Fitness of *Erysiphe necator* with G143A-Based Resistance to Quinone Outside Inhibitors

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Abstract

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Management of grape powdery mildew (*Erysiphe necator*) using quinone outside inhibitors (QoIs) has eroded in an increasing number of regions due to resistance development. To determine persistence of resistance when QoIs are withdrawn, competition assays were conducted on unsprayed grape plants (*Vitis vinifera* 'Chardonnay') by cycling mixtures of resistant and sensitive isolates characterized as genetically diverse based on microsatellite analyses. Under laboratory conditions, %G143A, quantified by quantitative polymerase chain reaction (qPCR), increased significantly, indicating competitiveness of the resistant fraction. To confirm competitiveness in the field, trials using potted plants were conducted. Percent G143A tended to decrease in

one growing season, probably due to spore migration and mixing of populations with natural background inoculum. In a second season, QoI resistance persisted at high frequency for 4 weeks. Resistant populations were also found to persist in one vineyard without QoI application for four consecutive years. The frequency was still about 25% in the fourth year, with higher frequency (36%) in a hotspot section. QoI-resistant populations with >5% G143A also harbored Y136F in the *cyp51* gene that confers some resistance to sterol demethylation inhibitors, another fungicide class for powdery mildew control. Double resistance could have been partly responsible for persistence of QoI resistance at this location.

Grapevine powdery mildew caused by *Erysiphe necator* (syn. *Uncinula necator* (Schw.) Burr.) is a major disease in grapes affecting vine vigor, fruit yield, wine quality, and winter-hardiness of canes (12,24,39). Grapevine susceptibility varies, but no commercially grown cultivars of the European grape species *Vitis vinifera* are known to be resistant to *E. necator* (12,13,45). Therefore, disease management relies heavily on the use of protective and systemic fungicides, in conjunction with cultural practices such as canopy management to reduce shade and promote air circulation, and avoidance of over-fertilization.

Quinone outside inhibitors (QoIs) constitute a fungicide class used for powdery mildew control in many crops including grapes (2). QoIs represented a breakthrough in chemical control with their broad-spectrum and protectant, eradicant, and systemic activities (2). However, resistance developed in a number of pathogens not long after introduction of QoIs (20,21). Practical resistance, which indicates observed loss of control in field conditions (4), in *E. necator* was first reported in New York and Pennsylvania (49). Widespread resistance was later reported present in Virginia (3,7), Michigan (36), and Europe (21). QoI resistance poses a challenge for effective disease control; thus, it is important to determine whether it is possible to devise strategies to extend the usefulness of QoIs as a major component of disease management in grapevines.

The binding of the QoI molecule to the outer quinol-oxidation site of the cytochrome *bc₁* enzyme complex disrupts ATP production (15). Mutations in the cytochrome *b* gene (*cyt b*) are associated with QoI resistance, resulting in amino acid substitutions that can disrupt fungicide binding. Three mutations—G143A, F129L, and G137R—are associated with naturally occurring QoI-resistant

pathogens (34). The G143A mutation has been detected in a number of pathogens (21), including *E. necator* isolates from the United States (3,7,36), and is associated with high resistance factors (26). The F129L substitution confers lower levels of resistance (37,47) in *Pyricularia grisea* (31), *Pythium aphanidermatum* (26), *Pyrenophora* sp. (43), and *Alternaria solani* (38). G137R is also associated with a similar resistance level as F129L and was found at low frequency in field isolates of *P. tritici-repentis* (43).

In this report, we focused on G143A as the major mechanism of QoI resistance and the only mechanism reported so far for *E. necator*. Although the mutation is claimed to have no effect on enzyme activity, suggesting that resistant individuals may not suffer a significant fitness penalty (1,6,9,26,29,33,34), QoI resistance can compromise fitness in some pathogens (15,17,18,25,35,46,50). Only a few studies have been presented on the fitness of laboratory-induced QoI-resistant mutants or of field isolates of fungal pathogens (9,18,29,35,46). Fitness can be defined as the survival and reproductive success of an allele, individual, or group (40). Understanding the fitness of resistant mutants can contribute to our ability to predict sensitivity shifts in field pathogen populations that are impacted by chemical selection pressures. If there is sufficient fitness penalty associated with resistance, withdrawal of the fungicide may result in a decline in frequency of resistance to the point that the fungicide could be reintroduced for occasional use (15). On the other hand, if there is no significant fitness penalty, anti-resistance strategies such as alternating fungicides from groups with different modes of action may only serve to delay resistance development (19), and once practical resistance develops, the fungicide has lost efficacy. Conflicting reports on the fitness of QoI-resistant pathogens (9,25,46) indicate that this may be species-specific, dictated in part by the pathogen's genetic background and conditions of disease development.

Currently, little is known regarding fitness compromise and resistance stability in *E. necator*. In the present study, the hypothesis that a fitness cost is associated with G143A-based QoI resistance in *E. necator* was tested using competition assays, where artificially mixed populations of QoI-resistant (with A143-allele) and QoI-sensitive isolates (with G143 allele) were inoculated on fungi-

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cide-free grape plants to determine whether the ratio (%G143A) of sensitive to resistant would change, either up or down, in the absence of QoI applications. The persistence of resistance in one commercial vineyard that has stopped using QoIs was also monitored for several years.

