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Strobilurin (QoI) Resistance in Populations of *Erysiphe necator* on Grapes in Michigan

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Abstract

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Powdery mildew, caused by *Erysiphe necator*, is the most common and destructive disease of grapes (*Vitis* spp.) worldwide. In Michigan, it is primarily controlled with fungicides, including strobilurins (quinone outside inhibitors [QoIs]). Within the United States, resistance to this class of fungicides has been reported in *E. necator* populations in some east coast states. Among 12 *E. necator* isolates collected from five Michigan vineyards in 2008, one carried the G143A single-nucleotide mutation responsible for QoI resistance. This isolate was confirmed to be resistant in a conidium germination assay on water agar amended with trifloxystrobin at 0.001, 0.01, 0.1, 1, 10, or 100 µg/ml and salicylhydroxamic acid (100 mg/liter). The mutant isolate was able to germinate on media amended with 100 µg/ml trifloxystrobin, whereas a representative wild-type isolate did not germinate at concentrations higher than 0.1 µg/ml. In 2009, 172 isolates were collected from a total of 21 vineyards (juice and wine grapes): three vineyards with no fungicide application history (baseline sites), six research vineyards, and 12 commercial vineyards. QoI resistance was defined as the effective concentration that inhibited 50% of conidial germination (EC_{50}) > 1 µg/ml. Isolates from baseline sites had EC_{50} values mostly below 0.01 µg/ml, while isolates that were highly resistant to trifloxystrobin (EC_{50} > 100 µg/ml) occurred in five research and three commercial wine grape vineyards at frequencies of 40 to 100% and 25 to 75% of the isolates, respectively. The G143A mutation was detected in every isolate with an EC_{50} > 1 µg/ml. These results suggest that fungicide resistance may play a role in suboptimal control of powdery mildew observed in some Michigan vineyards and emphasizes the need for continued fungicide resistance management.

Powdery mildew (*Erysiphe necator* Schwein.) is the most destructive and widely distributed disease of grapes worldwide (16). Many wine grape cultivars (*Vitis vinifera* L. and *Vitis* L. interspecific hybrids) are highly susceptible. Considerable reductions in fruit and wine quality can occur even when infections are relatively mild, e.g., 1 to 5% of the berries infected in a cluster (4,20,21). Juice grapes (*V. labrusca* L.) are more tolerant to powdery mildew than many wine grape cultivars, but reductions in juice quality in terms of sugar content, color, and acidity have also been reported (10). Traditionally, management of powdery mildew has relied on the application of sulfur, synthetic fungicides, or a combination of both (16). The sterol demethylation inhibitor (Fungicide Resistance Action Committee [FRAC] code 3) and quinone outside inhibitor (QoI, strobilurin [FRAC code 11]) fungicides are widely used around the world. In Michigan, QoI fungicides may be applied throughout the growing season, but the total number of applications per hectare per season is limited to reduce the risk of fungicide resistance (23). Since the introduction of fungicides with a site-specific mode of action, the development of fungicide resistance has become an important issue in crop protection because the repeated use of these products selects for those rare fungicide-resistant isolates with genetic mutations that survive and proliferate in the population (13).

Fungicides in the QoI class are site-specific and act by blocking mitochondrial respiration in a wide range of fungi (Ascomycetes, Basidiomycetes, and Deuteromycetes) as well as Oomycetes; they specifically bind to the ubiquinol oxidation center (Q_o site) of cytochrome b, obstructing the electron transfer to cytochrome c_1, which then prevents the synthesis of adenosine-5′-triphosphate (ATP) (2). In total, at least 11 point mutations have been identified in the cytochrome b (*CYTB*) gene that confer different levels of resistance to QoI fungicides in a variety of organisms (11). In plant-pathogenic fungi, three single-nucleotide mutations of the *CYTB* gene (G143A, F129L, and G137R) and the activation of the alternative oxidation (AOX) pathway have been reported as the mechanisms through which these fungi acquire resistance to QoI fungicides (12,13). In the United States, QoI resistance in *E. necator* has been previously reported in Maryland, New York, North Carolina, Pennsylvania, and Virginia (3,22), based on the nucleotide sequence change that leads to the G143A mutation and the resistant phenotype (3,6).

The objectives of this study were to screen a subpopulation of *E. necator* isolates collected from Michigan vineyards for resistance to trifloxystrobin, a synthetic fungicide in the QoI class that is commonly used in disease management programs throughout the state. This survey was designed in response to concerns from grape growers regarding inadequate control of powdery mildew in their vineyards. The baseline sensitivity of *E. necator* to trifloxystrobin was determined for a subsample of individuals isolated from sites with no history of strobilurin fungicide applications.

Materials and Methods

Collection of *E. necator* isolates. *Preliminary study of QoI resistance*. In October 2008, a total of 12 single-conidium isolates were collected in Michigan from two commercial vineyards (three isolates from a 'Concord' vineyard in Watervliet and three from a 'Cabernet Sauvignon' vineyard in Traverse City), two Michigan State University (MSU) research vineyards (three isolates from 'Concord' in Fennville and one isolate from 'Pinot Noir' in Traverse City), and an MSU greenhouse (two isolates from potted 'Chardonnay' plants in East Lansing).

Main study of QoI resistance. A more in-depth sampling was performed in September and October 2009; isolates were collected

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from 21 juice and wine grape vineyards in Michigan. The vineyards were located in southwest, central, and northwest Michigan. Three sites with no history of fungicide applications (from this point forward referred to as "baseline sites"), six MSU research vineyards, and 12 commercial vineyards were included in the sample. Baseline sites were located in East Lansing (abandoned 'Concord' vineyard) and Traverse City ('Pinot Gris' and 'Vignoles' vineyards managed according to the principles of biodynamic agriculture). MSU research vineyards were located at the Clarksville Research Center (CRC, 42° 52′ 18″ N, 85° 15′ 31″ W) in Clarksville; Northwest Michigan Horticultural Research Center (NWMHRC, 44° 52′ 59″ N, 85° 40′ 28″ W) in Traverse City; Southwest Michigan Research and Extension Center (SWMREC, 42° 5′ 22″ N, 86° 21′ 36″ W) in Benton Harbor; and Trevor Nichols Research Center (TNRC, 42° 35′ 38″ N, 86° 9′ 19″ W) in Fennville, MI. Commercial vineyards were mostly located in Southwest Michigan (two 'Chancellor', six 'Concord', one 'Frontenac', and one mixed *V. vinifera* vineyard). One 'Frontenac' vineyard in Highland was also sampled as well as a young 'Chardonnay' vineyard near Traverse City (Table 1).

