Sensitivity of *Venturia inaequalis* Populations to Anilinopyrimidine Fungicides and Their Contribution to Scab Management in New York

Wolfram Köller, W. F. Wilcox, and D. M. Parker, Department of Plant Pathology, Cornell University, New York State Agricultural Experiment Station, Geneva 14456

ABSTRACT

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Sensitivities of *Venturia inaequalis* isolates to the anilinopyrimidine fungicides (APs) pyrimethanil and cyprodinil were determined for nine populations by measuring the growth of colonies formed from germinating conidia derived from single scab lesions. At the discriminatory pyrimethanil dose of 0.2 $\mu\text{g ml}^{-1}$, the mean relative growth range measured for eight *V. inaequalis* populations ($n = 39$ to 74) never treated with AP fungicides varied from 18.1 to 48.2, translating into an approximately sixfold difference in mean baseline sensitivities. For the composite of all 469 isolates tested, sensitivities to pyrimethanil and to the sterol demethylation inhibitor (DMI) myclobutanil were significantly correlated. When isolates were organized into subpopulations based on their sensitivities to an individual fungicide, sensitivities to both fungicides declined in parallel through the highly and moderately sensitive spectra of subpopulations, but they diverged for isolates in subpopulations least sensitive to either fungicide. The result suggested that at least one of the multiple genes conferring DMI resistance also lowered the sensitivity to AP fungicides. The relative contribution of AP fungicides to scab management was evaluated at an experimental orchard representative of the Great Lakes region of the United States. Frequencies of DMI-resistant isolates of *V. inaequalis* had progressed to the stage of practical resistance at the site, and the sensitivity to pyrimethanil was similar to several commercial orchard populations never treated with APs. For management programs at the experimental site involving the AP fungicides cyprodinil and pyrimethanil and conducted from 1996 to 2000, the level of fruit and terminal leaf scab control was inferior to that of nonspecific protectants such as mancozeb or captan. For the control of scab on cluster leaves, the efficacy of AP fungicides equaled the performance of nonspecific protectants. This modest contribution of AP fungicides to scab management might have been caused by a lack of the extended cool temperature conditions that were conducive to AP performance in northern Europe in previous studies, and/or by the reduced sensitivity to AP fungicides in this DMI-resistant *V. inaequalis* population.

The class of anilinopyrimidine (AP) fungicides represented by pyrimethanil, mepanipyrim, and cyprodinil was introduced in the early 1990s (22). They are in current use for the management of gray mold caused by *Botrytis* spp. and tree fruit diseases such as apple scab and brown rot (15,35,41,44). In addition, cyprodinil is employed in the control of cereal diseases such as eyespot (15). APs were shown to act as weak inhibitors of spore germination, appressorium formation, and cuticle penetration, with disease development arrested after the respective hosts had been invaded (8,9,20,31). This physical mode of

action suggested a primary target site in postpenetration steps of pathogen development.

Thus far, the biochemical mode of AP action has only been investigated for *Botrytis cinerea* (teleomorph *Botryotinia fuckeliana*). Inhibitory potencies of APs under in vitro test conditions were low on growth media containing complex components such as yeast or malt extract, whereas growth on minimal media increased sensitivities of *B. cinerea* isolates more than 100-fold (17,34). On such minimal media, AP action was strongly antagonized by methionine and the methionine precursor homocysteine, suggesting a target site in methionine biosynthesis (13,36). The interference of cyprodinil with the regulation of methionine biosynthesis rather than the direct inhibition of candidate enzymes has been suggested recently (47). Alternatively, pyrimethanil and mepanipyrim were found to inhibit the secretion of extracellular enzymes (37,38).

Mepanipyrim employed at relatively high doses was also reported to affect steps of intracellular protein trafficking in human cell cultures (39,40), a finding in support of a potential target site of APs in protein secretion. Although the relative importance of these different mode-of-action models has not been elucidated, strict cross resistance among all members of the AP class (5,16) suggests a common mode of action for *B. cinerea*.

The development of practical resistance to APs has been documented for *B. cinerea*. Here, the selection of phenotypes highly resistant to APs was identified as a rapid population response (2,11,12,16,17, 32,33). A single gene governed this trait of high-level resistance (5,16), and a mutational change in the regulation of methionine biosynthesis was suggested as a potential mechanism of resistance (47).

