Prevalence and Stability of Qualitative QoI Resistance in Populations of *Venturia inaequalis* in the Northeastern United States

Zachary A. Frederick and Sara M. Villani, Department of Plant Pathology and Plant-Microbe Biology, Cornell University, Geneva, NY 14456; Daniel R. Cooley, Department Plant, Soil, and Insect Science, University of Massachusetts, Amherst 01003; Alan R. Biggs, Kearneysville Tree Fruit Research and Education Center, West Virginia University, Kearneysville 25443; and Jessica J. Raes and Kerik D. Cox, Department of Plant Pathology and Plant-Microbe Biology, Cornell University

Abstract

Frederick, Z. A., Villani, S. M., Cooley, D. R., Biggs, A. R., Raes, J. J., and Cox, K. D. 2014. Prevalence and stability of qualitative QoI resistance in populations of *Venturia inaequalis* in the northeastern United States. Plant Dis. 98:1122-1130.

Quinone-outside-inhibitor (QoI) fungicides are a safe and effective means of managing apple scab caused by *Venturia inaequalis*. To determine the prevalence of both quantitative (partial) and qualitative (complete) QoI resistance in *V. inaequalis* in the northeastern United States, we sampled single-lesion conidial isolates ($n = 4,481$) from 120 commercial and research orchards from 2004 to 2011 with a range of exposure to QoI fungicides from none to several applications a year. In all, 67% of these orchard populations of *V. inaequalis* were sensitive to QoI fungicides, 28% exhibited QoI practical resistance, and 5% were not sensitive QoI fungicides but had not become practically resistant. Isolates with qualitative QoI resistance, conferred by the G143A *cytochrome b* gene mutation, were found in 13 of the 34 QoI-resistant or-

chard populations. To evaluate the stability of the G143A mutation, 27 isolates were selected from different orchard populations to represent the scope of regional populations. These isolates were subcultured continuously in the presence or absence of the QoI fungicide trifloxystrobin. All isolates that initially possessed qualitative resistance maintained the resistant genotype (G143A) for six transfers over 6 months in both the absence and presence of trifloxystrobin. Given the observed QoI resistance in orchard populations of *V. inaequalis* and the stability of the G143A mutation in individual isolates, apple scab management paradigms must encompass strategies to limit selection of QoI resistance in the sensitive orchard populations remaining in the region.

Apple scab, caused by the ascomycete fungal pathogen *Venturia inaequalis*, is one of the most economically important diseases of apple (2,14,23). The disease is most severe in temperate climates where weather conditions during spring are conducive to infection (12,23). Infections occur when temperatures are over 1°C and leaves are wet for at least 6 h (24). Marketable fruit losses can generally range from 50 to 100% of the total fresh market harvest if proper chemical management programs are not used in commercial orchards in temperate zones during a season with typical weather conditions (2,12,14).

Despite the existence of host resistance in a few cultivars, successful management of apple scab is primarily achieved through the use of protectant and systemic fungicides (1,23). Of these fungicides, quinone-outside-inhibitors (QoIs), have proven to be safe and highly effective for apple scab management (9,26). The antifungal activity of QoIs was first noted in the 1970s (3), and they were first used in apple for apple scab management in the mid- to late 1990s (18,27).

QoI fungicides prevent fungal respiration by inhibiting electron transfer at the ubiquinol-oxidation (Qo) site of the mitochondrial cytochrome bc₁ enzyme III complex (5,9,22). One of the most common mechanisms of QoI resistance described for fungal pathogens is a single point mutation that codes for alanine instead of glycine at position 143 (G143A) in the *cytochrome b* (*cyt b*) gene (5), which imparts a vertical, qualitative, or complete resistance response whereby isolates are highly resistant to QoI fungicides (23). Previously, qualitative QoI resistance was reported in several orchard populations of *V. inaequalis* in Michigan (20), and was previously reported in a single *V. inaequalis* population in New

York (7). Other point mutations, both naturally and artificially induced, also confer qualitative resistance to QoI fungicides in other fungal pathogens (15,16,22); however, none of these has been reported in *V. inaequalis* to date. Additionally, there is an alternative respiration or cyanide insensitive pathway (9), which may influence the response of fungi to QoI fungicides (8,33). This pathway allows fungi to bypass the inhibited respiration pathway and persist through exposure to QoIs, as shown for *Magnaporthe grisea* (25), *Septoria tritici* (34), and *Botrytis cinerea* (30). This alternative respiration pathway becomes operative in *V. inaequalis* following exposure to high doses of QoI fungicides (27). Given the importance of qualitative resistance phenomena in several pathosystems, information on the stability of the G143A mutation in the absence of selective pressure is of key importance for understanding the persistence of qualitative resistance in *V. inaequalis*. Although investigations into the stability of such *cyt b* gene mutations have been carried out for a number of plant-pathogenic fungi (25,30,34), the stability of the G143A mutation in *V. inaequalis* is largely unknown. Information on the stability of this mutation within isolates of *V. inaequalis* would be the first step in understanding the persistence of qualitative resistance in an orchard population.

In addition to qualitative resistance, there appears to be a quantitative (polygenic or partial) resistance response to QoI fungicides in *V. inaequalis* whereby a collection of isolates may display a range of insensitivity phenotypes below the point at which the alternative respiration pathway becomes activated (18). Such responses would include those resulting from unknown factors imparting a minor level of fungicide insensitivity outside of the context of *cyt b* gene mutation. However, little is known about the molecular basis of quantitative resistance to QoI fungicides in *V. inaequalis* or other pathosystems. It was been hypothesized in this seminal report that this quantitative resistance precedes QoI qualitative resistance in *V. inaequalis* in commercial orchard populations (18). In such a scenario, isolates with quantitative resistance could emerge in a population prior to the onset of QoI practical resistance (18), which is defined as greatly diminished levels of

Corresponding author: K. D. Cox, E-mail: kdc33@cornell.edu

Accepted for publication 21 February 2014.

<http://dx.doi.org/10.1094/PDIS-10-13-1042-RE>
© 2014 The American Phytopathological Society

disease control (i.e., a control failure) following proper QoI fungicide application under field conditions (17).

To date, the prevalence and stability of qualitative resistance in populations of *V. inaequalis* in the northeastern United States is not well understood. Moreover, as practical resistance to QoI fungicides is reported to authors, the contribution of isolates with quantitative resistance or qualitative resistance to the development of practical resistance is warranted. Hence, the objectives of this study were to (i) determine the prevalence of quantitative and qualitative QoI resistance in orchard populations of *V. inaequalis* in the northeastern United States and (ii) assess the stability of qualitative QoI resistance in *V. inaequalis* in the absence of QoI-induced selection pressure.

Materials and Methods

In vitro sensitivity of orchard populations of *V. inaequalis* to the QoI fungicide trifloxystrobin. From 2004 to 2011, isolates of *V. inaequalis* were collected from 120 apple orchard populations located in the northeastern United States, including populations from 94 commercial, 16 research, and 10 baseline orchards in multiple states (Table 1). Baseline orchards are defined as orchard plantings never having exposure to site-specific fungicide chemistries, including the anilinoimidazoles, sterol biosynthesis or demethylation inhibitors, QoIs, benzimidazoles, dodine, and succinate dehydrogenase inhibitors. Of the 120 total orchard populations, 24 were sampled in multiple years (Table 1).

To obtain isolates for each of the 120 orchards, 50 to 100 symptomatic leaves were arbitrarily collected from trees throughout the planting. From each collection of leaves, 50 distinct individual leaf lesions arising from single-spore infections, composed of clonal conidia (18,24), were identified and processed for the evaluation of quantitative resistance (18) to the QoI fungicide trifloxystrobin. Each sporulating leaf lesion was excised with a cork borer (7 mm in diameter), placed in 1.2 ml of distilled water, and shaken for 60 s to rinse conidia from the lesion. The clonal conidia in the resulting suspension were considered a single isolate, which was termed a single-lesion conidial isolate, as described by Köller et al. (18,19).