Materials and Methods

Sources and maintenance of fungal isolates and plants. Thirty-two single-spored powdery mildew isolates collected between 2005 and 2010 from diseased plants in various locations in Virginia and a few from neighboring states were used in the present study (Table 1). Isolates deployed in the field competition trials came only from Virginia sources. Powdery mildew isolates were inoculated onto young Chardonnay leaves obtained from greenhouse-grown plants, and incubated in 60-mm petri dishes with 7 ml of water agar. The leaf cultures were maintained in plastic boxes at room temperature with alternating 12-h light and 12-h dark from fluorescent lights. They were transferred to new leaves when the older leaves aged and died. Further details of culture maintenance, host leaf preparation, and spore transfers are described in Colcol et al. (8).

Dormant canes of *Vitis vinifera* 'Chardonnay' were cut into 1- or 2-internode sections (10 to 15 cm long) and placed in water with a rooting hormone until shoots and roots formed. Rooted cuttings were transplanted to a peat-based potting mix and grown under mist for 1 week in the greenhouse. Potted plants were transferred to the greenhouse bench after roots became established. Shoots were cut back weekly until inoculation to maintain a plant height of about 20 cm from the soil surface. The resulting plants were

used for competition assays under laboratory and field conditions. Larger plants grown for at least 1 year in 2-, 3-, and 5-gallon (7.56-, 11.34-, and 19 liter) nursery pots were used in field trials.

Characterizing isolates by QoI bioassay, microsatellite analysis, and G143A quantitation. Isolates were first characterized using three methods. (i) *Bioassay*. Bioassays for azoxystrobin (Abound, Syngenta) were carried out based on the protocol of Colcol et al. (8). Briefly, leaves treated or not treated with various doses of azoxystrobin were inoculated using a vacuum-operated chamber patterned after the design of Reifschneider and Boiteux (41), then the EC₅₀ was calculated as described in Colcol et al. (8). (ii) *DNA extraction and real-time polymerase chain reaction (PCR) quantitation of %G143A using allele-specific primers*. To extract DNA, fungal material was collected into a cryo-tube from each isolate grown on grape leaves for 21 to 24 days or until profuse sporulation. Tubes were centrifuged for 10 min at 10,000 rpm. Six 2.5-mm glass beads (Biospec Products) were added to the pellet. Spore and hyphal disruption was done three times using the FastPrep-24 instrument (MP Biomedicals) with tube immersion in liquid nitrogen after the first and second bead-beating. Disrupted spores were resuspended in 300 µl of Buffer RLT (Qiagen) immediately after the third bead-beating. DNA extraction was performed using the Biosprint 15 DNA Plant Kit (Qiagen) according to the manufacturer's protocol. Amplifications were performed in the iCycler iQ instrument (Bio-Rad) using the SYBR Green I fluorescent dye detection method described in Baudoin et al. (3). Two reactions were performed on the same run for each DNA sample using allele-specific primers. The first reaction amplified the wild-type allele using the forward primer PMWd (5'-TACGGG

Table 1. Quinone outside inhibitor (QoI) sensitivity profile and microsatellite-based haplotype groupings of single-spored isolates of *Erysiphe necator* used in competition experiments on *Vitis vinifera* 'Chardonnay' host plants

Isolate	Source location	%G143A ^b	EC50 ^c	Haplotype ^a	
				Group	Allele size
AMP1	Nelson Co., VA	99.9	34	1	<u>252</u> -236-187
AVP7	Loudon Co., VA	99.9	>30	2	251-236-190
AW9-1	Franklin Co., VA	99.9	3	6	251-236- <u>196</u>
BP-P1	Accomack Co., VA	99.9	6	7	251-233- <u>193</u>
GRP20	King George Co., VA	99.9	>20	13	236-233- <u>196</u>
HUP1	Halifax Co., VA	99.9	—	3	251-236- <u>193</u>
IVP11	Westmoreland Co., VA	99.9	23	8	266-233- <u>193</u>
JRP4	Hanover Co., VA	99.9	>60	9	239-233-187
LIP3	Fauquier Co., VA	99.9	9*	4	252- <u>228</u> - <u>193</u>
MDMRP4	Prince George Co., MD	99.9	>3	10	239- <u>228</u> - <u>193</u>
PRP4	Madison Co., VA	99.9	>30*	11	266-236-208
ROP6	Rockbridge Co., VA	99.9	56	12	<u>250</u> -242-190
VAHP4	Patrick Co., VA	99.9	>3	5	266- <u>228</u> - <u>196</u>
WI9-1	Frederick Co., VA	99.9	>1	—	—
BLP4	Montgomery Co., VA	0.02	0.03	18	239-222-208
BLP6	Montgomery Co., VA	0.01	0.01*	18	239-222-208
BLP11	Montgomery Co., VA	—	—	17	236-222-202
CB9-1	Montgomery Co., VA	0	<0.1	18	239-222-208
CL9-3	Montgomery Co., VA	0.02	—	19	239-236-198
CMP1	Floyd Co., VA	0.02	0.03	23	266-236- <u>193</u>
GRP1	King George Co., VA	0	<0.3*	6	251-236- <u>196</u>
FH9-1	Rappahannock Co., VA	0.01	<0.1	15	269-243-202
HCGP4	Anne Arundel Co., MD	—	0.06	—	—
LI9-1	Fauquier Co., VA	0.02	<0.1	20	269-239- <u>196</u>
MVP9	Halifax Co., VA	0.1	0.06	21	239-233- <u>193</u>
PBP1	New Kent Co., VA	0.03	0.06	22	245-236- <u>193</u>
SCCP4	Surry Co., NC	0.02	0.1	16	252- <u>231</u> -187
SHP6	Surry Co., NC	0.02	<0	22	239-233- <u>193</u>
SHP7	Surry Co., NC	0	0.1	13	236-233- <u>196</u>
SNP1	Augusta/Albemarle Co., VA	0.03	<0.01	14	254-222-211
SUP9	Greene Co., VA	0	0.5*	—	—
VIP6	Fairfax Co., VA	0.01	0.2	22	266-236- <u>193</u>

^a Based on microsatellite analysis for three loci, *EnMS1*, *EnMS3*, *EnMS7*; primers and method adapted from Frenkel et al. (22). Haplotype groups arbitrarily assigned by authors indicated as numbers in first column, followed by tri-loci allele sizes. Allele sizes not reported elsewhere are underlined. Dashes (—) indicate culture was lost before assay.