Reports describing AP resistance of other pathogens are scarce in comparison to the information available for *B. cinerea*. In the control of cereal eyespot caused by *Pseudocercospora herpotrichoides* (teleomorph *Tapesia yallundae* and *Tapesia acuformis*) with cyprodinil, experimental field trials over several years gave no indication for the selection of isolates leading toward practical resistance to this fungicide (1). However, one isolate expressing a resistance factor >50 in in vitro tests was identified. As with *B. cinerea*, resistance was governed by a single gene (1).

APs are also in worldwide use for the management of apple scab caused by *Venturia inaequalis* (Cooke) G. Wint. (6,7,30). In sensitivity tests involving mixed conidia populations of *V. inaequalis* collected from experimental orchards, it was found that up to 43 AP treatments applied over several years had no measurable effect on population sensitivities (31), a finding interpreted as an indication for a low risk of resistance. In other surveys of population sensitivities also conducted with mixed conidia populations, sensitivity shifts in response to treatments with cyprodinil were described as significant, leading to the recommendation that the number of AP applications should be restricted to four per season (30). In tests determining the pyrimethanil sensitivities of single *V. inaequalis* isolates, the range of sensitivities

Corresponding author: W. Köller
E-mail: WK11@cornell.edu

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was broad and separated by a factor of >100, a result attributed to the growth medium employed (45).

In this study, we describe the AP sensitivity of *V. inaequalis* baseline populations in New York, a partial correlation of isolate responses to the AP pyrimethanil and the sterol demethylation inhibitor (DMI) myclobutanil, and the contributions of cyprodinil and pyrimethanil to the management of apple scab in a DMI-resistant New York experimental orchard representative of the Great Lakes region of apple production in the United States.

MATERIALS AND METHODS

Materials. Pyrimethanil, cyprodinil, and myclobutanil were obtained from their respective manufacturers as technical products used in sensitivity tests or as formulated products used in orchard trials. Potato dextrose agar (PDA) and Bacto agar were purchased from Difco Laboratories (Detroit, MI). Disposable petri dishes (100 × 15 mm) were from Fisher Scientific (Pittsburgh, PA). All other chemicals were from Sigma Chemical Company (St. Louis, MO).

Origin of *V. inaequalis* isolates. The populations described in this study originated from 12 different apple orchards:

Baseline isolates. For sensitivity tests aimed at defining an appropriate discriminatory test dose for pyrimethanil, 22 monoconidial isolates collected in 2001 (Michigan) and 2002 (Pennsylvania) and described previously (26) were used. They were selected according to their typical baseline sensitivities to fenarimol or myclobutanil and to trifloxystrobin (26).

Wild-type populations. Isolates originated from apple trees never treated with fungicides and located in the western part of New York. Population 1 originated from backyard trees in Tompkins County, which were never treated with fungicides. The backyard was separated from commercial apple orchards by a distance of approximately 10 km. Population 2 originated from the Montezuma National Wildlife Refuge, Seneca County, as described previously (48). Diseased leaves were collected from both locations in June 2003.

Abandoned. Isolates originated from an abandoned apple orchard. Commercial apple production and use of fungicides had been discontinued for over 20 years. The abandoned site was located near Highland, NY. Diseased leaves were collected in June 2003.

Vicinity. Diseased leaves were collected in June 2003 from a row of apple trees on the campus of the New York State Agricultural Experiment Station, Geneva, NY. The trees had never been treated with fungicides, but they were located in the vicinity (500 m distance) of an experimental apple orchard described previously (27).

Commercial orchards. The commercial orchards sampled were located in the Lake

Ontario region of Wayne County (NY and W) and the Hudson/Champlain Valley region of New York (B, R, CP). The orchards were chosen because the measured incidence of fruit scab in 2002 was substantially above a commercially acceptable level (>10%). All five orchards had been treated for over 10 years with DMI fungicides, but none had been treated with AP fungicides. Diseased leaves were collected from trees left unsprayed until the first leaf scab developed, according to a sampling procedure described previously (27).