The individual isolates were evaluated for QoI resistance phenotypes using a microscopy-aided mycelial relative growth assay for *V. inaequalis* previously published by Köller et al. (18,19). To summarize, this procedure involved applying 100- μ l aliquots of each single-lesion conidial suspension (10^4 conidia/ml) onto the surface of potato dextrose agar (PDA; Difco Laboratories) amended with a discriminatory dose of trifloxystrobin (0.02 μ g/ml analytical standard; Sigma-Aldrich; 18) and to PDA without trifloxystrobin. This discriminatory dose was chosen based on previously published evaluations of the quantitative (polygenic or partial) resistance response to QoI fungicides in *V. inaequalis* (18). Quantitative (polygenic or partial) resistance response was described (18) as insensitivity phenotypes observed when isolates were exposed to doses of QoIs below the point at which the alternative respiration pathway becomes activated. These resistance responses would encompass the contributions of both unknown minor gene factors and the *cyt b* gene mutations, if present. Streptomycin sulfate (50 μ g/ml, Sigma-Aldrich) and chloramphenicol (50 μ g/ml, Sigma-Aldrich) were added to all media to suppress bacteria and yeast fungi. Cultures were incubated for 7 days at 23 to 25°C. Mean percent relative growth (%RG) for each isolate was determined by measuring the mean growth of five colonies, each originating from a single conidium, in the presence of trifloxystrobin relative to the mean growth of five such colonies on the PDA medium without trifloxystrobin. All colonies were measured at the point of their largest expansion using a SPOT Idea digital camera with the SPOT Imaging Basic software package (Diagnostic Instruments Inc.) attached to an Olympus SZX12 stereo scope ($\times 70$ to 120; Olympus America Inc.).

Quantitative QoI sensitivity responses in the form of mean %RG values were generated for each isolate in each of the 120 orchard populations. Each orchard population was subsequently classified

as having or lacking practical resistance to trifloxystrobin by comparing the distribution of sensitivity responses (mean %RG values of isolates) for the orchard population in question with standard orchard populations with known practical resistance or sensitivity to QoI fungicides. This method for comparing and classifying the practical resistance status for populations of *V. inaequalis* on the basis of quantitative fungicide resistance response was previously described by Köller et al. (19). Practical resistance or sensitivity was determined directly by examining fungicide application practices for each standard orchard and assessing apple scab symptom development following QoI use. Orchard population 33 had disease incidence of 0.0% apple scab on fruit and leaves at harvest following four applications of Flint WG (a.i. trifloxystrobin) in 2005 and was used as the sensitive standard population (mean isolate growth rate 0.71 mm/day). Orchard population 118 had disease incidence of 40.6% apple scab on fruit at harvest following four applications of Flint WG (a.i. trifloxystrobin) and was used as the resistant standard (mean isolate growth rate 0.74 mm/day). Comparisons of isolate sensitivity distributions for each of the 120 orchard populations to the practical resistant and sensitive standards were made using the nonparametric Kolmogorov-Smirnov (K-S) two-sample test in SAS (version 9.3; SAS Institute). The distributions of sensitivity classifications for orchard populations in different apple production regions (New York, Mid-Atlantic, and Midwest) were compared in an exploratory manner using χ^2 tests in SAS (version 9.3; SAS Institute).

Specifically, the classification of orchard populations using the K-S two-sample test was conducted by comparing distributions of trifloxystrobin sensitivity (i.e., quantitative resistance) responses for the orchard populations being evaluated ("test orchards") with both the resistant and the sensitive standards, as previously described (19). First, we determined whether or not the distribution of responses for a given test orchard was significantly different from the distribution of responses for the standard population with practical resistance ($Pr > Ks_{a_{Resistant}}$). Second, we determined whether or not the distribution of responses for a given test orchard was significantly different from the distribution of responses for the standard population that was sensitive ($Pr > Ks_{a_{Sensitive}}$; Table 1). The outcomes of the K-S tests were used in conjunction to classify the population. When the two tests used in conjunction there can be three possible outcomes: (i) the test population is significantly different from the resistant population ($Pr > Ks_{a_{Resistant}} < 0.05$) but not the sensitive population ($Pr > Ks_{a_{Sensitive}} > 0.05$), (ii) the test population is significantly different from the sensitive population ($Pr > Ks_{a_{Sensitive}} < 0.05$) but not the resistance population ($Pr > Ks_{a_{Resistant}} > 0.05$), or (iii) the test population is significantly different from both population standards ($Pr > Ks_{a_{Resistant}} < 0.05$ and $Pr > Ks_{a_{Sensitive}} < 0.05$). In outcomes i and ii, classification is simple. If the distribution of test population is not significantly different from the sensitive or resistant population, it is resistant or sensitive respectively. However, outcome iii represents the possibility that the distribution of responses from the test orchard can be significantly more sensitive or more resistant than the standard or be intermediate to both standards. In the case of outcome iii, one would use a visual depiction of responses such as a histogram for the test population and two standards to determine whether or not the test orchard was more or less sensitive or fell in the between the two standards. To simplify the classification for outcome iii, the population mean %RG for the test and standard populations was used. Presented are a series of examples for the classification of orchards in outcome iii. If the test population mean %RG was greater than that of the resistant standard, then the test orchard is significantly more resistant than the resistant standard. Similarly, if the test population mean %RG was lower than that of the sensitive standard, then the test orchard is significantly more sensitive than the sensitive standards. Finally, if the test population mean %RG was intermediate to that of the sensitive and resistant standard, the orchard has classified as being reduced sensitive (Table 1).

Determination of QoI qualitative resistance in orchard populations of *V. inaequalis*. Because practical resistance can also

Table 1. Practical resistance classification, number of isolates, percent relative growth, sensitivity, number of isolates with G143A resistance, year collected, and location from which *Venturia inaequalis* populations were evaluated for trifloxystrobin resistance