^b Measured by quantitative polymerase chain reaction (qPCR); 99.9% G143A = resistant; <1% G143A = sensitive; dashes (—) indicate culture was lost before assay.

^c Values with asterisk (*) are data from Colcol (7); dashes (—) indicate culture was lost before assay.

CAGATGAGCCTATGCGG-3'), and the second reaction amplified the mutant allele using the forward primer PMMt (5'-TACGGG CAGATGAGCCTATGCGC-3'). The reverse primer PMR (5'-ACCTACTTAAAGCTTTAGAAAGTTTCC-3') was the same for both reactions. Reactions were prepared in 25- μ l volumes containing 12.5 μ l of 2 $\times$ iQ SYBR Green Supermix (Bio-Rad), 5 μ l template DNA, and 2.5 μ l of each primer (1 μ M stock). PCR amplifications were carried out using the following parameters: preheating at 95°C for 4 min, followed by 35 cycles at 95°C for 10 s, 58°C for 15 s, and 72°C for 15 s. Data collection was carried out at 72°C. The absence of nontarget products was confirmed by automated melting curve analysis. G143A frequencies were computed from Ct (threshold cycle) values for each DNA sample using the equation derived by Sirven and Beffa (44): %G143A = $100 \times [1 \div (1 + 2^{CT(\text{mutant}) - CT(\text{wildtype})})]$. Isolates with $EC_{50} > 1$ μ g/ml were designated as resistant, while isolates with $EC_{50} < 1$ μ g/ml were considered sensitive. The genotype of resistant isolates revealed a very high frequency of the A143 allele (>99%), whereas that of sensitive isolates was very low (<1%). (iii) *Microsatellite analysis*. This was conducted to characterize diversity among isolates. The protocol developed by Frenkel et al. (22) using primers for three *E. necator* microsatellite loci, *EnMS1*, *EnMS3*, and *EnMS7*, was utilized. Reactions were carried out in the MasterCycler (Eppendorf) with the following cycling conditions: initial denaturation at 95°C for 2 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, and a final extension step at 72°C for 5 min. Two microliters of the pooled PCR products from the three amplifications were added to 9.8 μ l Hi-Di formamide (Applied Biosystems) and 0.2 μ l GeneScan Liz500 size standard (Applied Biosystems), then heated at 95°C for 5 min. Fragment analysis was conducted in the 3130xl Genetic Analyzer (Applied Biosystems). The tri-loci allele sizes were scored, and each unique tri-locus genotype was assigned a haplotype number.

Competition assays under laboratory conditions. Two types of assays were conducted: assays employing pairs of one resistant and one sensitive isolate (paired) or a combination of several resistant and sensitive isolates (bulk). A total of six independent assays were set up, with each assay consisting of one or several inocula and each inoculum sprayed onto two or three plants. Thirteen populations composed of different combinations of resistant and sensitive isolates were used. In paired inoculations, nine pairs of resistant and sensitive isolates were selected. No two isolates collected from the same vineyard source were paired together to diversify inoculum sources and utilize several genetic backgrounds. For bulked inoculations, three to eight isolates of the same phenotype were pooled and designated 'R' for resistant or 'S' for sensitive inoculum. To prepare R only or S only inoculum, powdery mildew-infected grape leaves (three per isolate) were placed in 20 ml of sterile water with 0.005% Tween 20 (STW) and vigorously shaken to dislodge fungal material. Five milliliters of the suspension adjusted to 10^4 spores/ml were sprayed on a powdery mildew-free and fungicide-free small grape plant. To prepare mixed inoculum, R and S suspensions were combined to achieve various spore ratios (5R:95S, 40R:60S, 50R:50S), and these suspensions were sprayed on small plants. Inoculated plants were incubated in plastic cylinders with a diameter of 10 cm under a 12-h light cycle for 2 weeks at room temperature; humidity in the cylinders was near saturation at night, and filtered ambient air was pumped through the cylinders during the day to lower the humidity. The remaining suspension for each inoculum was concentrated by membrane filtration through a 0.22 μ m PES membrane filter (Tisch Scientific), resuspended in 0.5 ml STW in 2-ml conical tubes, then frozen at -50°C. This material served as the DNA sample at cycle 0. Fungal material was collected from infected plants after 14 days of incubation (cycle 1) by shaking all powdery mildew-infected leaves in STW. Five milliliters of the resulting suspension were applied to a new plant to start cycle 2, while the remaining suspension was concentrated by vacuum filtration and stored at -50°C. Up to four cycles of inoculation were conducted, depending on the experiment, and spores were harvested at the end

of each cycle for G143A quantitation by real time PCR as described above.