Each vineyard in 2009 was sampled following a diagonal pattern. Two leaves were collected from a single vine in 10 different rows evenly spaced and proportional to the size of the vineyard. In vineyards with less than 10 rows, more vines were sampled per row. A total of 20 powdery-mildew-infected leaves were collected per site. Samples were cold-transported to the laboratory in plastic resealable bags.

Maintenance of *E. necator* isolates. An ethanol-sterilized eyelash attached with Parafilm M to a glass Pasteur pipet was used to transfer a single conidium from each field sample onto a single, detached, disease-free *V. vinifera* 'Chardonnay' leaf placed in a petri dish containing 25 ml of water agar as previously described (8). A total of 20 conidial transfers were attempted per site. The leaves were taken from potted, 1-year-old, bare-root vines grown in 16-cm plastic pots containing commercial potting soil (BACCTO, Michigan Peat Company, Houston, TX, USA), selecting young leaves at the third and fourth node positions from the shoot tip. The vines were started and kept in a controlled-environment growth chamber (model E15, Conviron, Winnipeg, Manitoba, Canada) at 25°C with a photoperiod of 16 h of light (fluorescent light intensity of 230 mE at a height of 30 cm) and 8 h of darkness. In total, 172 single-conidium isolates were obtained in 2009 from both juice (*Vitis labrusca* L.) and wine (*V. vinifera* L. and interspecific hybrids) grapevines (Table 1). Powdery mildew isolates were maintained on 'Chardonnay' leaves by transferring two to three conidial chains every 3 weeks onto three separate areas of a leaf (in duplicate), until fungicide sensitivity and molecular analyses were performed. To prevent cross contamination between isolates, all transferring utensils were dipped in ethanol for 1 min and allowed to dry between inoculations. All transfers were conducted in a laminar flow hood, which was also sprayed with ethanol between isolates. Noninoculated leaves served as controls of contamination. Between three and seven transfers were made per isolate before DNA extraction and bioassays were performed.

Detection of the G143A mutation. For every *E. necator* isolate collected in 2008 and 2009, DNA was extracted from conidia scraped from 3-week-old colonies growing on detached 'Chardonnay' leaves. Conidia were suspended in 1 ml of lysis buffer (2% CTAB, 3% SDS, 25 mM EDTA, 200 mM Tris-HCl pH 8.5, and 250 mM NaCl) heated at 65°C for 1 h. DNA in the supernatant was extracted and stored as previously described (18).

Preliminary study of QoI resistance. In the study of isolates collected in 2008, 5 µl of DNA was used in 25-µl polymerase chain reactions (PCR) amplifying the ribosomal internal transcribed spacer (ITS) region (as a control of fungal DNA amplification) and a fragment of the mitochondrial cytochrome b (*CYTB*) gene containing the G143A single-nucleotide mutation responsible for strobilurin resistance (13). The primer sets used in each case were ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), and the Amplification Refrac-

Table 1. Origin, G143A detection, and trifloxystrobin sensitivity of *Erysiphe necator* isolates collected in Michigan during 2009

Vineyard site[a]		Location[b]		Grape host[c]		G143A detection[d]		Trifloxystrobin sensitivity[e]	
No.	Type	City	Region	Type	Cultivar	Total tested	Total positive	Total tested	EC_{50} range (µg/ml)
1	Baseline	East Lansing	C	Labrusca	Concord	14	0	14	<0.001 to 0.005
2	Baseline	Traverse City	NW	Vinifera	Pinot Gris	8	0	8	<0.001 to 0.159
3	Baseline	Traverse City	NW	Hybrid	Vignoles	17	0	15	<0.001 to 0.006
4	Research	Fennville	SW	Hybrid	Aurora	8	8	4	57.3 to >100
5	Research	Fennville	SW	Hybrid	Chancellor	8	8	7	22.2 to >100
6	Research	Clarksville	C	Hybrid	Chardonel	4	4	3	>100
7	Research	Traverse City	NW	Vinifera	Mixed	9	9	5	0.1 to >100
8	Research	Benton Harbor	SW	*Vitis* spp.	Mixed	7	0	2	<0.001
9	Research	Clarksville	C	Hybrid	Vignoles	7	7	5	0.251 to >100
10	Commercial	Lawton	SW	Hybrid	Chancellor	5	5	4	0.316 to >100
11	Commercial	Lawton	SW	Hybrid	Chancellor	6	6	2	1.7 to >100
12	Commercial	St. Joseph	SW	Labrusca	Concord	4	0	4	<0.001
13	Commercial	Lawton	SW	Labrusca	Concord	8	1	3	<0.001
14	Commercial	Berrien Springs	SW	Labrusca	Concord	5	0	5	<0.001
15	Commercial	Lawton	SW	Labrusca	Concord	9	0	7	<0.001 to 0.002
16	Commercial	Lawton	SW	Labrusca	Concord	10	0	7	<0.001
17	Commercial	Lawton	SW	Labrusca	Concord	8	0	4	<0.001 to 0.002
18	Commercial	Highland	C	Hybrid	Frontenac	10	0	10	<0.001 to 0.003
19	Commercial	Lawton	SW	Hybrid	Frontenac	6	6	4	0.088 to >100
20	Commercial	Berrien Springs	SW	Vinifera	Mixed	10	0	8	<0.001
21	Commercial	Traverse City	NW	Vinifera	Chardonnay	9	0	4	<0.001
Total						172	54	125	

[a] Vineyard site: baseline = no history of fungicide applications, research = Michigan State University research vineyard, commercial = Michigan grape grower's vineyard.

[b] Location = nearest city and region where vineyards were located (SW = Southwest Michigan, C = Central Michigan, NW = Northwest Michigan). These are all in the lower peninsula of Michigan.

[c] Hybrid = *Vitis* L., interspecific hybrid.

[d] A total of 172 isolates were tested using the Amplification Refractory Mutation System (ARMS)-SYBR Green Q-PCR standardized by Baudoin et al. (3). A positive detection was defined as an isolate with >95% of mitochondrial DNA with the G143A allele of the *CYTB* gene. The results for each site are shown as the number of isolates tested and the number of isolates that tested positive.