Desig. ^a	Classification ^b	<i>n</i>	RG (%) ^c	Pr > Ksa _{Resistant} ^d	Pr > Ksa _{Sensitive} ^d	Number with G143A ^e	Year	State
1	Sensitive	32	0.0 ± 0.0	<0.0001	<0.0001	0	2009	New York
2	Sensitive	30	19.8 ± 4.7	0.0125	0.3876	0	2007	New Hampshire
3	Sensitive	27	14.5 ± 8.0	<0.0001	0.006	0	2007	Massachusetts
4	Sensitive	30	12.5 ± 6.4	<0.0001	0.0072	0	2007	Rhode Island
5	Sensitive	30	0.5 ± 0.5	<0.0001	<0.0001	0	2007	New York
5a	Sensitive	26	10.4 ± 3.6	<0.0001	0.0101	4	2011	New York
6	Sensitive	31	12.8 ± 4.1	0.0002	0.0566	0	2005	Massachusetts
6a	Sensitive	29	14.6 ± 4.6	0.0002	0.1618	0	2006	Massachusetts
7	Sensitive	30	23.2 ± 5.2	0.0242	0.586	0	2006	Vermont
8	Sensitive	16	7.6 ± 4.5	0.0002	0.018	0	2007	Maine
9	Sensitive	30	26.1 ± 3.5	0.0002	0.3876	0	2005	Massachusetts
10	Sensitive	30	2.8 ± 2.8	<0.0001	0.0001	0	2007	New York
11	Sensitive	30	17.9 ± 4.3	0.0015	0.586	0	2007	Maine
12	Sensitive	30	29.2 ± 4.0	0.0033	0.799	0	2005	New York
12a	Sensitive	30	18.6 ± 5.6	0.0005	0.3876	0	2006	New York
12b	Sensitive	30	18.6 ± 3.6	0.0007	0.2365	0	2007	New York
12c	Sensitive	32	6.3 ± 3.6	<0.0001	0.0009	3	2009	New York
12d	Sensitive	50	12.7 ± 4.3	<0.0001	0.0007	7	2008	New York
13	Sensitive	30	11.3 ± 2.7	<0.0001	0.0165	0	2004	New York
13a	Sensitive	30	1.67 ± 1.62	0.0004	0.586	0	2005	New York
13b	Sensitive	32	0.0 ± 0.0	<0.0001	<0.0001	0	2006	New York
13c	Sensitive	30	3.0 ± 1.8	<0.0001	0.0004	0	2007	New York
13d	Sensitive	34	0.9 ± 0.5	<0.0001	<0.0001	0	2008	New York
13e	Sensitive	22	0.1 ± 0.1	<0.0001	0.0003	0	2010	New York
13f	Sensitive	25	4.51 ± 2.7	<0.0001	0.0021	0	2011	New York
14	Sensitive	36	8.2 ± 2.2	<0.0001	0.0008	0	2008	Rhode Island
15	Sensitive	24	33.6 ± 5.2	0.0222	0.1379	0	2007	Massachusetts
16	Sensitive	30	2.2 ± 1.7	<0.0001	0.0001	0	2007	New Hampshire
17	Sensitive	19	35.5 ± 3.4	0.003	0.1207	0	2007	New York
18	Sensitive	30	27.6 ± 5.3	0.0366	0.9525	0	2005	New York
18a	Sensitive	31	4.1 ± 2.0	<0.0001	0.0023	0	2006	New York
18b	Sensitive	30	15.5 ± 3.6	0.0002	0.0354	0	2007	New York
18c	Sensitive	32	0.5 ± 0.5	<0.0001	<0.0001	0	2009	New York
19	Sensitive	21	10.6 ± 3.2	0.0002	0.0114	0	2008	Rhode Island
20	Sensitive	30	34.8 ± 4.9	0.0366	0.2365	0	2007	Rhode Island
21	Sensitive	30	20.6 ± 3.3	<0.0001	0.1344	0	2006	Massachusetts
22	Sensitive	32	4.4 ± 3.1	<0.0001	0.0003	0	2009	Maine
23	Sensitive	30	12.2 ± 3.3	0.0005	0.0354	0	2007	Rhode Island
23a	Sensitive	15	0.0 ± 0.0	<0.0001	0.0015	0	2009	Rhode Island
24	Sensitive	30	12.1 ± 5.4	<0.0001	0.0165	0	2006	New York
25	Sensitive	22	25.13 ± 6.3	0.0674	0.5995	0	2009	Michigan
26	Sensitive	47	20.7 ± 3.5	0.0012	0.4843	0	2008	West Virginia
26a	Sensitive	39	18.1 ± 6.4	<0.0001	0.0177	0	2010	West Virginia
27	Sensitive	30	33.1 ± 7.0	0.0242	0.0713	0	2006	New York
28	Sensitive	33	7.8 ± 3.6	<0.0001	0.0036	0	2009	New York
29	Sensitive	30	5.0 ± 3.7	0.0354	0.004	0	2005	New York
29a	Sensitive	30	5.1 ± 3.7	<0.0001	0.0004	0	2006	New York
30	Sensitive	30	2.3 ± 1.2	<0.0001	0.0001	0	2007	West Virginia
31	Sensitive	30	33.0 ± 5.1	0.0657	0.2365	0	2005	New York
32	Sensitive	30	5.9 ± 2.8	<0.0001	0.0029	0	2005	Massachusetts
32a	Sensitive	30	7.6 ± 2.9	<0.0001	0.0165	0	2006	Massachusetts
33	Sensitive	30	25.9 ± 4.4	0.0025	1	0	2005	New York
34	Sensitive	36	0.1 ± 0.1	<0.0001	<0.0001	0	2009	New York
34a	Sensitive	16	5.5 ± 5.1	<0.0001	0.0068	0	2011	New York
35	Sensitive	30	16.2 ± 4.2	0.0005	0.1344	0	2006	New York
36	Sensitive	22	32.7 ± 5.3	0.0263	0.7232	0	2007	New York
37	Sensitive	30	36.3 ± 4.3	0.0056	0.1494	0	2007	Massachusetts

(continued on next page)

^a Designation. Orchard populations that were reexamined additional times in later years are denoted by the presence of letters (e.g., 18, 18a, 18b, and so on). Rows highlighted in bold represent sensitive and resistance standard orchard populations.

^b Orchard classification in terms of practical resistance status was determined in the study by comparing the distribution of quantitative quinone-oxidoreductase (QoI) fungicide sensitivity responses (isolate percent mean relative growth values on analytical-grade trifloxystrobin at 0.02 µg/ml) for a given *V. inaequalis* orchard population with standard orchard populations with and without practical resistance to QoI fungicides resistant using a Kolmogorov-Smirnov two-sample test.

^c Quantitative resistance responses for orchard population. Quantitative (polygenic or partial) resistance response result from multiple unknown factors imparting a minor level of fungicide insensitivity outside of the context of *cytochrome b* (*cyt b*) gene mutation. This response is measured by the mean percent growth of *V. inaequalis* isolates on medium amended with analytical-grade trifloxystrobin at 0.02 µg/ml relative to that on nonfungicide medium (%RG). Values are means and standard errors of *n* isolates for each population, with five single-conidium colonies for each isolate.

^d Probability that the distribution of responses for a given test orchard was significantly different from the distribution of responses for the standard orchard population with (Pr > Ksa_{Resistant}) or without (Pr > Ksa_{Sensitive}) practical resistance to trifloxystrobin. Ksa is the test statistic for the Kolmogorov-Smirnov two-sample test. See the Materials and Methods section for an explanation of orchard classification using both probabilities.

^e Number of isolates in a population with qualitative resistance, which is a vertical or complete resistance response whereby isolates are highly resistant to QoI fungicides resulting from a *cyt b* gene mutation.

Table 1. (continued from preceding page)