Competition assay under field conditions. Field inoculation trials were conducted in 2011 and 2012 at the Virginia Tech Glade Road Research Center in Blacksburg, VA. The 2011 field trial was conducted from 13 June to 29 September. Five sites were selected, and three large plants (in 2-, 3-, or 5-gallon pots) were placed per site. Sites in shady places at the edge of groups of trees were each separated by over 100 m, and locations were chosen so as to maximize barriers such as bushes, trees, and buildings between them to minimize cross-infection by windblown spores. Treatments consisted of three controls (noninoculated, three resistant isolates or R inoculum only, and three sensitive isolates or S inoculum only) and a mixed (R/S) inoculum. The R/S-inoculated plants were divided over two separate sites. The inocula were prepared as described for the laboratory trial, but using a 10R:90S spore ratio for the R/S inoculum and 10 powdery mildew-infected leaves for each isolate. This ratio was used to simulate a situation where the powdery mildew population is just starting to build up resistance and hence, a low initial %G143A, and because in preceding laboratory experiments the R:S ratio had tended to rise. The inoculum (150-ml suspension, 10^4 spores/ml) was sprayed on site using a Preval Sprayer (Precision Valve Corporation). Water was applied to noninoculated control plants. New mildew-free plants were added one or two times to each site to provide additional leaf tissue for infection. Infection on new tissues was allowed to occur naturally. Infected leaves were collected during two sampling periods, 4 to 8 July and 19 to 27 September, about 3 and 16 weeks after inoculation. Only young, infected leaves were collected each time either from the originally infected plants or from newly added plants. Each infected leaf was treated as one leaf population. To produce enough fungal material for DNA extraction from each leaf sample, the infected leaf was rubbed onto disease-free grape leaves from greenhouse-grown plants, which were then incubated under a 12-h light regimen at room temperature for 14 to 20 days. Because direct rubbing of infected leaves collected from the field sometimes generated heavy contamination which hastened leaf necrosis, spores from the rub-inoculated leaves were transferred from all sporulating colonies with an acupuncture needle and deposited onto fresh leaves. Once profuse sporulation was achieved, fungal material was scraped from the leaf surface into a 2-ml conical tube and stored in STW at -50°C until DNA extraction and qPCR.

The 2012 field trial was conducted from 31 May to 29 June 2012. The procedure was the same as for the 2011 trial with the following modifications. (i) The R/S spore ratio was increased to 50R:50S consisting of the same resistant and sensitive isolates deployed in 2011 to duplicate the ratio used in the laboratory, and because the R:S ratio in the 2011 field experiment had declined. (ii) The suspension (250 ml) was applied to four large grape plants for each treatment (50R:50S, R only, S only). Plants sprayed with R/S inoculum were again divided over two sites. (iii) Inoculated leaves were allowed to dry, then whole plants were wrapped with perforated plastic bags for 12 to 16 h to promote spore germination. (iv) Three small grape plants per site were included and received the same treatment as large plants. After unbagging, two of the small plants were each enclosed in a plastic cylinder, transported to the laboratory, and incubated under laboratory conditions to check for growth and phenotype of the inoculum. The third small plant was incubated further for 1 week in the field before transport to the laboratory. The onset of visible powdery mildew and disease severity were closely monitored by visual inspection of leaves 5 days after inoculation, and every 3 days thereafter until disease rating. New plants were also added 1 week before the first sampling on 15 June to provide new tissues for infection. The second sampling was conducted on 28 to 29 June from infection on this second batch of plants. Twenty leaf populations were obtained from each treatment for each sampling date. Quantitative PCR was carried out with DNA extracted from leaf populations using the method described above.

Field monitoring of G143A. The Afton Mountain Vineyard (Afton, VA) was selected because: (i) it had withdrawn QoIs from the spray program from 2008 to 2011; (ii) powdery mildew infection occurred every sampling year from 2008 through 2011; (iii) QoI resistance was documented in this powdery mildew population in 2007, after which QoI use was terminated. The vineyard received two to four QoI applications (azoxystrobin, pyraclostrobin, or trifloxystrobin) per season prior to 2008. Demethylation inhibitors (DMIs) were also applied one to four times per season. Infected leaf samples were collected by the vineyard manager, placed in Ziploc bags protected by a cardboard box, and mailed to the laboratory. Data for 2007 from an earlier report (7) were included as baseline population profile. Each value in Table 2 represents a leaf population. For 2008, four single-spore cultures were established from different bagged-leaves collected from a hotspot section (see Fig. 1). Samples from 2009 included leaf populations in addition to two single-spore isolates. These were obtained from different bags of leaves collected randomly from the same hotspot section (Fig. 1). The 2010 samples (from the same hotspot section) and 2011 samples (from different rows, Fig. 1) consisted of only leaf populations. Ten bagged samples for 2011 were submitted for analysis, with two to three leaves per bag. Leaves per bag were pooled.

To generate enough fungal material from leaf populations, the same rubbing method was used as described for the field competition assays. Care was taken to sample all growth on infected leaves. New growth that came from the same leaf sample was treated as one “leaf population”. Spores for DNA extraction and qPCR were harvested by scraping as described for the field competition assays.