Experimental. This research orchard at the New York State Agricultural Experiment Station was planted in 1974 and consists of alternating 'McIntosh' and 'Cortland' trees grafted onto MM. 106 rootstock. The orchard serves as an experimental site for fungicide efficacy trials. DMI fungicides have been used in the management of apple scab in randomized plots since 1976 (48), and AP fungicides were tested first in 1994. Diseased leaves were collected in June 2003 from nontreated 'McIntosh' trees.

Tests of fungicide sensitivities. Sensitivities of *V. inaequalis* isolates to pyrimethanil, cyprodinil, and myclobutanil were tested for mycelial colonies developing from germinating conidia after 7 days of incubation at 20°C, following a procedure described previously (26). Conidia representing baseline isolates collected in 2001 and 2002 were prepared from mycelia as described previously (42). For all conidia samples collected in 2003, distinct lesions were cut from approximately 60 diseased leaves per sampling site, leaf disks were placed into Eppendorf tubes, and conidia were suspended by shaking (26).

Suspensions of conidia (100 µl) were spread over the surfaces of either PDA or Czapek's medium (25) amended with Bacto agar (1.5%). The suspensions contained approximately 10⁴ conidia for tests conducted on fungicide-amended media and approximately 10³ conidia for non-amended controls. All agar media contained antibiotics as described previously (48). Sizes of colonies formed after incubation for 7 days at 20°C were measured after magnification as described previously (26). For each test, the sizes of five colonies were measured at the points of their largest expansion. Relative growth (RG) values were defined as mean colony expansion (treated)/mean colony expansion (nontreated) × 100.

In order to determine the effective dose for 50% growth inhibition (ED₅₀) for pyrimethanil and cyprodinil, agar media were amended with the fungicides dissolved in acetone to yield final concentrations of 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 µg ml⁻¹. For conidial suspensions derived from scab lesions sampled in 2003, the discriminatory test doses employed were 0.2 µg ml⁻¹ for pyrimethanil

as deduced from mean ED₅₀ values of baseline populations, and 0.1 µg ml⁻¹ for myclobutanil as described previously (26,29). All final acetone concentrations were adjusted to 0.01% (vol/vol).

Evaluation of fungicide performance.

In the experimental orchard, fungicide treatments were arranged in a randomized complete block design with four replications. Each experimental unit consisted of a single 'McIntosh' tree. Spray solutions were applied dilute (2,800 liters/ha) to runoff with a handgun. Unless indicated otherwise, test fungicides were applied during the primary scab season, starting soon after bud break and ending 1 to 2 weeks after the completion of bloom (52–56). Test fungicides were cyprodinil (Vangard 75WP), pyrimethanil (Scala 40SC), trifloxystrobin (Flint 50WG), mancozeb (Dithane 75DF), and captan (Captan 50 WP). Disease management programs were continued with four to five cover sprays of benomyl (Benlate 50WP; 210 g a.i./ha) mixed with captan (Captan 50W; 1,700 g a.i./ha), which were applied to all treated trees using an airblast sprayer. Apple scab incidence on leaves was evaluated by examining all leaves on 25 fruit clusters per tree in June or July, on all leaves of 10 shoots per tree in August, and on 100 fruits per tree at the same two times.

Data analysis. As described previously (29), sensitivities of *V. inaequalis* isolates comprising distinct subpopulations were compared by independent *t* test analysis. Categorical sensitivity data were compared by fitting log-linear models with numbers of isolates grouped into separate sensitivity categories. ED₅₀ values were calculated from the log-linear correlation of fungicide concentrations and RG values. Additive interactions between mixture components were analyzed by applying the formula $E_{xp} = X + Y - XY/100$, with E_{xp} as the percentage of control expected from additive effects of the two mixture components, and with X and Y as the percentages of control provided by the mixture components applied individually (43).

RESULTS

Dose responses of baseline isolates to pyrimethanil. ED₅₀ values of baseline isolates to pyrimethanil ($n = 22$) were distributed narrowly, ranging from 0.12 to 0.3 µg ml⁻¹ (mean ED₅₀ = 0.16 ± 0.4). This narrow range of sensitivities was accompanied by a steep dose response for all isolates tested, as illustrated in Figure 1 for three representative isolates. For pyrimethanil at 1 µg ml⁻¹, the inhibition of colony expansion was ≥95% for 20 of the 22 isolates tested, while strong (>80%) yet incomplete inhibition at increasingly higher doses was observed for the remaining two isolates tested, as illustrated in Figure 1 for one isolate.