Desig. ^a	Classification ^b	n	RG (%) ^c	Pr > Ksa ^{Resistant} ^d	Pr > Ksa ^{Sensitive} ^d	Number with G143A ^e	Year	State
38	Sensitive	19	3.8 ± 3.9	<0.0001	0.0019	0	2005	New York
39	Sensitive	30	34.0 ± 7.4	0.0543	0.0713	0	2005	West Virginia
39a	Sensitive	30	11.3 ± 4.1	<0.0001	0.0204	0	2006	West Virginia
40	Sensitive	16	18.0 ± 3.9	0.003	0.616	0	2004	New York
41	Sensitive	30	20.5 ± 3.8	0.011	0.0713	0	2006	West Virginia
42	Sensitive	30	17.0 ± 6.5	<0.0001	0.1344	0	2005	New York
43	Sensitive	30	2.7 ± 1.7	<0.0001	0.0001	0	2006	Vermont
44	Sensitive	31	8.3 ± 3.8	<0.0001	0.0023	0	2005	West Virginia
45	Sensitive	30	28.9 ± 6.9	0.0657	0.586	0	2005	New York
46	Sensitive	30	12.7 ± 3.9	0.0004	0.0354	0	2005	Ohio
47	Sensitive	30	25.4 ± 9.9	<0.0001	0.0713	0	2007	New York
48	Sensitive	30	16.4 ± 4.4	0.0037	0.1344	0	2007	Rhode Island
49	Sensitive	27	0.5 ± 0.4	<0.0001	<0.0001	0	2009	Rhode Island
50	Sensitive	30	14.0 ± 3.2	<0.0001	0.0354	0	2005	Ohio
51	Sensitive	30	0.00 ± 0.0	<0.0001	<0.0001	0	2009	New York
52	Sensitive	30	11.2 ± 4.2	<0.0001	0.0165	0	2005	New Hampshire
53	Sensitive	27	4.4 ± 4.4	<0.0001	0.0005	0	2008	Pennsylvania
54	Sensitive	34	0.1 ± 0.1	<0.0001	<0.0001	0	2009	Indiana
55	Sensitive	20	0.0 ± 0.0	<0.0001	0.0004	0	2009	Maine
56	Sensitive	26	19.6 ± 8.1	0.0072	0.1849	0	2006	Massachusetts
57	Sensitive	30	8.4 ± 3.0	<0.0001	0.0165	0	2005	West Virginia
58	Sensitive	30	6.0 ± 2.4	<0.0001	0.0029	0	2006	New York
59	Sensitive	30	5.3 ± 2.2	<0.0001	<0.0001	0	2007	New York
59a	Sensitive	34	0.6 ± 0.3	<0.0001	<0.0001	0	2008	New York
59b	Sensitive	33	0.3 ± 0.3	<0.0001	0.0003	0	2009	New York
60	Sensitive	30	27.7 ± 4.7	0.0061	0.586	0	2007	Massachusetts
60a	Sensitive	34	3.4 ± 1.6	<0.0001	0.0001	0	2008	Massachusetts
61	Sensitive	27	12.6 ± 0.3	<0.0001	0.0448	0	2008	Rhode Island
62	Sensitive	29	0.00 ± 0.0	<0.0001	<0.0001	0	2009	Maine
63	Sensitive	32	28.1 ± 4.0	0.0035	0.4994	0	2005	Ohio
64	Sensitive	30	8.5 ± 3.7	<0.0001	0.0165	0	2005	New York
64a	Sensitive	30	3.7 ± 2.3	<0.0001	0.0008	0	2006	New York
64b	Sensitive	30	4.1 ± 2.4	<0.0001	0.0011	0	2007	New York
65	Sensitive	41	1.6 ± 1.6	<0.0001	<0.0001	0	2009	Michigan
66	Sensitive	30	15.0 ± 4.4	<0.0001	0.0703	0	2007	Rhode Island
67	Sensitive	30	5.0 ± 2.2	<0.0001	0.0029	0	2006	New York
68	Sensitive	24	20.2 ± 4.6	0.0013	0.2348	0	2005	Massachusetts
69	Sensitive	20	20.7 ± 6.5	0.024	0.2904	0	2006	Massachusetts
70	Sensitive	33	8.5 ± 3.1	<0.0001	0.0096	0	2009	New York
71	Sensitive	29	35.5 ± 7.3	0.0145	0.2775	0	2007	New York
72	Sensitive	30	34.0 ± 3.7	0.0048	0.2365	0	2005	West Virginia
73	Sensitive	30	23.5 ± 4.6	0.0048	0.799	0	2005	West Virginia
74	Sensitive	29	23.0 ± 3.9	0.0006	0.8397	0	2006	Rhode Island
75	Sensitive	30	31.7 ± 4.9	0.0269	0.9525	0	2006	Massachusetts
76	Sensitive	30	31.8 ± 9.1	0.0598	0.586	0	2005	Wisconsin
77	Sensitive	33	19.9 ± 3.1	<0.0001	0.1737	0	2004	New York
78	Sensitive	21	18.6 ± 5.3	0.0164	0.7232	0	2010	Maine
79	Sensitive	26	17.86 ± 4.9	0.0052	0.3752	1	2011	Maine
80	Sensitive	25	0.316 ± 0.31	<0.0001	<0.0001	1	2011	Vermont
81	Sensitive	24	32.70 ± 3.8	0.0133	0.2656	1	2011	Maine
82	Sensitive	38	11.1 ± 4.0	<0.0001	0.0013	4	2008	Rhode Island
83	Sensitive	16	2.39 ± 2	<0.0001	0.0048	2	2011	North Carolina
31a	Resistant	17	68.7 ± 13.0	0.2329	0.003	8	2008	New York
33a	Resistant	30	76.7 ± 3.9	0.0002	<0.0001	0	2007	New York
48a	Resistant	31	28.8 ± 4.9	0.0125	0.9525	10	2008	Rhode Island
62a	Resistant	24	12.03 ± 5.8	<0.0001	0.0134	8	2011	Maine
81a	Resistant	30	46.7 ± 4.0	0.1333	0.0354	0	2007	Vermont
81b	Resistant	35	25.6 ± 6.1	0.0012	0.3934	10	2008	Vermont
84	Resistant	30	67.6 ± 4.9	0.0088	<0.0001	0	2005	New York
85	Resistant	31	75.6 ± 3.3	0.0001	<0.0001	0	2005	New York
86	Resistant	30	43.2 ± 7.1	0.8005	0.0165	0	2006	New York
87	Resistant	30	44.2 ± 6.1	0.1225	0.0129	0	2007	New York
88	Resistant	30	50.1 ± 5.2	0.1032	0.0165	0	2007	New Hampshire
89	Resistant	30	51.8 ± 8.6	0.8005	0.0029	0	2006	New York
90	Resistant	30	71.0 ± 5.1	0.0033	<0.0001	0	2007	New York
91	Resistant	31	44.1 ± 5.5	0.6083	0.0855	0	2005	Ohio
92	Resistant	30	51.6 ± 5.3	0.2145	0.0165	0	2007	Rhode Island
93	Resistant	30	54.5 ± 3.9	0.0217	0.0072	0	2007	New York
94	Resistant	30	45.0 ± 4.1	0.0543	0.0165	0	2007	Connecticut
95	Resistant	30	57.1 ± 7.8	0.1988	0.0011	0	2006	New York
96	Resistant	30	67.9 ± 5.4	0.0111	<0.0001	0	2005	New York
97	Resistant	31	41.0 ± 4.1	0.0493	0.1344	0	2005	Ohio

(continued on next page)

occur solely on the basis of isolates with qualitative or vertical responses resulting from *cyt b* gene mutations, both qualitative and quantitative resistance phenomena need to be considered. The prevalence of QoI qualitative resistance was also investigated for each orchard population of *V. inaequalis*. To determine presence of qualitative resistance in individual isolates, an in vitro germination assay and polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) analysis were used. All single-lesion conidial isolates from each of the orchard populations sampled were subjected to the in vitro germination assay by placing 200 µl of the conidial suspension (see above) on PDA (Difco Laboratories) amended with trifloxystrobin (0.2 µg/ml) to suppress quantitative resistance response, and amended with salicylhydroxamic acid (SHAM; 100 µg/ml, analytical standard; Sigma-Aldrich) to inhibit the alternative respiratory pathway, as suggested by Köller et al. (18). The combination of SHAM with a high dose QoI fungicide such as trifloxystrobin induces a synergistic mycelial growth inhibition that effectively eliminates quantitative QoI resistance responses from masking the effects of *cyt b* mutations (9,29). Plates were incubated for 14 days at 23 to 25°C and evaluated for the growth of *V. inaequalis* colonies (radial growth >1 mm in diameter), which indicates the presence of qualitative QoI resistance for that isolate (18). In addition to the evaluation of the quantitative resistance response as described above, populations were also considered to have practical resistance to QoI fungicides when more than 25% of the population members possessed qualitative resistance, as previously suggested (20).