Detecting Y136F mutation in leaf populations. All leaf populations from the vineyard profiling from 2009 to 2011 were also subjected to a genotyping protocol for the Y136F mutation associ-

ated with resistance to DMIs (8). The assay included *EnCyp51*-specific forward (5'-ACTAATTAACTCCGGTCTTTGGA-3') and reverse (5'-ACTCGACCATTTACGGACCTTTT-3') primers and two allele-specific TaqMan MGB probes (Applied Biosystems), each labeled with a different reporter dye at the 5'-end (VIC-TTGGACAATCAAATACAAC-3' and FAM-TTTGGACAA TCATATACAAC-3'). Each 20-μl reaction consisted of 10 μl of 2× TaqMan Universal PCR Master Mix (no AmPErase UNG, Applied Biosystems), 0.5 μl of 40× TaqMan CYP51 Genotyping Assay mix (containing the primers and probes, Applied Biosystems) and 2 μl DNA template (5 to 8 ng/μl). Reactions were carried out in the StepOne Plus instrument (Applied Biosystems). The fluorescence of reporter dyes was normalized to the fluorescence of the passive reference dye (ROX). The StepOne Plus Real-time PCR software plots the normalized intensities of the reporter dyes in each sample well on an Allelic Discrimination Plot. Samples were assigned one of the following based on their position on the plot (our phenotype assignment in parentheses): ‘homozygous allele1/allele1’ (pure wild-type), ‘homozygous allele2/allele2’ (pure mutant), or ‘heterozygous allele1/allele2’ (mix).

Statistical analysis. To address if there was a change over time in %G143A in competition assays under laboratory conditions, the following generalized linear mixed model was fit using SAS 9.2 (SAS Institute), assuming that %G143A followed a β -distribution (16):

$$\log \left[\frac{E(Y_{ijk})}{1 - E(Y_{ijk})} \right] = \mu + \alpha_i + \alpha_{j(i)} + X_{ijk}\beta, \alpha_i \sim N(0, \sigma_1^2), \alpha_{j(i)} \sim N(0, \sigma_2^2)$$

where $[1 + \exp(-\mu)]^{-1}$ is the overall mean of G143A at day zero, α_i is the effect for population i of %G143A, $\alpha_{j(i)}$ is the effect for replication j within population i , X_{ijk} is k th measurement of time in days for rep j within population i , β is the effect of time on G143A,

Table 2. Percent G143A and occurrence of CYP51 genotypes in *Erysiphe necator* leaf populations in one vineyard that withdrew quinone outside inhibitor (QoI) fungicides in 2008

	2007 ^b	2008	2009	2010	2011
Number resistant isolates ^a / number tested	5/5	4/4	2/2	–	–
			%G143A CYP51 ^c	%G143A CYP51 ^c	%G143A CYP51 ^c
%G143A profile of leaf populations ^d	–	–	99 Mix 95 Mix 55 Mix 42 Mix 21 Mix 5 – 5 WT 0.1 WT	100 Mix 100 Mix 100 Mix 100 Mix 100 Mix 99 Mix 90 Mix 43 Mix 27 Mix 5 WT 1 WT	100 Mix 100 Mix 100 Mix 68 Mix 65 Mix 65 Mix 59 Mix 43 Mix 23 Mix 22 – 16 Mix 10 Mix 7 – 5 – 2 WT 0.01 WT 0 WT 0 WT 0 WT 0 WT 0 WT 0 WT 0 WT 0 WT 0 WT

^a Resistant = 99.9% G143A; dashes (–) indicate no single-spore isolates were tested.

^b Reported by Colcol (7).

^c Based on a SNP genotyping method using multiplex polymerase chain reaction (PCR) with TaqMan chemistry; ‘mix’ has both Y136 wildtype allele and F136 mutant allele; WT = only wildtype allele Y136 was present; dashes (–) indicate lack of DNA sample for analysis.

^d Leaf population = all powdery mildew growth on the leaf; each %G143A value represents a leaf population.

σ_1^2 is the variance of the distribution of possible populations for G143A, and σ_2^2 is the variance of the distribution of possible replications from each population. Note, σ_2^2 was assumed to be the same for all populations.

Results

Genetic diversity and phenotypic stability of single-spore isolates. All resistant isolates ($EC_{50} > 1$) used in this study possessed a high level of the G143A mutation (99.9%), while all sensitive isolates ($EC_{50} < 1$) had very low levels (<1%), even after 2 years of subculturing on fungicide-free host leaves (Table 1). This indicates that the QoI-resistance phenotype of single-spore isolates of *E. necator* based on the mitochondria-borne G143A is stable in culture on fungicide-free host leaves. We were able to reproduce the allele size and numbers reported by Frenkel et al. in their microsatellite analysis of *E. necator* from the northeastern United States (22) with some exceptions (Table 1). Those alleles not reported before may represent genotypes that were not sampled in the other study. The *EnMS1* locus was confirmed to be relatively hypervariable as reported previously (22). We retained this in our analysis since it differentiated several of the isolates that shared alleles for the other two loci. In general, the diversity among the isolates was very high as the analysis generated 23 unique tri-locus haplotypes for 29 isolates (Table 1). Twelve unique haplotypes were found within the resistant group and nine within the sensitive group. Two unique haplotypes (6 and 13) were shared by a resistant isolate and a sensitive isolate taken from distant locations. Three sensitive isolates (BLP4, BLP6, CB9-1) obtained from sites in Blacksburg and Christiansburg (Montgomery County), VA,

which are relatively close, shared the same alleles for the three loci; the same was true for three other sensitive isolates (PBP1, SHP6, VIP6) from sources that were more distant from each other.