[e] A total of 125 isolates were tested in an in vitro conidium germination assay to test sensitivity to trifloxystrobin (Flint, 50% active ingredient). The number of isolates tested and the range of EC_{50} values are shown for each site.

tory Mutation System (ARMS) primer sets for cytochrome b developed by Baudoin et al. (3) to detect the wild-type and mutant allele G143A, respectively (expected product size approximately 100 base pairs). To detect the G143A mutation with traditional PCR, 25-µl reactions (with final primer concentrations of 600 nM each) were prepared and run under the following conditions: an initial denaturation step at 95°C for 15 min, followed by 40 amplification cycles (denaturation at 94°C for 15 s, annealing at 60°C for 30 s, extension at 72°C for 30 s), and a final elongation step at 72°C for 10 min. PCR products were then run in a 1% agarose Tris-acetate-ethylenediaminetetraacetic acid (TAE) gel at 100 V for 45 min in 1× TAE buffer, and stained with ethidium bromide (0.5 µg/ml). To verify *CYTB* gene amplification, the products obtained with both the wild-type and mutant primer sets were cloned for two representative isolates using the pGEMT-T Easy Kit (Promega, Madison, WI, USA). Then, 5 µl of the ligation reaction was used to transform DH5α chemically competent cells (Invitrogen, Carlsbad, CA, USA) using the manufacturer's protocols. Inserts were sequenced using T7 (5′-AATACGACTCACTATAG-3′) and Sp6 (5′-ATTTAGGTGACACTATAG-3′) primers in the MSU Genomics Technology Support Facility (GTSF). Contiguous sequences were constructed with the DNASTAR Lasergene 8 program (DNASTAR Inc., Madison, WI, USA) and were analyzed using the GenBank BLASTn program. The total DNA of these two *E. necator* isolates was diluted to a concentration of 5 ng/µl and used as a reference to standardize the detection of the G143A mutation for the 2009 powdery mildew collection by quantitative PCR. DNA concentrations were determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). Detection of the point mutation was performed with 15-µl reactions (with final primer concentrations of 300 nM each) using the ARMS-SYBR Green Q-PCR standardized by Baudoin et al. (3) and the Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems Inc., Foster City, CA, USA) in the MSU GTSF. This was done at three different concentrations: the standard concentration (5 ng/µl) and two 10-fold serial dilutions (1:10 and 1:100 vol/vol) of the DNA extracts of each reference isolate. Amplification plots and disassociation curves were inspected for each pair of primers to analyze the quality of each overall reaction and final PCR product, respectively.

Main study of QoI resistance. The DNA extracts of the 2009 powdery mildew collection were diluted to a concentration of 5 ng/µl for Q-PCR. Detection of the G143A mutation was performed as previously described using the ARMS-SYBR Green Q-PCR standardized by Baudoin et al. (3). The percentage of G143A in the total mitochondrial DNA of each isolate was calculated using the cycle threshold (Ct) value obtained from the amplification with both wild-type and mutant primer sets as described by Colcol (5) using the following equation: $100 \times \{1/[1 + 2^{(\text{mutant Ct} - \text{wild type Ct})}]\}$. Whenever the percentage of the G143A allele in any given isolate was higher than 95%, the detection was considered positive. Amplification plots and disassociation curves were also inspected for every isolate that was evaluated.

Differences in detection of the G143A mutation were also assessed by performing chi-square analysis. Chi-square (χ^2) values were determined for the hypothesis that the total number of positive detections was evenly distributed over each type of vineyard (baseline, research, or commercial), region in Michigan (northwest, central, or southwest), and type of grape (juice or wine).

Fungicide sensitivity. A conidium germination bioassay was performed on microscope slides covered with 3 ml of water agar amended with the commercial formulation of the fungicide Flint (trifloxystrobin, 50% active ingredient; Bayer Crop Sciences, Kansas, MO, USA) at a trifloxystrobin concentration of 0.001, 0.01, 0.1, 1, 10, or 100 µg/ml. The medium was also supplemented with 100 mg/liter of salicylhydroxamic acid (SHAM, 99%, Sigma-Aldrich, St. Louis, MO, USA) dissolved in methanol to block the alternative oxidation (AOX) pathway (25). Germination controls included powdery mildew conidia on slides with water agar only and water agar amended with SHAM. Conidia were transferred from a 2-week-old *E. necator* colony onto the agar slides with an ethanol-sterilized, fine-hair paintbrush. Inoculated slides were placed inside 150 × 15 mm petri dishes. After 24 h of incubation under fluorescent light at ambient temperature (22 to 24°C), 50 conidia were microscopically observed for germination in each of three replicate samples per treatment. A conidium was considered germinated if the germ tube was equal in length to at least half the width of the conidium. From the preliminary study in 2008, two representative isolates with differential patterns of amplification using the G143A primer set for detection of the G143A mutation (Supplemental Fig. 1) were tested to corroborate the results obtained using the molecular approach. A total of 125 isolates were evaluated in 2009 using this germination assay.

Qualitative analysis of trifloxystrobin sensitivity. For this analysis, an isolate was declared resistant if the average number of germinated conidia in a particular treatment was at least two standard deviations above the mean in the control treatment (i.e., agar slides amended with SHAM). At each trifloxystrobin concentration evaluated, either a "1" (germination occurred hence the isolate was considered resistant) or a "0" (germination did not occur hence the isolate was considered sensitive) was assigned to every isolate tested.

Quantitative analysis of trifloxystrobin sensitivity. Inhibition percentages were calculated relative to germination of conidia in the SHAM control. The effective concentration of trifloxystrobin that inhibited 50% of the conidial germination (EC_{50}) was calculated by linear regression analysis of the germination percentage of *E. necator* conidia according to the logarithm of the active ingredient concentration. When the control treatment with SHAM only had a larger inhibitory effect than the lowest fungicide concentration (i.e., agar slides amended with trifloxystrobin at 0.001 µg/ml and SHAM at 100 mg/liter), the latter was used as the reference point for calculating the EC_{50}. This effect was observed in 28% (48 of 172) of the isolates tested. The reproducibility of the bioassay for trifloxystrobin sensitivity was evaluated by testing six isolates (three from baseline sites and three from MSU research vineyards) three times. For every repetition of the bioassay, new stock solutions of both SHAM and trifloxystrobin were prepared.

Responses of *E. necator* to trifloxystrobin and SHAM. Due to an inhibitory effect of SHAM on 28% of the isolates, the responses of *E. necator* to SHAM were further characterized for a sensitive isolate (collected from a baseline vineyard) and a resistant isolate (collected from an MSU research vineyard). Different concentrations of SHAM (12.5, 25, 50, and 100 mg/liter) were evaluated on agar slides amended with 0, 0.001, 0.1, and 10 µg/ml trifloxystrobin. Treatment differences were analyzed by one-way analysis of variance (ANOVA) using the statistical computer package SigmaPlot (version 11.0, Systat Software Inc., San Jose, CA, USA). Differences were analyzed further using Fisher's protected LSD as the all-pairwise multiple comparison procedure, and treatment effects were declared significant at $P \leq 0.05$.