To confirm the molecular basis of qualitative resistance for a subset (33 to 100%) of *V. inaequalis* isolates from each population, PCR-RFLP was performed as described by Fontaine et al. (10), with several modifications. Prior to DNA extraction, colonies were grown from a single germinating conidium on trifloxystrobin-amended media (0.02 µg/ml) for 5 weeks at 23 to 25°C. Isolation of *V. inaequalis* tissue for DNA extraction was accomplished by gently scraping mycelium from the surface of the medium using a scalpel. Mycelia from each isolate (100 mg) were ground using liquid nitrogen and total DNA was extracted using an Omega Bio-Tek Plant DNA extraction kit (Omega Bio-Tek) following the manufacturer's protocol. To determine the presence of the *cyt b* G143A mutation, PCR was performed to amplify a 950-bp am-

plicon from the *cyt b* gene using *V. inaequalis*-specific primers ViCytB-5F (5'-GGACCAAGTAATCACT GGTGTATGG-3') and ViCytB-6071R (5'-TTGAAAGCTAGGCTAGGGCGAACA-3') (7). PCR reactions were made in 25-µl reaction volumes and contained 1× PCR buffer, 0.4 µM each primer, 2.5 mM MgCl₂, 200 µM each dNTP, 0.625 U of GoTaq Flexi DNA polymerase (Promega Corporation), and 1 µl of extracted DNA. This PCR reaction was performed using an iCycler thermal cycler (Bio-Rad Laboratories) with the following cycling protocol: initial denaturation for 3 min at 94°C; 30 cycles consisting of 30 s at 94°C, 30 s at 53°C, and 30 s at 72°C; and a final extension step of 5 min at 72°C. PCR products were then separated on a 1.0% agarose gel in 1× TAE buffer (44.5 mM Tris-borate and 1 mM EDTA, pH 8.0) for 45 min at 100 V and imaged on a KODAK Gel Logic 200 Imaging System (Eastman Kodak Company). RFLP analysis was performed on PCR products using 5 U of Fnu4HI restriction enzyme (New England Biolabs) on 0.5 µg of DNA from the PCR product. The reaction was incubated at 37°C for 12 h. After incubating, a final inactivation step of 25 min at 65°C was performed with an iCycler thermal cycler (Bio-Rad Laboratories) to inactivate the Fnu4HI restriction, as required by the manufacturer's protocol, and DNA fragments were analyzed by electrophoresis on a 1.0% agarose gel (Invitrogen).

Stability of QoI qualitative resistance in isolates of *V. inaequalis* in the absence of QoI-induced selective pressure. A subset of 28 isolates from orchards surveyed in 2010 and 2011 with a range of quantitative resistant phenotypes was evaluated for the presence and stability of the *cyt b* G143A mutation (Table 2). To establish the presence of the *cyt b* G143A mutation, isolates were subjected to the in vitro germination assay, PCR, and RFLP analysis described above. To test for stability of the mutation within isolates, colonies were first grown on PDA amended with trifloxystrobin and SHAM (PDA-TSHAM), transferred to medium amended with trifloxystrobin (0.02 µg/ml), and allowed to grow for 5 weeks at 23 to 25°C to maintain selective pressure. Once *V. inaequalis* colonies were 5 weeks old, two agar plugs were removed from the periphery of the colony with a 5-mm-diameter cork borer and transferred to new trifloxystrobin-amended medium and nonamended PDA. At each of six successive 5-week time points, each isolate was transferred to either fresh PDA or fresh

Table 1. (continued from preceding page)

Desig. ^a	Classification ^b	n	RG (%) ^c	Pr > Ksa _{Resistant} ^d	Pr > Ksa _{Sensitive} ^d	Number with G143A ^e	Year	State
98	Resistant	30	49.7 ± 6.9	0.2145	0.0713	0	2007	Massachusetts
99	Resistant	30	78.8 ± 5.4	0.017	<0.0001	0	2005	New York
100	Resistant	30	45.2 ± 3.0	0.0147	0.0032	0	2007	Rhode Island
101	Resistant	30	48.4 ± 5.5	0.1032	0.0072	0	2004	New York
102	Resistant	30	47.4 ± 6.9	0.4249	0.0354	0	2007	New York
103	Resistant	30	70.9 ± 4.4	0.0019	<0.0001	0	2005	New York
104	Resistant	30	46.3 ± 5.3	0.0269	0.0165	4	2007	New York
105	Resistant	19	41.8 ± 9.6	0.6632	0.0546	5	2010	West Virginia
106	Resistant	20	31.64 ± 7	0.2391	0.5121	5	2011	Maine
107	Resistant	32	21.3 ± 5.6	0.0001	0.086	9	2009	Michigan
108	Resistant	20	50.95 ± 5.87	0.285	0.0107	6	2009	Michigan
109	Resistant	15	10.73 ± 6.1	0.0007	0.0815	5	2011	Maine
110	Resistant	15	94.19 ± 4.9	0.0001	<0.0001	6	2011	Vermont
111	Resistant	24	19.8 ± 27.7	<0.0001	0.0466	9	2011	Maine
112	Resistant	25	58.4 ± 12.7	0.27	0.002	13	2008	New York
113	Resistant	34	54.7 ± 7.2	0.1799	0.0033	21	2010	New York
114	Resistant	35	67.7 ± 9.4	0.0002	<0.0001	22	2008	New York
115	Resistant	18	3.04 ± 2.2	<0.0001	0.0035	13	2011	Virginia
116	Resistant	21	69.92 ± 7.2	0.0449	0.0001	18	2011	Massachusetts
117	Resistant	21	100.35 ± 3.2	<0.0001	<0.0001	20	2011	New York
118	Resistant	37	49.9 ± 2.4	1	<0.0001	11	2012	New York
18d	Reduced sensitive	25	36.3 ± 11.6	0.2404	0.5774	1	2010	New York
19a	Reduced sensitive	30	39.8 ± 3.0	0.0088	0.0165	0	2007	Rhode Island
30a	Reduced sensitive	30	33.9 ± 6.6	0.0016	0.0308	0	2006	West Virginia
35a	Reduced sensitive	30	34.3 ± 4.1	0.0088	0.2365	0	2004	New York
118	Reduced sensitive	28	39.1 ± 3.6	0.0118	0.0428	0	2006	Massachusetts
119	Reduced sensitive	30	39.2 ± 3.6	0.0366	0.0165	0	2007	New York
120	Reduced sensitive	30	35.3 ± 4.4	0.3074	0.1344	0	2005	New York

trifloxystrobin-amended (0.02 µg/ml) medium to mimic the absence of selection pressure or constant selection pressure, respectively. By the end of the experiment, each isolate had undergone six transfers over the course of 6 months. At the outset and at the time of each transfer, the presence of the *cyt b* G143A mutation was assessed for each isolate from each medium using PCR-RFLP analysis, as described above.

Eight isolates that were not resistant to trifloxystrobin were included in the study as controls. Because these isolates would not grow on trifloxystrobin-amended media, they were first grown on nonamended PDA for 5 weeks at 23 to 25°C, then transferred to both trifloxystrobin-amended and nonamended PDA. These isolates were also evaluated for the presence of the *cyt b* G143A mutation from each medium using PCR-RFLP analysis at each evaluation point.

Results

Trifloxystrobin sensitivity of *V. inaequalis* orchard populations. From 2004 to 2011, 120 individual orchard populations of *V. inaequalis* were evaluated throughout the northeastern United States (Table 1). Although 27 orchard populations were represented by fewer than 25 isolates due to loss of isolate viability during collection and processing, the majority of orchard populations were represented by 25 or more isolates (Table 1).

During the course of the testing efforts, there were 34 (28%) orchard populations of *V. inaequalis* determined to have less sensitivity (i.e., practical resistance) to trifloxystrobin on the basis of statistical comparison of their quantitative resistance phenotype distributions with the known standards with or without practical resistance (Tables 1 and 3). The mean %RG of quantitative resistant orchard populations was 41.0 to 100.4%, with commercial orchards from Pataskala, OH (41.0 ± 4.1%) and Newfield, NY (100.4 ± 3.2%) representing the end points of the range. Of the 34 orchard populations with resistance to trifloxystrobin, 32 were commercial orchards (Table 1) with a QoI use history of approximately 2 to 3 times per season, with a total of 15 or more QoI applications. The remaining two orchard populations with practical

resistance to trifloxystrobin were collected from research orchards where QoI fungicides were used over the course of the planting history (Tables 1 and 3).

Of the 120 orchard populations examined, only 6 (5%) orchard populations of *V. inaequalis* were found to have reduced sensitivity. The mean %RG for these orchard populations was 33.9 to 39.2%, with a research orchard (33.9 ± 6.6%) and a commercial orchard (39.2 ± 3.6%) representing the end points of the range. Of the orchard populations that had reduced sensitivity to trifloxystrobin, five were commercial orchards with a QoI use history of approximately 2 to 3 times per season, with a total of 10 to 15 QoI applications according to pesticide application records. The remaining orchard population classified as having reduced sensitivity was a research orchard treated with QoI fungicides over the planting history (Table 3).