Competitiveness under laboratory conditions. Growth rates (mm/day) of six QoI-sensitive and five QoI-resistant isolates were compared based on diameters of colonies grown from spores inoculated on detached grape leaves. The interaction effect between phenotype (R or S) and rating interval was significant ($P = 0.03$); thus, the growth rate from day 6 to 7 and from day 7 to 8 were not averaged. Mean growth rates of the sensitive and resistant groups were not statistically different for either rating interval (1.2 ± 0.05 mm/day for sensitive and 1.0 ± 0.06 mm/day for resistant from day 6 to 7; 0.8 ± 0.05 for sensitive and 0.9 ± 0.05 for resistant from day 7 to 8).

Out of the six independent competition assays, two are shown in Figure 2A and B. In paired (Fig. 2A) and bulked inoculations (Fig. 2B), the percent G143A tended to increase, and a decline was observed only rarely. When all data were fitted into the generalized linear mixed model, the increase in percent G143A over time in mixed populations and in the absence of the fungicide was significant ($P = 0.03$, Fig. 2C).

Competitiveness under field conditions: 2011 trial. In the first site (R/S-1), starting with a low proportion of resistance (10R:90S spore ratio, with measured A143 allele frequency at 5%), the resistant fraction increased to 14% but dropped to <1% after another 10 to 11 weeks of infection (Fig. 3, 2011). An opposite trend was observed in the second site (R/S-2), with the population decreasing first to 0.1% followed by an increase back to 5% by the second sampling. In the S population, G143A remained low at <1%

Fig. 1. Distribution of QoI resistance of *Erysiphe necator* in a Chardonnay block at Afton Mountain Vineyard (Afton, VA) in 2011. Values show %G143A of populations from the row where infected leaf samples were taken. Each value represents the population on one leaf. Percent G143A was analyzed by qPCR. Shaded areas on the left side of the vineyard block and on front row are hot spot sections with yearly incidence of powdery mildew.

G143A. The frequency of G143A in the R population dropped to <10% by 8 July, and remained at that level until the end of the season. The noninoculated plants developed powdery mildew later than inoculated plants, but disease development was substantial by 4 to 8 July (>30% of leaf surfaces with powdery mildew). G143A on noninoculated control plants was low at <1% G143A on the average (mid-season and late-season sampling); however, one leaf population with a 3% G143A was detected (data not shown), suggesting that spore migration from one inoculated site to another could occur. In the 2011 field inoculation, there was neither an obvious decline nor increase in percent G143A for mix-inoculated plants. In plants inoculated with only resistant spores, %G143A unexpectedly declined from 100% to about 11% (Fig. 3). Powdery mildew collected from the noninoculated plants (background infection) remained low in %G143A, and severe powdery mildew developed on these.

Competitiveness under field conditions: 2012 trial. Visible powdery mildew was first observed 11 days after inoculation only on powdery mildew-inoculated plants for both small plants transported back to the lab and field plants, confirming that the sprayed inoculum was able to cause infection under field conditions. Only a few leaves in the noninoculated control plants showed visible powdery mildew growth (<1% surface area) by the final evaluation date on 15 July, and samples taken in late June to mid-July were free of mildew, except for one leaf sample (5% G143A). Thus, spore migration probably did occur over 100 m despite presence of barriers. On average, %G143A in R and S populations remained constant throughout the experiment (Fig. 3, 2012), except for one R and one S leaf population, with 70 and 13% G143A, respectively (data not shown). Initial R/S inoculum consisted of 63% G143A and increased to 84 and 93% G143A on the small plants at the two sites (1 June 2012, Fig. 3). On large plants, %G143A of the R/S population increased to an average of 98% for both sites 2 weeks after inoculation, and dropped slightly to about 80% after an additional 2 weeks. Throughout the monitoring period, high proportions of G143A did not disappear from the population in 2012.

Persistence of QoI resistance in a vineyard population. Only a few single-spore isolates were obtained in 2007, the last year that QoIs were applied to the vines according to the spray record of the vineyard manager, and tested for QoI resistance by Colcol (7). All four 2007 isolates were resistant, with almost 100% G143A (Table 2). In 2008 to 2009, the first 2 years that QoIs were not included in the spray program, all six isolates obtained were still QoI-resistant. A large number of leaf populations were added in 2009, and we worked with leaf populations only thereafter. These samples were easier to manage in the laboratory and provided a more precise estimate of allele frequencies than a similar number of single-spore isolates. We detected the presence of mixed populations on individual leaves. A physical map of the leaf populations sampled in 2011 showed that resistant populations were confined to the front rows of the vineyard block up to row 14 (middle of the Chardonnay block), with a decreasing percent G143A (Fig. 1). Percent G143A in leaf populations sampled from row 18 to 31 was nil. The vineyard manager reported that the 2007 to 2010 samples had all been collected in the front rows or the hotspot sections. These findings support the persistence of QoI resistance in the pathogen population even without QoI selection pressure. In addition, the Y136F mutation in the demethylase protein (CYP51) of *E. necator* was detected in leaf populations with >5% G143A and in all completely QoI-resistant populations (>99% G143A) but absent in all QoI-sensitive populations in all sampling years (Table 2).