Results

Preliminary study of QoI sensitivity in 2008. Amplification and sequencing of the ITS region confirmed that the DNA samples belonged to *E. necator* and no other eukaryotic microorganisms or plant tissue were present in the DNA extracts. The ARMS primer sets (CYTB wild type and CYTB G143A mutant) and conditions standardized by Baudoin et al. (3) allowed for the amplification of a fragment of the cytochrome b (*CYTB*) gene by traditional PCR for detection of the presence or absence of the G143A mutation. Using the wild-type primers, a single PCR product (~100 bp) was amplified. Using the G143A primers, a distinct amplification pattern was observed for a single isolate (out of 12 total) in a 1% agarose TAE gel. Background amplification was observed in reactions with all other isolates and in the water control with bands of approximately 400, 350, and a faint band at 100 bp. However, in isolate 16, a strong PCR band of about 100 bp was observed with no background amplification. This *E. necator* isolate originated

from a commercial 'Cabernet Sauvignon' vineyard in Traverse City, MI.

The detection of the G143A mutation was further confirmed by ARMS-SYBR Green Q-PCR with final DNA concentrations of 500, 50, and 5 pg/µl. The G143A primers led to earlier amplicon detection in the reaction with the resistant isolate as compared to the wild-type primers (Supplemental Fig. 2B). In a susceptible isolate, detection occurred earlier with the wild-type primers. We did not observe any evidence of background amplification in the Q-PCR assays. We believe that higher primer concentrations in the traditional PCR (600 nM per primer) than in the Q-PCR assays (300 nM per primer) could have contributed to nonspecific amplifications. Additionally, different DNA polymerases and thermocyclers were used for the two different types of PCR assays.

The in vitro germination bioassay on agar slides amended with trifloxystrobin and SHAM showed that the isolate with the G143A mutation was able to germinate at the highest trifloxystrobin concentration tested (89% germination at 100 µg/ml, relative to the SHAM control). A representative wild-type isolate was chosen based on the *CYTB* genotype screening. This isolate was collected from a 'Concord' MSU research vineyard in Fennville and was unable to germinate at a trifloxystrobin concentration greater than 0.1 µg/ml. The estimated EC_{50} value for the sensitive isolate was 0.03 µg/ml, while the value for the resistant isolate could not be calculated because it exceeded the highest concentration tested. The DNA from these two *E. necator* isolates was used as reference controls for the ARMS-SYBR Green Q-PCR detection of the G143A mutation in the 2009 powdery mildew collection.

Qualitative analysis of QoI sensitivity in 2009. Twenty-one vineyards were sampled in Michigan during 2009. In total, 125 isolates were evaluated for sensitivity to trifloxystrobin in conidium germination bioassays. The qualitative germination analysis demonstrated that the majority of the isolates germinated at 0.001 µg/ml trifloxystrobin (Table 2). In general, as we increased the concentration of trifloxystrobin in the assay, the proportion of isolates that were able to germinate decreased considerably. At the highest fungicide concentrations (10 and 100 µg/ml), only 21% of the isolates germinated (Table 2).

None of the 14 *E. necator* isolates collected from the abandoned 'Concord' vineyard in East Lansing (one of the baseline sites) were able to germinate at trifloxystrobin concentrations higher than 0.001 µg/ml. Among the 23 baseline isolates collected from the 'Pinot Gris' and 'Vignoles' biodynamic vineyards near Traverse City, 35% were able to germinate at 0.01 µg/ml trifloxystrobin and 9% germinated at 0.1 µg/ml trifloxystrobin, which corresponds to 21.6 and 5.4% of all baseline isolates, respectively (Table 2). At the highest trifloxystrobin concentration evaluated (100 µg/ml), only 11.3% of the isolates from commercial vineyards germinated, whereas 73.1% of the isolates from research vineyards did (Table 2).

When comparing isolates from juice grapes (*V. labrusca* L.) and wine grapes (*V. vinifera* L. and *Vitis* L. interspecific hybrids), 32.1% of the isolates from wine grapes (26 of 81 isolates) were able to germinate at 100 µg/ml trifloxystrobin (Table 2), whereas no isolates from juice grapes were able to germinate at concentrations higher than 0.01 µg/ml trifloxystrobin. In fact, only 6.8% of the juice grape isolates (3 of 44 isolates) germinated at 0.01 µg/ml trifloxystrobin (Table 2).

Quantitative analysis of QoI sensitivity in 2009. *Reproducibility of the conidium germination bioassay.* The effective concentrations of trifloxystrobin at which conidial germination was inhibited by 50% (EC_{50}) were estimated for six isolates (three from baseline sites and three from research vineyards) in three repetitions of the germination bioassay on agar slides amended with trifloxystrobin and SHAM. The calculated coefficients of variation ranged from 23 to 141% (Table 3). All three *E. necator* isolates from baseline sites had a mean EC_{50} value lower than 0.001 µg/ml, while two of three isolates from research vineyards had a mean EC_{50} value higher than 100 µg/ml. Due to potential errors that may result from extrapolating beyond the range of concentrations tested, no EC_{50} values are reported here for those *E. necator* isolates with a value lower than 0.001 or higher than 100 µg/ml, but they were grouped in the EC_{50} categories of "≤0.001 µg/ml" or "≥100 µg/ml", respec-

Table 2. Percentage of *Erysiphe necator* isolates collected in Michigan vineyards in 2009 that germinated at different concentrations of trifloxystrobin evaluated (0.001, 0.01, 0.1, 1, 10, and 100 µg/ml)[a]

		Number of isolates	Percentage of isolates that germinated at each trifloxystrobin concentration (µg/ml)					
			0.001	0.01	0.1	1	10	100
Type of site	Baseline	37	86.5	21.6	5.4	0.0	0.0	0.0
	Commercial	62	61.3	25.8	16.1	12.9	11.3	11.3
	Research	26	88.5	84.6	84.6	76.9	73.1	73.1
Type of grape	Juice[b]	44	63.6	6.8	0.0	0.0	0.0	0.0
	Wine	81	80.2	53.1	42.0	34.6	32.1	32.1
Total		125	74.4	36.8	27.2	22.4	20.8	20.8

[a] Germination was considered to have occurred when the average number of germinated conidia at a certain fungicide concentration was at least two standard deviations above the mean in the control treatment.
[b] Juice grapes are cultivars of *Vitis labrusca* L. Wine grapes are cultivars of *V. vinifera* L. and *Vitis* L. interspecific hybrids.

