *Erysiphe necator* (grape powdery mildew) in a Virginia vineyard. Plant Disease.