Discussion

E. necator isolates harboring high proportions of G143A (almost 100%), and therefore considered to be QoI-resistant, retained the phenotype even after more than 50 consecutive transfers on fungicide-free grape leaves, indicating the stability of resistance when grown in isolation. This means that the G143A-carrying mitochondria in the resistant fungal cell are not lost through repeated subculturing, probably because they have become homoplasmic in

most isolates. This is in contrast with a minority of isolates of *Blumeria graminis* f. sp. *tritici* which were heteroplasmic, and lost the mutation and regained the wild-type allele after many transfers on fungicide-free leaves (18). The absence of a difference in growth rate between our resistant and sensitive groups grown on untreated leaves in the laboratory suggests that the G143A mutation in cytochrome *b* has no harmful effect on the growth of *E. necator*. However, the overall fitness of one population relative to another does not depend on only one fitness parameter; a combination of param-

Fig. 2. A and B, Actual changes in percent G143A determined by qPCR of artificial mixtures of QoI-resistant and QoI-sensitive isolates of *Erysiphe necator* cycled on fungicide-free grape plants. Each cycle represents 14 days of incubation. **A,** Percent G143A over time for four pairs of isolates. Each pair was a unique combination of a sensitive and a resistant isolate inoculated on two plants. Each line represents one inoculated plant. The starting proportion of the mutant allele (A143A) in each population varied from 5 to 65%. **B,** Percent G143A over time in a second experiment using one bulked inoculum consisting of eight resistant and eight sensitive isolates. Several starting ratios of the mutant allele were used (5 to 55%, represented by the different lines). In both A and B, powdery mildew was harvested after one cycle and inoculated to new plants to start another cycle. DNA for qPCR was extracted from a fraction of the harvested material from each cycle. Extractions that yielded poor DNA quality are represented by lines with missing data point. **C,** Plot of the conditional fitted values of %G143A over cycle of the inocula used in experiments A (▲) (two replications averaged) and B (●) (all combinations combined in one line). Inoculum samples were treated as random effect factor. Analysis was based on the generalized linear mixed model, assuming a β -distribution for %G143A, SAS v. 9.2 ($P = 0.03$).

eters (growth, latent period, spore production, infection efficiency) factored into a fitness index would be more informative (9). Fitness refers to the survival and reproductive success of an allele, individual, or group (40), and the relative ability to survive and reproduce is critical to the persistence of resistant individuals in a population once they have been selected (34). If resistant isolates are as fit as sensitive isolates, they are expected to persist in the population even after the fungicide application is stopped. Experimental results show that vegetative and reproductive fitness costs associated with fungicide resistance vary with fungal species and with fitness parameters measured (10). The fungicide in question can also be an important determining factor in the stability or instability of resistance in certain pathogens. For example, benzimidazole-resistant isolates of *Cercospora beticola* did not exhibit any fitness penalty, but strains resistant to fentin fungicides were less competitive than sensitive strains (30).

In our competition experiments, we followed the same principle that Schepers (42) applied in fitness testing by utilizing many combinations of resistant and sensitive isolates in order to reduce side-effects brought about by different genetic backgrounds. Fitness is a result of interacting factors in the whole genome, which implies that not all observed differences in fitness can be attributed to the resistance phenotype. Ideally, two isogenic isolates which differ only in fungicide sensitivity are compared. This approach is not feasible with *E. necator* because of the lack of such isolates and a method to generate resistance under controlled conditions; hence, the use of several isolates with different genetic backgrounds in fitness testing. High genetic diversity in our isolates was confirmed by microsatellite analysis, which agrees with the conclusions of Frenkel et al. (22) for *E. necator* in the eastern part of the United States. It is interesting to note that a few QoI-sensitive isolates collected from locations near each other (BLP4, BLP6, and CB9-1) belonged to the same haplotype group, and thus may be clones. The occurrence of the same haplotypes from sites at much greater distances from each other was noted for a few other isolates (e.g., PBP1, VIP6, and SHP6). This could be due to long-distance dispersal (27) but also occasional cross-contamination during 6 years of culture maintenance.

The beta distribution is a flexible distribution for modeling variables on open intervals, thus, it is a natural choice for modeling our response variable (%G143A) in the competition assays under laboratory conditions. The error structure on population and replication within population was chosen to account for genetic relat-

edness of samples from the same lineage. The data structure is simple and has only one covariate of interest. Based on residual diagnostic plots of model performance, we found the model to be sufficient at describing the data without adding extra complexity to the random or fixed effects in the model.

Based on the model analysis for %G143A, our resistant isolates tended to be more competitive than sensitive isolates over several generations under laboratory conditions and in the absence of fungicide, indicating a lack of significant fitness penalty. In other species, a fitness cost may sometimes be compensated for by mutations in another locus (or other loci) to restore or even increase fitness (28). In *Saccharomyces cerevisiae*, the G143A mutant was shown to have lower respiration and growth (48). However, a secondary mutation in *cyt b* and over-expression of certain nuclear genes with mitochondria-related function restored the respiratory fitness of the resistant mutant. This compensatory effect could possibly occur in plant pathogens since heterologous expression of one of the nuclear genes from *Botrytis cinerea* in *S. cerevisiae* produced the same result (48). We further observed in in vitro competition studies that increases in %G143A were delayed or slow in populations starting out with low fractions of the mutant allele, i.e., populations with 5:95 R:S ratio (Fig. 2A and B, other data not shown). Based on this observation, we hypothesized further that there is a density effect where it takes a certain proportion of mutants for rapid changes to occur in the absence of selection pressure, but we have no firm data confirming that this is the case.