Table 3. Reproducibility of the *Erysiphe necator* conidium germination bioassay using agar slides amended with different concentrations (0.001, 0.01, 0.1, 1, 10, or 100 µg/ml) of the fungicide trifloxystrobin and 100 mg/liter SHAM[a]

	Origin of isolate				
Isolate	Type of site	Location	Host cultivar	Mean EC_{50} (µg/ml)[b]	CV (%)[c]
1	Baseline	Traverse City	Vignoles	<0.001	37
2	Baseline	Traverse City	Vignoles	<0.001	75
3	Baseline	Traverse City	Pinot Gris	<0.001	23
4	Research	Fennville	Aurora	1.2	129
5	Research	Clarksville	Vignoles	>100	138
6	Research	Clarksville	Chardonel	>100	141

[a] Data represent the averages of three separate experiments.
[b] Mean EC_{50} value of trifloxystrobin (Flint, 50% active ingredient). The values "<0.001 µg/ml" or ">100 µg/ml" denote cases where the numerical value lay beyond the resolution range of the test but actual numbers (outside the range) were used to calculate the coefficient of variation.
[c] Coefficient of variation (CV = [standard deviation/mean] × 100) for the EC_{50} values based on estimates of three separate bioassays.

tively. The mean EC_{50} value for the third isolate from research vineyards was estimated as 1.2 µg/ml (Table 3).

Trifloxystrobin sensitivity ranges in the subpopulation of 2009. The calculation of EC_{50} values generated a series of fungicide sensitivity ranges (Table 1, Fig. 1). *E. necator* isolates from the baseline sites had EC_{50} values ranging from less than 0.001 µg/ml to 0.159 µg/ml (Fig. 1). Isolates collected from commercial vineyards and MSU research centers had EC_{50} values that ranged from less than 0.001 µg/ml to more than 100 µg/ml (Fig. 1). Isolates with EC_{50} values higher than 100 µg/ml were found in 5 of 6 MSU research vineyards and 3 of 12 commercial vineyards (Table 1).

The percentage of *E. necator* isolates that were highly sensitive to trifloxystrobin (EC_{50} values lower than 0.001 µg/ml) was 73 and 79% in the baseline and commercial vineyards, respectively (Fig. 1A and B). This proportion dropped to 8% in the MSU research vineyards, where 62% of the isolates had EC_{50} values higher than 100 µg/ml (Fig. 1C).

Detection of G143A mutation in the 2009 collection. Total DNA was extracted from 172 *E. necator* isolates collected in Michigan during 2009. All of these isolates were analyzed for the presence of the G143A mutation in the *CYTB* gene using the ARMS-SYBR Green Q-PCR detection system developed by Baudoin et al. (3). The mutant allele was detected at very low percentages (between 0.005 and 1.75% of the total mitochondrial DNA per single-conidium isolate) in the isolates obtained from all baseline sites. However, in the majority of MSU research vineyards (5 of 6 sites) and in some commercial vineyards (4 of 12 sites), isolates having a high percentage of the G143A allele present (between 95.3 and 99.99% of the total mitochondrial DNA per single-conidium isolate) were found (Table 1). Calculations of the %G143A allele in each isolate indicated that the proportion of the mutant allele was close to but never reached 100%; this suggests that isolates that tested positive for the mutation (%G143A > 95%, as defined in the Materials and Methods) still possess residual wild-type mitochondria (A. Baudoin, *personal communication*).

Among the site types (baseline, commercial, or research), the proportion of isolates that exhibited the mutation (%G143A > 95% of the total mitochondrial DNA) was significantly different (Table 4). No positive detections occurred in isolates from baseline vineyards. In research vineyards, 84% of the isolates tested positive, whereas in commercial vineyards, only 20% of the isolates tested positive (Table 4). By region (northwest, central, or southwest), no significant differences in the proportion of isolates having a positive detection for the G143A mutation were observed. The mutation was more frequently detected in isolates from wine grapes than from juice grapes (Table 4). A high percentage of the G143A mutation was detected in the mitochondrial DNA of only one of eight isolates from a 'Concord' vineyard in Lawton, MI (Table 1). However, this isolate was lost before a QoI-resistant phenotype could be confirmed by the in vitro germination assay. This vineyard was within 2 km of a wine grape vineyard where resistant isolates were found.

Relationship between the bioassay and Q-PCR G143A detection. A total of 124 isolates from the 2009 *E. necator* collection were evaluated for QoI resistance using both the conidial germination assay and the molecular detection by ARMS-SYBR Green Q-PCR. The proportion of isolates with the G143A mutation progressively increased at higher EC_{50} values of trifloxystrobin (Fig. 2). None of the 87 isolates that had an EC_{50} value of less than 0.001 to 0.01 µg/ml contained more than 5% of the G143A mutation in their mitochondrial DNA. However, 1 out of 4 isolates with EC_{50} values of 0.0101 to 0.1 µg/ml and 4 out of 5 isolates with EC_{50} values in the range of 0.101 to 1.0 µg/ml tested positive for the G143A mutation. Further investigation into the presence of the mutation in sensitive isolates is required to determine the relevance of these genotypes with respect to disease management. The mutation was also detected at very high percentages in all of the 28 *E. necator* isolates with estimated EC_{50} values greater than 1.0 µg/ml (Fig. 2).

***E. necator* responses to SHAM.** The calculation of EC_{50} values was complicated as some *E. necator* isolates were inhibited by SHAM at 100 mg/liter in the agar slides amended with SHAM only. The conidial germination rate of 35% (13 of 37) of the isolates from baseline sites was reduced by 31 to 98% compared to the germination on slides with nonamended water agar. Likewise, germination of 20% (7 out of 35) of the isolates from MSU research vineyards was reduced by 21 to 65%, and the germination of 23% (14 out of 62) of the isolates from commercial vineyards was reduced by 19 to 97% (data not shown). The remaining per-