In order to examine the impact of growth medium on in vitro sensitivities of

V. inaequalis isolates, as reported for *B. cinerea* isolates (17,34), we tested the sensitivities of the same 22 isolates on minimal Czapek's medium. The mean colony growth in the absence of fungicide was reduced to 47% ($P < 0.001$) of the growth supported by PDA. However, at the discriminatory pyrimethanil dose of $0.2 \mu\text{g ml}^{-1}$, the mean RG value was 21 ± 19 and not different ($P = 0.99$) from the value of 21 ± 22 determined on PDA. The result suggested that the composition of the two growth media examined had no significant effect on the inhibitory potency of pyrimethanil. PDA was, therefore, employed in the sensitivity tests described below.

Pyrimethanil and cyprodinil sensitivities of *V. inaequalis* populations. For the quantification of population sensitivities, pyrimethanil was employed at a discriminatory dose of $0.2 \mu\text{g ml}^{-1}$, reflecting a concentration slightly higher than the mean ED_{50} of the baseline isolates described above. The mean RG values determined for wild-type populations 1 and 2 were 16.8 ($n = 46$) and 20.3 ($n = 28$), respectively. These mean RG values were not significantly different ($P = 0.22$), thus the data were combined to represent a wild-type composite population never treated with fungicides (Table 1 and Fig. 2).

Fig. 1. Dose responses of three representative baseline isolates of *Venturia inaequalis* to pyrimethanil. Pyrimethanil concentrations are given in $\mu\text{g ml}^{-1}$.

The mean RG values determined for populations sampled from an abandoned orchard (Abandoned), from a row of non-managed apple trees in the vicinity of an experimental orchard (Vicinity), and from five commercial orchards located in different regions of New York ranged from 19.0 to 48.2 (Table 1). None of these populations had been treated with AP fungicides prior to sampling, and thus they reflected baseline populations. The RG value determined for an experimental orchard where AP performance had been tested since 1994 was 42.0, and thus fell into the upper range of baseline sensitivities (Table 1). With the exception of one population sampled from a commercial orchard (NY), all mean RG values were significantly greater than for the wild-type population (Table 1).

The mean RG values of two populations (R and CP) were either not different from or higher than the RG value determined for the experimental orchard with its prolonged history of AP use (Table 1). Data from these three populations were combined to provide a composite for comparison with the wild-type population. The frequency distributions of the two isolate sensitivities overlapped (Fig. 2), as is typical for quantitative responses of populations to fungicide treatments (21,23). Both the mean RG values (18.1 and 44.3) and the frequency distribution of isolate sensitivities were significantly different ($P < 0.001$).

In order to quantify sensitivity differences represented within the two composite populations, ED_{50} values for both pyrimethanil and cyprodinil were determined for two separate mixtures of conidia derived from six scab lesions each, representing RG values of 27 ± 3 and 82 ± 7 , respectively. For pyrimethanil, the ED_{50} values of these two mixtures of conidia were $0.11 \mu\text{g ml}^{-1}$ and $0.64 \mu\text{g ml}^{-1}$, respectively (Fig. 2). ED_{50} values of cyprodinil measured for the same mixtures of conidia were $0.004 \mu\text{g ml}^{-1}$ and $0.024 \mu\text{g ml}^{-1}$, respectively, and thus reflected the same sixfold difference observed for pyrimethanil.

Myclobutanil sensitivity of *V. inaequalis* populations. All commercial orchards investigated in this study had been treated with DMI fungicides for up to 15 years, and adaptive responses of respective *V. inaequalis* populations to prolonged treatments with DMIs were anticipated (29). Therefore, the sensitivity to the DMI myclobutanil was tested for each orchard population.