During the course of the study, the majority (67%; 80 of 120) of orchard populations of *V. inaequalis* were found to be sensitive to trifloxystrobin on the basis of statistical comparison (K-S test) of quantitative resistance phenotype distributions to known standards with or without practical resistance (Tables 1 and 3). The mean %RG for sensitive orchard populations was 0.0 to 36.3%, with a baseline orchard (0.0 ± 0.0%) and a sensitive commercial orchard (36.3 ± 4.3%) representing the end points of the range. Of the 80 orchard populations that were sensitive to trifloxystrobin, 58 were from commercial apple orchards, 12 were from research orchards, and 10 were from baseline orchards. Baseline orchards had isolates with mean %RGs of 0.0 to 18.0%, with orchards from Geneva, NY (0.0 ± 0.0%) and Montezuma, NY (18.0 ± 3.9%) representing the end points of the baseline range.

Orchard populations were pooled by orchard type and region, with Connecticut, Massachusetts, Maine, New Hampshire, Rhode Island, and Vermont in New England (48 populations); Indiana, Michigan, Ohio, and Wisconsin in the Midwest (13 populations); North Carolina, Pennsylvania, Virginia, and West Virginia in the Mid-Atlantic (13 populations); and New York (46 populations). The percent resistant populations in New York (42%) was nearly double that in the Mid-Atlantic (23%) and higher than that in New

Table 2. Characteristics of *Venturia inaequalis* single-lesion conidial isolates used for the stability of the cytochrome *b* (*cyt b*) G143A mutation assay

Isolate	Isolate classification ^a	Mean %RG ^b	<i>cyt b</i> Genotype ^c	Origin
11.1.11	Resistant	132.65	A143	Montague, MA
11.25.11	Resistant	132.65	A143	Montague, MA
5.44.11	Resistant	124.23	A143	Newfield, NY
5.40.11	Resistant	108.49	A143	Newfield, NY
14.4.10	Resistant	108.49	A143	Geneva, NY
11.13.11	Resistant	100.63	A143	Montague, MA
12.19.10	Resistant	100.63	A143	Geneva, NY
6.47.10	Resistant	92.81	A143	Newfield, NY
8.33.10	Resistant	90.47	A143	Geneva, NY
6.40.10	Resistant	90.18	A143	Newfield, NY
11.11.11	Resistant	83.88	A143	Montague, MA
11.45.10	Resistant	83.88	A143	Geneva, NY
11.17.11	Resistant	72.8	A143	Montague, MA
11.20.11	Resistant	66.5	A143	Montague, MA
41.3.10	Resistant	54.26	A143	Geneva, NY
2.7.10	Resistant	51.69	A143	Shepherdstown, WV
41.11.10	Resistant	48.51	A143	Geneva, NY
27.35.10	Resistant	45.54	A143	Geneva, NY
28.24.11	Resistant	26.49	A143	Buckfield, ME
37.22.10	Sensitive	52.47	G143	Sanford, ME
11.22.11	Sensitive	46.93	G143	Montague, MA
25.6.11	Sensitive	46.93	G143	Manchester, ME
11.8.11	Sensitive	45.54	G143	Montague, MA
28.17.11	Sensitive	41.46	G143	Buckfield, ME
3.35.10	Baseline	0	G143	Geneva, NY
32.11.11	Baseline	0	G143	Geneva, NY
32.42.11	Baseline	0	G143	Geneva, NY

^a Classification of individual isolates as Resistant was determined by whether or not the isolate had more than 48% relative growth (RG) and grew on trifloxystrobin (0.2 µg/ml)- and SHAM (100 µg/ml)-amended medium.

^b Mean percent growth of *V. inaequalis* on medium amended with analytical-grade trifloxystrobin at 0.02 µg/ml relative to that on nonfungicide medium (%RG). Values are means and standard errors of five randomly selected single-conidium microcolonies for each isolate.

^c Amino acid at position 143 in the *cyt b* gene of *V. inaequalis*: A indicates resistant (A143) allele and G indicates sensitive (G143) allele.

England and the Midwest (both 31%). These regional differences were not significant, as evaluated by a χ^2 analysis ($P = 0.542$). Overall, 33% of all populations were either resistant or had reduced sensitivity to trifloxystrobin.

Percentage of *V. inaequalis* isolates with qualitative QoI resistance in orchard populations. Of the 34 orchard populations of *V. inaequalis* that were considered to have practical resistance to trifloxystrobin based on the quantitative resistance analysis, 14 also had >25% of isolates with qualitative resistance conferred by the *cyt b* G143A mutation. The remaining 20 orchard populations with practical resistance to trifloxystrobin had few or no isolates that possessed the *cyt b* G143A mutation (<18%), suggesting that other resistance mechanisms were responsible for these orchards' resistance. None of the six orchard populations that were found to have reduced sensitivity or were shifted toward trifloxystrobin resistance had any isolates with the *cyt b* G143A mutation. Of the 80 trifloxystrobin-sensitive orchard populations, 8 did have isolates with the *cyt b* G143A mutation (Table 1). Although there were isolates with qualitative resistance in these populations, they were not sufficiently abundant (2 to 14% of the isolates) to exceed the threshold of 25%.

During the course of the study, the majority of orchard populations of *V. inaequalis* were found to be sensitive to trifloxystrobin based on the quantitative resistance responses for population members. Considering the number of commercial orchard sites evaluated, there appears to be widespread sensitivity to QoI fungicides for orchard populations in New York and most of the other apple production regions in the eastern United States. At the specific locations with sensitive orchard populations, QoI fungicides are still an effective means of managing apple scab. However, such broad generalizations should be made with caution, because it is clear from the study that orchard population sensitivity is highly specific to the local operation and production practices.

Evaluation of the stability of the G143A mutation in isolates of *V. inaequalis*. Qualitative QoI resistance to trifloxystrobin imparted by the *cyt b* G143A mutation as determined by growth and germination on PDA-TSHAM was maintained through six con-

Table 3. Practical resistance classification, state, orchard type, and number of orchard populations (*n*) of *Venturia inaequalis* examined for practical resistance to the quinone-outer-membrane-inhibitor (QoI) fungicide trifloxystrobin

^a Orchard classification in terms of practical resistance status was determined in the study by comparing the distribution of quantitative QoI fungicide sensitivity responses (isolate percent mean relative growth values on analytical-grade trifloxystrobin at 0.02 µg/ml) for a given *V. inaequalis* orchard population with standard orchard populations with and without practical resistance to QoI fungicides resistant using a Kolmogorov-Smirnov two-sample test.

every year. For the 21 orchard populations that were tested in more than 1 year, 13 remained in the same QoI sensitivity classification but 4 orchard populations changed classification to resistant and 4 changed to sensitive. Fungicide application records collected for the study indicate that QoI fungicides were used in the orchards where resistance classification changed from sensitive to resistant. However, the application records also indicate QoI use in the 4 orchards where the classifications changed from resistant to sensitive and in the 18 orchards that had a consistent sensitivity classification. It is important to note that members with qualitative resistance were not absent in the four populations that were classified as sensitive when tested at a later date. Although the orchard population changes are interesting observations, it would be erroneous to make any generalizations regarding QoI use and temporal development of resistance without formal experimentation.

As with similar studies, the present study focused on many populations of *V. inaequalis* from commercial orchards (9,10,13,18, 20,29). A unique factor in the current study was the evaluation of a large number of baseline orchard populations (8%; 10 of 120) that had never been exposed to a QoI fungicide or any other site-specific chemistry. None of the isolates in the baseline orchard populations had the *cyt b* G143A mutation but did vary in quantitative resistance response. Under the current sampling protocol, we cannot rule out the possibility that the mutation could be found in populations that are unexposed to QoI fungicides. Although we do not mean to suggest that the QoI, or any other fungicide class, is inherently mutagenic, the lack of mutation in baseline populations does provide indirect evidence supporting one of the hypotheses of van den Bosch et al. (32), who suggested that stresses induced by fungicide applications may predispose population members to more rapidly develop mutations that serve as the initiation point for selection of fungicide resistance.