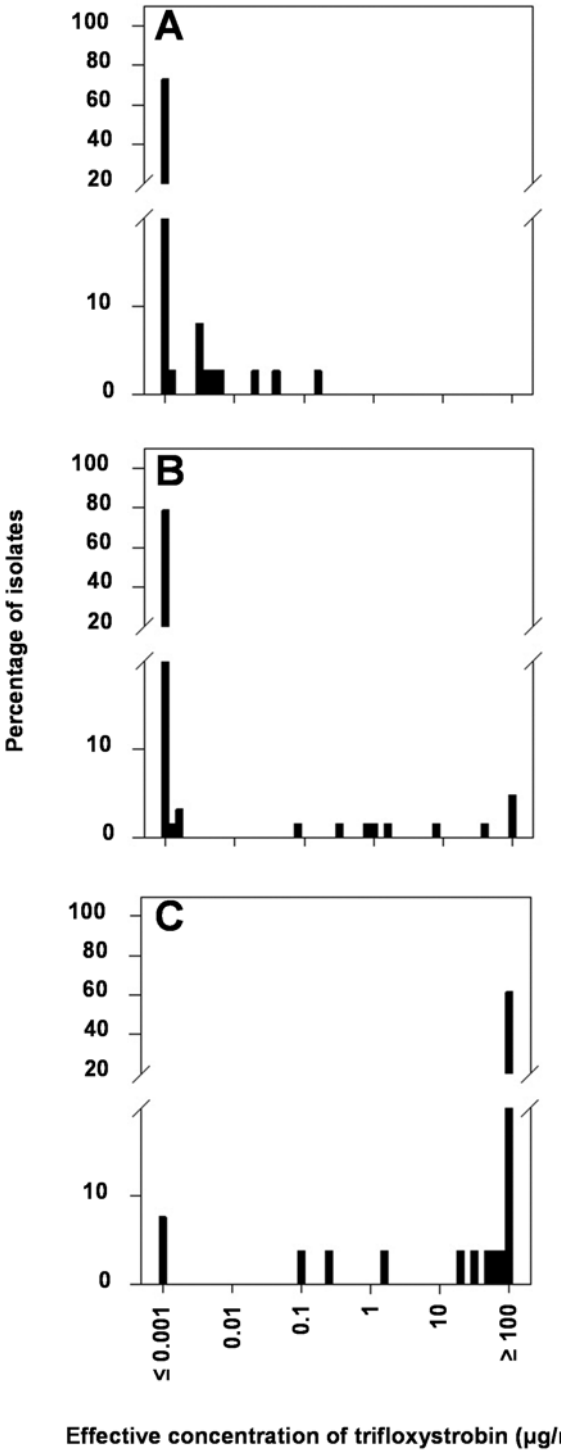

Fig. 1. Distribution of effective concentration values of trifloxystrobin that inhibited 50% of the conidial germination (EC_{50} in micrograms per milliliter) in *Erysiphe necator* isolates collected in Michigan vineyards during 2009. Isolates were obtained from **A,** baseline sites (n = 37), **B,** commercial vineyards (n = 62), and **C,** Michigan State University research vineyards (n = 26). Percentages were calculated according to the total number of isolates tested within each type of site. Data shown are averages of three replicates per isolate.

centages in each category correspond to isolates that were not inhibited by the addition of SHAM to the media.

To further analyze the inhibitory effect of SHAM on the germination of *E. necator*, two representative isolates (one trifloxystrobin-resistant, one sensitive, as determined from the in vitro bioassay) were tested on water agar slides amended with 0, 12.5, 25, 50, and 100 mg/liter SHAM. In the treatments with SHAM only (no trifloxystrobin), an inhibitory effect of the chemical was observed at all concentrations (Fig. 3). However, the effect was very slight at lower concentrations of SHAM (i.e., 12.5 to 50 mg/liter), and it was only significant at 100 mg/liter SHAM for the trifloxystrobin-sensitive isolate (Fisher's protected LSD, $P < 0.001$). Germination was reduced to less than 50% in this treatment (Fig. 3A). For the trifloxystrobin-resistant isolate, the inhibition by SHAM alone was not as pronounced as for the sensitive isolate, but it was significant at 50 and 100 mg/liter (Fisher's protected LSD, $P < 0.001$). The conidial germination percentage of the resistant isolate in these SHAM treatments was 81 and 84%, respectively (Fig. 3B).

The combined effect of SHAM and trifloxystrobin was further analyzed for these two *E. necator* isolates with contrasting fungicide-sensitivity phenotypes. Depending on the amount of SHAM utilized in the germination assay, different responses to the fungicide were observed (Fig. 3). The greatest inhibitory effect was observed when 100 mg/liter SHAM was added to the fungicide assays for both isolates. Conidium germination of the sensitive isolate was inhibited to different degrees at 0.001 µg/ml trifloxystrobin depending on the amount of SHAM added to the media (Fisher's protected LSD, $P < 0.001$), but germination was completely inhibited at higher trifloxystrobin concentrations regardless of the amount of SHAM in the media (Fig. 3A). In the case of the resistant isolate, the two highest SHAM concentrations inhibited germination the most (Fig. 3B). In the 100 mg/liter SHAM plus 0.1 and 10 µg/ml trifloxystrobin treatments, germination of the resistant *E. necator* isolates was reduced by 47 and 80%, respectively, compared to the respective controls (Fisher's protected LSD, $P < 0.001$, Fig. 3B).

Discussion

Strobilurin (QoI) fungicides have been used for disease management in grapes in the United States for more than a decade (7). Due to their broad-spectrum activity and good weathering ability, growers have adopted them relatively quickly. Resistance to QoI fungicides was detected in certain plant pathogens, notably *Blumeria graminis* (DC.) Speer in cereals, within a few years of first use (2,13). In addition, resistant isolates of *E. necator* have been detected previously in grapes in the United States (3,22). In most cases, QoI resistance was associated with the G143A mutation (2,13). An outbreak of powdery mildew in Michigan vineyards in 2006 despite regular fungicide applications led growers to question whether fungicide resistance had developed. However, poor control may also result from high disease pressure, use of ineffective fungicides, improper application timing, poor spray coverage, or a combination of these. This study represents the first assessment of fungicide (QoI) sensitivity in the *E. necator* population on grapes in Michigan using conidium germination (a phenotypic approach) and Q-PCR assays to detect the G143A mutation (a genotypic approach).

In grapes in Michigan, *E. necator* isolates with reduced sensitivity to trifloxystrobin were mostly found in research vineyards and to a lesser extent in commercial vineyards. Of the latter, wine grape vineyards were more likely to have resistant strains than juice grape vineyards. All of these isolates appear to have acquired resistance to trifloxystrobin through the G143A mutation. The detection of resistant strains does not necessarily equate to field-scale resistance with a concomitant loss of control due to the fact that pathogen populations are heterogeneous in a field scenario. However, targeted sampling of vineyards with severe powdery