Under field conditions, the competitiveness of our resistant isolates was not as obvious. The low %G143A in the noninoculated plants and severe powdery mildew development on these same plants suggest that the drop in %G143A in R-inoculated plants could be largely due to the influx of sensitive strains and some spore migration from one treatment to another. This huge decline was not observed in the 2012 field inoculation trial. We did not conduct a test to detect background infection, but it appears plausible that spore migration between treatments and/or immigration from outside sources was responsible for this decline. Mere mixing of inoculum among treatments would have been expected to result in approximately 30% G143A, while the actual percentage in July and September was clearly lower than 30%. There are no commercial vineyards nearby, but small plantings are likely to exist in residential neighborhoods several hundred meters to a kilometer away, and wild grapevines are present in wooded areas and seem likely to provide a source of unsprayed powdery mildew. The effect of

Fig. 3. Changes in %G143A of *Erysiphe necator* populations consisting of QoI-resistant (R), sensitive (S), or mix (R/S) isolates inoculated on QoI-free potted plants (*Vitis vinifera* 'Chardonnay') under 2011 and 2012 field conditions. Noninoculated plants were sprayed with water. Initial %G143A was based on the spore inoculum ("Inoc"). Percent G143A was quantified by SYBR Green real-time polymerase chain reaction from 15 to 20 leaf samples per treatment per sampling date.

background infection may have been pronounced in the first field trial because of the longer interval between inoculation and sampling (4 weeks), and between first and second sampling periods (10 to 11 weeks), allowing for more time for mixing of populations, or may have been due to differences in weather conditions and wind patterns. Wind speed data from an automated weather station located about 3 km to the WSW of the experimental site indicated that average wind speed in 2011 for the first part of the experiment (through 8 July) was 0.66 m/s with maxima during 15-min intervals averaging 1.79 m/s. In 2012, the average from 31 May through 29 June was 0.90 m/s with maxima during 15-min intervals averaging 2.14 m/s; thus, 2012 was somewhat windier than 2011.

The persistence of resistant populations of *E. necator* for 4 years (2008 to 2011) was documented in a vineyard that has withdrawn strobilurins (QoI) from the spray program. The remaining resistant proportion would appear large enough, if strobilurin is reapplied to this population, to reproduce rapidly and spread the mutant allele quickly throughout the vineyard block again. Vineyard leaf samples collected in 2010 came solely from the front rows, and earlier samples came from hotspot sections (Fig. 1). The hotspot section suffered powdery mildew infection yearly, according to the vineyard manager. Pathogen leaf populations harboring high %G143A were detected from the hotspot sections every sampling year even without QoI application. The very heterogeneous populations from hotspot sections in 2009 to 2011 suggest considerable strain mixing. Sensitive leaf populations (<1% G143A) were regularly detected as well. These populations either remained as persistent as the resistant populations or could have been constantly introduced from outside sources. Wild grapes are known to grow in the wooded area adjacent to the vineyard, and these may be a reservoir of wild-type powdery mildew. In *E. necator*, little host specialization has been found among *Vitis* species, except for *V. rotundifolia* (5,23), and we have successfully cultured powdery mildew isolated from a wild species (*V. vulpina*) on *V. vinifera*, indicating that gene flow can occur from one species to another.

A vineyard map of our leaf sample data indicates that resistance was confined within the first 14 rows (Fig. 1), where both high and low levels of %G143A were detected. Only sensitive leaf populations were detected in the back rows. It is not clear how the hotspot section came about initially and why infection occurred in this area every year. A partial explanation may be the presence of a culvert near the R1 mark (Fig. 1) that made it difficult to spray that area (H. Corpora, *personal communication*). Although strobilurins were discontinued in this vineyard starting in 2008, DMIs remained part of the disease control program through all years of sampling. DMI resistance in *E. necator* operates by a different mechanism and, at least in part, involves a mutation (Y136F) in the CYP51-target molecule (8,11). The fact that Y136F was detected in all leaf populations with detectable G143A, and was absent in leaf populations without detectable G143A, raises the possibility that selection by DMI applications may have maintained the presence of G143A. The resistance phenotype of these isolates was not determined, but in a separate study, 17 out of 18 isolates with Y136F had higher resistance factors (median RF = 27) to one or more DMIs than all isolates lacking the mutation (median RF = 1) (L. Rallos, *unpublished data*). The occurrence of double resistance to DMI and QoI in *E. necator* was earlier reported in Europe (14), and DMI-resistant isolates of *Monilinia fructicola* have been found to be predisposed to accelerated development of QoI resistance (mechanism not investigated) (32). These phenomena can limit the use of DMIs and QoIs as rotational or tank mix partners. The durability of QoI resistance in this population was probably further enhanced by DMI-selection of double mutants.

Overall, QoI resistance in *E. necator* did not disappear quickly when QoI selection pressure was removed. Therefore, relying on sensitivity shifts is not a practical approach to resistance management. Once resistance develops to high frequencies, and when it is not associated with fitness penalties, using QoIs even in mixtures—a commonly recommended strategy to slow down re-

sistance development—will maintain the resistant allele in the population. It is also important to determine if resistance to other chemistries used in the vineyard is present before mitigating the situation. Our detection of *cyp51* mutants from Afton Mountain Vineyard indicates the concurrent presence of DMI resistance, which may explain the development of powdery mildew every year despite DMI applications. We have no evidence that the QoI and DMI resistances are linked, and our results suggest that even chance combinations of resistance can affect selection. QoIs have broad-spectrum activity and continue to be an integral component of grape disease control. It is thus important to understand the response of pathogen populations to changes in selection pressure in order to continuously develop strategies that will maximize the benefits and minimize the disadvantages (such as pathogen resistance build-up) resulting from the use of these chemical tools if their useful life is to be extended. Such studies on fitness cost may present ways to improve risk assessment of resistance development in some situations, but in this case served to demonstrate that, once resistance developed, the only viable course was to abandon the use of QoIs against *E. necator*.

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