A few of the orchard populations of *V. inaequalis* were classified as possessing reduced sensitivity to trifloxystrobin or shifting toward quantitative practical resistance, indicating that they should respond similarly to the resistant standard population when treated with trifloxystrobin. The distributions of these orchards were significantly different from both the sensitive and resistant standards, and their %RG values typically fell in the range of both sensitive and resistant distributions. One explanation for these odd distributions is that the populations were primarily composed of isolates with quantitative resistance and most of the populations didn't have any isolates with qualitative resistance, which usually confers a high degree of resistance to the isolates in which it occurs (20,31). Instead, a population with reduced sensitivity may be composed of individuals with resistance resulting from a myriad of unknown genetic factors working additively or synergistically to confer a range of fungicide sensitivity phenotypes (18). To date, little is known about the molecular basis of quantitative resistance to QoI fungicides in *V. inaequalis* or other pathosystems. Indeed, quantitative resistance to QoI fungicides has not been reported in any other pathosystem to date. In the six orchard populations with reduced sensitivity, there were several isolates with high %RG values but none of the isolates possessed the *cyt b* G143A mutation (Table 1).

We conclude from this study that populations of *V. inaequalis* with practical resistance to QoI fungicides are present in commercial apple plantings in the northeastern United States but not in the majority of orchard populations examined. Of the 40 QoI-resistant *V. inaequalis* populations, 17 had more than 25% of the isolates with the G143A mutation. In those cases, qualitative QoI resistance was likely responsible for the observed QoI-resistant phenotypes, which is in agreement with a previous study in Michigan (20). Köller et al. (18) suggested that QoI resistance occurs in two phases in orchard populations of *V. inaequalis*, beginning with occurrence of quantitative resistance that is later succeeded by development of the *cyt b* G143A mutation that confers a high degree of qualitative resistance. We found that, in 22 of the 40 *V. inaequalis* orchard populations that were classified as resistant to QoI fungicides, none of the isolates possessed the *cyt b* G143A mutation (Table 1). These 22 orchard populations comprising

quantitatively resistant isolates may be in the first phase of this process while the 18 orchards with qualitatively resistant isolates mentioned above may be in the second phase. Given that there is only a minor temporal aspect to the study, these results do not provide evidence for the two-phase development of QoI resistance in orchard populations, as previously proposed (18). However, our results do support the idea that genetic factors other than the *cyt b* G143A mutation are involved in QoI resistance. Interestingly, the factors do not include the F129L or G137R *cyt b* mutations because these have never been observed in a 37-sample subset of isolates from the current study (*data not shown*) or reported in another study on *V. inaequalis* populations in the Michigan (20).

In this study, we found that nearly all orchard populations with reduced sensitivity to QoI fungicides had received two or more QoI fungicide applications per season (*data not shown*) for four or more years, which suggests that frequent QoI usage creates strong selective pressure for resistance. For example, orchard 117 (Table 1) had a population mean %RG of 100.4, with 20 (Table 1) of the 21 isolates having the *cyt b* G143A mutation. In this extremely resistant orchard population, QoI fungicides had been applied three times per season for eight seasons according to pesticide application records. Similar levels of QoI resistance following intensive application schedules have been reported in other systems. It has been shown that more than 10 QoI applications in a kiwifruit planting had strongly selected for highly resistant isolates of *B. cinerea* populations (4), and six QoI applications annually in strawberry resulted in sufficient selective pressure for the development of resistant *B. cinerea* isolates (28). Intensive QoI usage for 9 years in Michigan was also sufficient for the development of *V. inaequalis* isolates with the *cyt b* G143A mutation in several orchard populations (20). By comparison, there were 6 years of QoI use prior to the identification of *V. inaequalis* isolates with the *cyt b* G143A mutation in orchard populations in France (10).

In the present study, it was demonstrated that the *cyt b* G143A mutation appears to be stable in isolates for six transfers in the absence of QoI selective pressure. Because a reversion or loss of mutation was never observed for any isolates during the course of the 6-month experiment, a reliable estimate of the duration allowing for a potential loss of the G143A mutation cannot fully be determined without further experimentation. Indeed, it has been reported that more than 30 similar clonal generations were needed before a loss of the G143A mutation or reversion to wild type were observed for the wheat pathogen *Blumeria graminis* f. sp. *tritici* (11). If the G143A mutation were to become absent over time in an individual or population, it might imply that there is a fitness cost associated with the mutation, and that mitochondria without the mutation would make the isolate more fit, so that individuals would have a selective advantage over resistant individuals. The results of other studies have shown little (21) or no (15) effects on fitness for qualitative QoI resistance in *M. oryzae* and *Alternaria alternata*, respectively. Hence, it is not surprising that the isolates in the present study maintained the mutation. Further analysis of *V. inaequalis* isolate fitness in regards to *cyt b* genotype and monitoring of changes in the proportion isolates with qualitative resistance in an orchard setting where QoI applications have ceased would provide better insight into the stability of the G143A mutation.

Overall, the results of this study suggest that orchard populations of *V. inaequalis* with quantitative and qualitative resistance to QoI fungicides are present in the northeastern United States but that resistance is restricted or specific to the individual plantings. Although we found that many of the orchard populations examined in this study are not yet completely resistant, the potential exists for the development of practical resistance depending on continued QoI fungicide use, as suggested by Chapman et al. (6). Köller et al. (18) affirmed that QoI resistance management for *V. inaequalis* could be accomplished by limiting the number of QoI applications made seasonally. Such curtailment of QoI use, if practiced, could slow the selection of population members with both qualitative and quantitative resistance. However, further research is needed to understand the persistence of qualitative and even quantitative QoI

resistance in the absence of QoI use over successive field seasons. From this point onward, apple producers in the northeastern United States should use QoI fungicides with caution until fungicide resistance testing is conducted on their local *V. inaequalis* orchard populations.

Acknowledgments

This work was supported, in part, by funds appropriated to New York State Agricultural Experiment Station, Cornell University. We thank W. Köller and D. Parker for providing isolates, for training the authors on many of the techniques utilized in the microbiological assays of the study, and for providing useful advice on the analysis and interpretation of data; our collaborators who assisted in the collection of field samples and fungicide use histories for evaluated orchards, including, J. Alicandro, P. Babcock, B. Baker, B. Bascombe, M. Belco, M. A. Bell, M. Biltonen, M. Borsick Herman, J. Bover, D. Breth, E. Brown, R. Bubier, J. Carroll, A. Clark, S. Clark, L. DeVoe, J. Dinardo, R. Drown, M. Ellis, M. Fargione, H. Faubert, H. Feller, M. Forrence, M. Forrence, M. Funk, Z. Goodband, D. Green III, A. Grobe, D. Hartley, L. Herring, A. Hill, C. Hodges, J. Ivison, C. Jaswell, E. Jensen, K. Karter, G. Koehler, E. Leadbeater, J. Lyman, A. Lynd, J. Matthews, J. Mitsiti, G. Morin, D. O'Connor, B. Olson, C. Petzoldt, D. Polseno, D. Rainer, D. Rosenberger, F. Santurri, R. Seem, M. Shannon, D. Simmons, B. Smith, R. Spitko, J. Steere, A. Sullivan, A. Teach, P. TenEyck, G. Thornton, D. Tower, F. Trail, B. Truncali, A. Tuttle, R. Wander, A. Wodehouse, S. Wood, M. Wright, K. Yoder, M. Zifchock, and S. Zonneville; and K. Abbot, W. Bacon, M. Ellis, J. Freier, N. Gottschall, C. Lehman, C. K. Poon, and M. Rosato for their assistance in the collection and preparation of and evaluation of isolates.