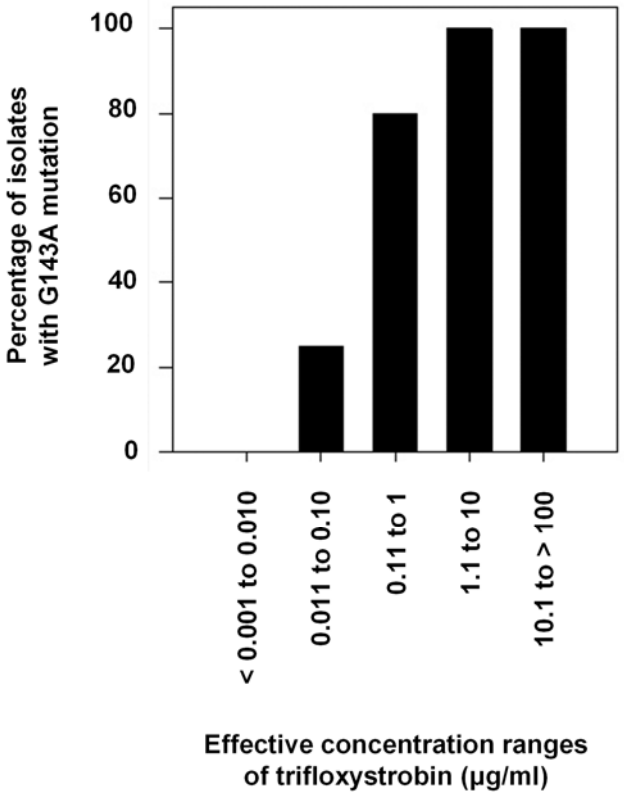

Fig. 2. Detection of the G143A mutation according to EC_{50} ranges calculated for *Erysiphe necator* isolates collected in Michigan vineyards during 2009. The percentage of isolates with the mutation was calculated based on a total of 124 isolates tested by an in vitro conidium germination bioassay as well as the Amplification Refractory Mutation System (ARMS)-SYBR Green Q-PCR test developed by Baudoin et al. (3).

Table 4. Chi-square analysis of the detection of the G143A mutation in the cytochrome b gene of *Erysiphe necator* isolates collected from grapes in Michigan in 2009 according to type of vineyard site (baseline, commercial, or Michigan State University research vineyard), region in the state of Michigan (northwest, central, or southwest), and type of grape (juice or wine)

Variables		Number of isolates with mutation/ total number of isolates tested[a]	Statistical test[b]		
			χ^2	df	*P* value
Site	Baseline	0/39	77.9	2	<0.0001
	Commercial	18/90			
	Research	36/43			
Region	Northwest	9/43	3.2	2	<0.2038
	Central	11/35			
	Southwest	34/94			
Grape	Juice	1/60	37.8	1	<0.0001
	Wine	53/112			

[a] Number of isolates with >95% of mitochondrial DNA with the G143A allele.
[b] The null hypothesis was that the proportions of isolates with > 95% of mitochondrial DNA with G143 allele are the same among the categories (type of site, region in Michigan, and type of grape).

mildew problems despite regular fungicide spray programs in the Traverse City area in 2010 resulted in the detection of resistant isolates in two out of three vineyards. Observations in MSU research vineyards over several years have also revealed lower-than-expected powdery mildew control with rotational programs of strobilurins and sterol inhibitors. Adding a protectant fungicide like ziram in the tank mix usually improved control (19).

In a study by Wong and Wilcox (24), the range of EC_{50} values determined for single-conidial-chain *E. necator* isolates collected from two baseline vineyards in the state of New York was 0.0058 to 0.052 µg/ml trifloxystrobin (mean 0.015 µg/ml, $n = 26$), which falls into the same EC_{50} range that we estimated for the baseline sites in Michigan. However, our powdery mildew baseline subpopulation included several isolates with EC_{50} values of less than 0.001 µg/ml (27 of 37 isolates, corresponding to 73%), but it should be noted that different assays (e.g., germination on agar slides versus mycelial growth on a leaf) could lead to somewhat different EC_{50} values. Wong and Wilcox (24) also found a strong correlation ($R^2 = 0.75$, $P < 0.001$) between azoxystrobin and trifloxystrobin EC_{50} values and proposed a single discriminatory dose of 0.031 µg/ml of azoxystrobin for the characterization of isolates within the range that was determined for sensitive isolates (24). From our results, 95, 84, and 8% of isolates from the baseline, commercial, and research vineyards, respectively, had EC_{50} values below the above-mentioned dose. Wong and Wilcox (24) also established an additional discriminatory dose of 2 µg/ml of azoxystrobin for the characterization of resistant isolates. When using this cut-off point, 0, 8, and 81% of the isolates from the baseline, commercial, and research vineyards in Michigan, respectively, were resistant to the QoI fungicide. The recommended rates per hectare for azoxystrobin, however, are two to four times higher than those for trifloxystrobin, which would suggest the need for a higher discriminatory dose when evaluating resistance to azoxystrobin.

In another study, 35 *E. necator* isolates were collected in 2002 from vineyards producing table grapes, raisins, and wine grapes in California and tested for trifloxystrobin sensitivity (among other fungicides) after the fungicide had been introduced to the market in 2001 (14). EC_{50} values ranged from 0.00003 to 0.343 µg/ml, with a mean value of 0.021 µg/ml. The range that we established for baseline isolates in Michigan was <0.001 to 0.159 µg/ml, with a mean value of 0.007 µg/ml trifloxystrobin (Table 3). While different methods were employed to obtain the EC_{50} values, both studies show similar ranges but different means. However, the Californian isolates were collected from commercial vineyards throughout the state and may have had some exposure to strobilurin fungicides, while our isolates came from a limited number of baseline sites with no known exposure history. In all, the EC_{50} values calculated for isolates from commercial vineyards in Michigan varied greatly (<0.001 to >100 µg/ml of trifloxystrobin).

In this study, the calculation of EC_{50} values was complicated by the inhibitory effect of SHAM on conidial germination of *E. necator*. However, the use of SHAM was necessary to block the alternative oxidation (AOX) pathway, which is not normally active *in planta* but occurs in culture media (1,15,25), to provide a more accurate estimate of fungicide sensitivity. In some fungi, the synthesis of the alternative oxidase is constitutive (always expressed); however, in most other fungi, the synthesis is induced when the main pathway has been inhibited (25). Inhibitory effects of SHAM were also reported for several foliar fungal pathogens of citrus, including *Colletotrichum acutatum* J.H. Simmonds, *Elsinoe fawcettii* Bitanc. & Jenkins, *Diaporthe citri* (Faw.) Wolf, and *Mycosphaerella citri* Whiteside (15). The authors also observed an increase in colony diameter when a low rate of the fungicide (i.e., 0.01 µg/ml pyraclostrobin or azoxystrobin) was added to the SHAM treatment when compared to SHAM without the fungicide amendment. In our study, the response to 100 mg/liter SHAM was stronger in the trifloxystrobin-sensitive than the trifloxystrobin-resistant isolate, which is not surprising as mutations in the *CYTB* gene would be able to circumvent the blockage of the Q_o site. This provides evidence that *E. necator* utilizes the alternative oxidation (AOX) pathway. In that sense, our results disagree with the study by Mondal et al. (15) in which no evidence was found of AOX occurring in the mycelium of fungal pathogens of citrus. However, this is probably because QoI fungicides affect mycelial growth to a lesser extent than spore germination (25). Wood and Hollomon (25) also discussed that, *in planta,* the energy requirement for a pathogen to sporulate and germinate is higher than for mycelial growth. Therefore, the AOX pathway is less likely to compensate for the high-energy requirements during the process of spore germination.