Literature Cited

- Agnello, A. M., Landers, A. J., Rosenberger, D., Cox, K. D., Robinson, T. L., Carroll, J. E., Breth, D., Kain, D., Bellinder, R., Stiles, W., Cheng, L., Hoying, S., and Curtis, P. 2012. Pest management guidelines for commercial tree-fruit production 2012. Cornell Coop. Ext. N.Y. State Agric. Exp. Stn. Geneva.
- Agrios, G. N. 2005. Plant Pathology, 5th ed. Elsevier Academic Press, Burlington, MA.
- Anke, T., Oberwinkler, F., Steglich, W., and Schramm, G. 1977. The strobilurins—new antifungal antibiotics from the Basidiomycete *Strobilurus tenacellus* (Pers. Ex Fr.) Sing. J. Antibiot. 30:806-810.
- Bardas G. A., Veloukas T., Koutita O., and Karaoglanidis G. S. 2010. Multiple resistance of *Botrytis cinerea* from kiwifruit to SDHIs, QoIs and fungicides of other chemical groups. Pest Manage. Sci. 66:967-973.
- Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., and Parr-Dobrzanski, B. 2002. The strobilurin fungicides. Pest Manage. Sci. 58:649-662.
- Chapman, K. S., Sundin, G. W., and Beckerman, J. L. 2011. Identification of resistance to multiple fungicides in field populations of *Venturia inaequalis*. Plant Dis. 95:921-926.
- Cox, K. D., Russo, N. L., Villani, S. M., Parker, D. M., and Köller, W. 2008. QoI qualitative resistance and *CYP51A1* upstream anomalies in NY populations of the apple scab pathogen *Venturia inaequalis*. (Abstr.) Phytopathology. 98:S42.
- Day, D. A., Whelan, J., Millar, A.H. Siedow, J. N., and Wiskich, J. T. 1995. Regulation of the alternative respiration pathway in plants and fungi. Aust. J. Plant Physiol. 22:497-509.
- Färber, R. B. K., Chin, K. M., and Leadbitter, N. 2002. Sensitivity of *Venturia inaequalis* to trifloxystrobin. Pest Manage. Sci. 58:261-267.
- Fontaine, S., Remuson, F., Fraissinet-Tachet, L., Micoud, A., Marmesse, R., and Melayah, D. 2009. Monitoring of *Venturia inaequalis* harbouring the QoI resistance G143A mutation in French orchards as revealed by PCR assays. Pest Manage. Sci. 65:74-81.
- Fraaije, B. A., Butters, J. A., Coelho, J. M., Jones, D. R., and Hollomon, D. W. 2002. Following the dynamics of strobilurin resistance in *Blumeria graminis* f. sp. *tritici* using quantitative allele-specific real-time PCR measurements with the fluorescent dye. Plant Pathol. 51:45-54.
- Holb, I. J., Heijne, B., and Jeger, M. J. 2003. Summer epidemics of apple scab: the relationship between measurements and their implications for the development of predictive models and threshold levels under different disease control regimes. J. Phytopathol. 151:335-343.
- Jobin, T., and Carisse, O. 2007. Incidence of myclobutanil- and kresoxim-methyl-insensitive isolates of *Venturia inaequalis* in Quebec orchards. Plant Dis. 91:1351-1358.
- Jones, A. L., Aldwinkle, H. S. 1990. Compendium of Apple and Pear Diseases. American Phytopathological Society, St. Paul, MN.
- Karaoglanidis, G. S., Luo, Y., and Michailides, T. J. 2011. Competitive ability and fitness of *Alternaria alternata* isolates resistant to QoI fungicides. Plant Dis. 95:178-182.
- Kim, Y. S., Dixon, E. W., Vincelli, P., and Farman, M. L. 2003. Field resistance to strobilurin (QoI) fungicides in *Pyricularia grisea* caused by mutations in the mitochondrial cytochrome b gene. Phytopathology 93:891-900.
- Köller, W. 1991. Fungicide resistance in plant pathogens. Pages 679-720 in: CRC Handbook of Pest Management in Agriculture, 2nd ed. CRC Press, Boca Raton, FL.
- Köller, W., Parker, D. M., Turechek, W. W., Avila-Adame, C., and Cronshaw, K. 2004. A two-phase resistance response of *Venturia inaequalis* populations to the QoI fungicides kresoxim-methyl and trifloxystrobin. Plant Dis. 88:537-544.
- Köller, W., Wilcox, W. F., Barnard, J., Jones, A. L., and Braun, P. G. 1997. Detection and quantification of resistance of *Venturia inaequalis* populations to sterol demethylation inhibitors. Phytopathology 87:184-190.
- Lesniak, K. E., Proffer, T. J., Beckerman, J. L., and Sundin, G. W. 2011. Occurrence of QoI resistance and detection of the G143A mutation in Michigan populations of *Venturia inaequalis*. Plant Dis. 95:927-934.
- Ma, B., and Uddin, W. 2009. Fitness and competitive ability of an azoxystrobin-resistant G143A mutant of *Magnaporthe oryzae* from perennial ryegrass. Plant Dis. 93:1044-1049.
- Ma, Z., and Michailides, T. J. 2005. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. Crop Prot. 24:853-863.
- MacHardy, W. E. 1996. Apple Scab: Biology, Epidemiology, and Management. American Phytopathological Society, St. Paul, MN.
- MacHardy, W. E., and Gadoury, D. M. 1989. A revision of Mills' s criteria for predicting apple scab infection periods. Phytopathology 79:304-310.
- Mizutani, A., Miki, N., Yukioka, H., Tamura, H., and Masuko, M. 1996. A possible mechanism of control of rice blast disease by a novel alkoxyiminoacetamide fungicide, SSF126. Phytopathology 86:295-300.
- Olaya, G., and Köller, W. 1999. Baseline sensitivities of *Venturia inaequalis* populations to the strobilurin fungicide kresoxim-methyl. Plant Dis. 83:274-278.
- Olaya, G., Zheng, D., and Köller, W. 1998. Differential Responses of Germinating *Venturia inaequalis* Conidia to Kresoxim-methyl. Pestic. Sci. 54:230-236.
- Samuel, S., Papayiannis, L. C., Leroy, M., Veloukas, T., Hahn, M., and Karaoglanidis, G. S. 2011. Evaluation of the incidence of the G143A mutation and cytb intron presence in the cytochrome bc-1 gene conferring QoI resistance in *Botrytis cinerea* populations from several hosts. Pest Manage. Sci. 67:1029-1036.
- Steinfeld, U., Sierotzki, H., Parisi, S., Poirey, S., and Gisi, U. 2001. Sensitivity of mitochondrial respiration to different inhibitors in *Venturia inaequalis*. Pest Manage. Sci. 57:787-796.
- Tamura, H., Mizutani, A., Yukioka, H., Miki, N., Ohba, K., and Masuko, M. 1999. Effect of the methoxyiminoacetamide fungicide, SSF129, on respiratory activity in *Botrytis cinerea*. Pestic. Sci. 55:681-686.
- Vallières, C., Trouillard, M., Dujardin, G., and Meunier, B. 2011. Deleterious effect of the QoI inhibitor compound resistance-conferring mutation G143A in the intron-containing cytochrome b gene and mechanisms for bypassing it. Appl. Environ. Microbiol. 77:2088-2093.
- Van Den Bosch, F., Paveley, N., Shaw, M., Hobbelen, P., and Oliver, R. 2011. The dose rate debate: does the risk of fungicide resistance increase or decrease with dose? Plant Pathol. 60:597-606.
- Zheng, D., Olaya, G., and Köller, W. 2000. Characterization of laboratory mutants of *Venturia inaequalis* resistant to the strobilurin-related fungicide kresoxim-methyl. Curr. Genet. 38:148-155.
- Ziogas, B. N., Baldwin, B. C., Young, J. E. 1997. Alternative respiration: a biochemical mechanism of resistance to azoxystrobin (ICIA 5504) in *Septoria tritici*. Pestic. Sci. 50:28-34.