Despite the inhibitory effects of SHAM on germination of *E. necator*, detection of the G143A mutation corroborates our phenotypic observations. Overall, the mutant allele was always found in isolates with EC_{50} values higher than 1 µg/ml trifloxystrobin, a dose that could be used as a discriminatory dose for future trifloxystrobin sensitivity screenings. A higher dose, such as the one proposed for azoxystrobin (2 µg/ml) by Wong and Wilcox (24), would allow better discrimination of resistant isolates because there would be fewer false positives detected. On average, powdery mildew isolates from grapes in Michigan were able to tolerate higher concentrations of trifloxystrobin than isolates collected from grapes in the Golan region in Israel (17) whose conidial germination was completely inhibited at 0.1 µg/ml of trifloxystrobin.

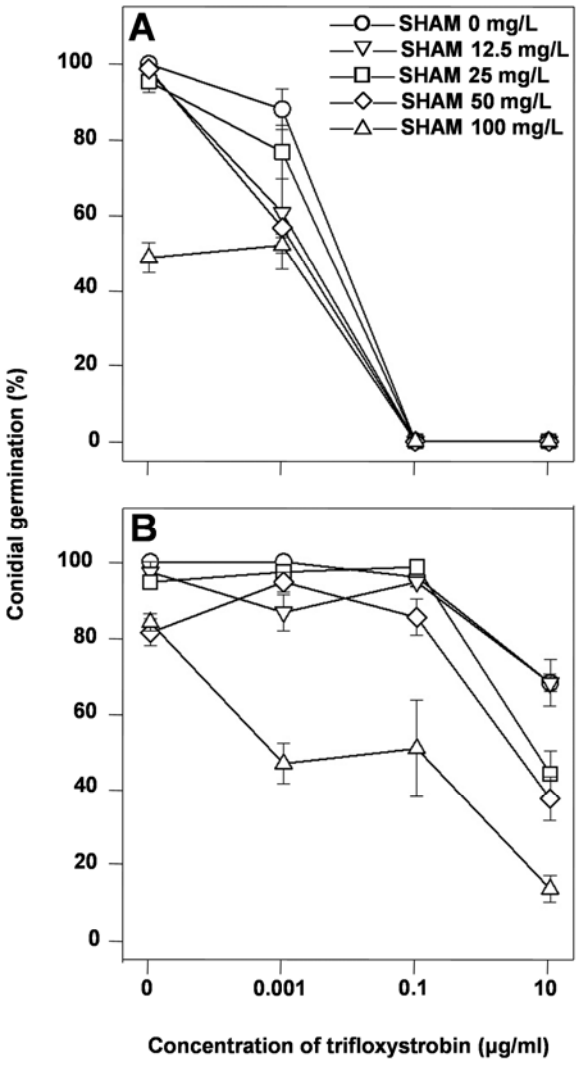

Fig. 3. Conidial germination in **A,** a trifloxystrobin-sensitive and **B,** a trifloxystrobin-resistant *Erysiphe necator* isolate collected from grapes in Michigan in 2008 as affected by different concentrations of salicylhydroxamic acid (SHAM; 0, 12, 25, 50, and 100 mg/liter) and trifloxystrobin (0, 0.001, 0.1, and 10 µg/ml). Data shown are averages of three replicates per isolate.

Their high sensitivity may be explained by a lack of exposure to QoI fungicides, which had just been introduced to the marketplace at that time. In addition, single-conidium isolates were not used in the assays, and mixtures of individuals that can occur within a single lesion may have affected their results. Most isolates from baseline sites in Michigan were also completely inhibited at a concentration of 0.1 μg/ml trifloxystrobin. The two isolates that were still able to germinate at that concentration originated from the baseline vineyards in Traverse City, which were situated less than 1 km from commercial wine grape vineyards that were managed conventionally. It is possible that airborne inoculum from those vineyards had reached the baseline sites.

Overall, our results provide evidence that QoI-resistant isolates are easily detectable in Michigan populations of *E. necator* and may be contributing to decreased efficacy of fungicide programs in some Michigan vineyards. This emphasizes the need for continued vigilance and fungicide resistance management. Although QoI resistance does not appear to be widespread in commercial vineyards, the survey exposed different scenarios in which QoI resistance is more likely to develop. For example, management programs that aim to delay the development of fungicide resistance are not normally followed in MSU research vineyards, which have been utilized for fungicide efficacy trials that included QoI fungicides for many years. Research plots would have been exposed to multiple strobilurin sprays per season over the past 10 years, which would have imposed relatively continuous selection pressure on local powdery mildew populations. Since fungicides in efficacy trials are usually applied to replicated small plots within the vineyard, the overall effect of selection on the powdery mildew population was larger than expected. Grape growers tend to be more conservative when applying strobilurin fungicides due to label restrictions and high application costs, i.e., fewer applications are made over the course of a growing season. This is especially true for juice grapes that are grown with a very small economic margin. Consequently, due to the lower application frequency, the selection pressure in commercial vineyards would be lower than in research vineyards. We observed that resistant isolates were more commonly found in wine grape than in juice grape vineyards. In general, juice grape cultivars (*V. labrusca*) are more tolerant to powdery mildew than the European *V. vinifera* cultivars (16); this intrinsic characteristic of the vines allows growers to considerably reduce the number of fungicide applications to juice grapes. In addition, due to the relatively higher cost of strobilurins compared to other commonly used fungicides, juice grape growers are less likely to use them. In many wine grape vineyards in Michigan, a total of 20 to 30 applications of strobilurins are estimated to have been made over the past 10 years since these fungicides first became available.

At this time, field-scale loss of powdery mildew control due to QoI resistance, although suspected in some locations, appears to be limited in Michigan, as growers usually rely on multiple chemical classes to control powdery mildew. The information obtained in this study will be used to develop more effective disease management programs to control powdery mildew and manage fungicide resistance in Michigan vineyards, including frequent rotation of fungicides with different modes of action. Sole reliance on strobilurins during periods of high fruit susceptibility, i.e., during the first 3 to 4 weeks after bloom, is discouraged. Newer fungicides with unique modes of action (e.g., metrafenone) (9) can be used in rotation or tank mixes with strobilurins. Older protectant fungicides, such as sulfur, oils, and bicarbonate salts, may take on a more important role (23). Further research is needed to confirm suspected resistance to sterol inhibitor fungicides in Michigan to improve recommendations for use of these materials in fungicide programs as well.

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