As expected, the wild-type population displayed the lowest mean RG value for myclobutanil (Table 1). The DMI sensitivity of the *V. inaequalis* population collected from an abandoned orchard was not different from the wild-type population, while the population from a row of apple trees in the vicinity of an experimental orchard departed significantly from the wild-type sensitivity (Table 1). The row of apple trees near the experimental orchard had never been treated with DMI fungicides. However, the experimental orchard nearby was tested as less DMI-sensitive than baseline during surveys conducted from 1992 to 1994 (27), suggesting an

Fig. 2. Distribution of isolate sensitivities to pyrimethanil for two *Venturia inaequalis* composite populations. The populations described are a wild-type population (hatched bars, $n = 74$) and a composite population characterized by significantly lower sensitivity (closed bars, $n = 159$). Sensitivities are expressed as relative growth (RG) at a discriminatory pyrimethanil dose of $0.2 \mu\text{g ml}^{-1}$. ED_{50} values ($\mu\text{g ml}^{-1}$) corresponding to RG values of 27 and 82 are provided above arrows.

Table 1. Sensitivity of *Venturia inaequalis* populations to pyrimethanil and myclobutanil

Population	n	AP history ^w	Pyrimethanil			DMI history ^w	Myclobutanil		
			Mean RG	P_{WT}^x	P_{EXP}^y		Mean RG	P_{WT}^x	P_{EXP}^y
Wild-type	74	–	18.1	–	hs ^z	–	20.1	–	hs
Abandoned	45	–	26.4	hs	hs	–	21.7	0.37	hs
Vicinity	39	–	31.6	hs	0.003	–	46.5	hs	0.003
Commercial NY	51	–	19.0	0.60	hs	+	47.9	hs	0.001
Commercial W	55	–	30.0	hs	hs	+	50.1	hs	0.003
Commercial B	46	–	31.0	hs	hs	+	63.0	hs	0.20
Commercial R	45	–	42.7	hs	0.77	+	33.3	hs	hs
Commercial CP	53	–	48.2	hs	0.03	+	59.5	hs	0.87
Experimental	61	+	42.0	hs	–	+	59.0	hs	–

^wPopulations had been (+) or had not been (–) treated with anilinoypyrimidine (AP) or demethylation inhibitor (DMI) fungicides prior to sampling diseased leaves subjected to sensitivity tests.

^x Comparison of mean relative growth (RG) values (t test) with the wild-type population.

^y Comparison of mean RG values (t test) with the population at the experimental orchard.

^z Highly significant ($P < 0.001$).

influx of inoculum into the row of trees in question.

The experimental orchard described in the present study was diagnosed as fully DMI-resistant in 1998 (W. Köller and W. F. Wilcox, unpublished data), and a level of DMI-resistance exceeding the threshold of practical resistance was described in 2003 (26). Mean myclobutanil RG values determined for two of the five commercial orchards tested (B and CP) were not different from the experimental orchard, and thus conferred the status of practical DMI resistance. Mean RG values of populations recovered from the other three commercial orchards (NY, W, R) departed significantly from wild-type sensitivity, but DMI resistance remained below the level established for our experimental orchard (Table 1).

Fig. 3. Correlation of relative growth (RG) values determined for pyrimethanil and myclobutanil. A, Mean population RG values. B, RG values for all individual isolates.

Interdependence of pyrimethanil and myclobutanil sensitivities. The significant variation of pyrimethanil sensitivity displayed by populations never treated with APs (Table 1) prompted us to analyze a potential relationship between isolate sensitivity to the AP pyrimethanil and the DMI myclobutanil. The correlation of mean pyrimethanil and myclobutanil RG values from the nine orchard populations was not significant (Fig. 3A), as reported previously for “bulk-sampled” populations of *V. inaequalis* retrieved from apple orchards in Europe (30). However, the correlation of pyrimethanil and myclobutanil sensitivities was significant when all isolate sensitivities determined in this study were included in the analysis (Fig. 3B).

In order to further analyze a potential relationship between isolate sensitivities to pyrimethanil and myclobutanil, mean RG values determined for myclobutanil were calculated for subpopulations delineated on the basis of decreasing RG values to pyrimethanil (Table 2). Because the two fungicides act independently, it was expected that myclobutanil sensitivities among these subpopulations would not be different. This assumption was not reflected in the respective data (Table 2). Myclobutanil RG values determined for the subpopulations most and least sensitive to pyrimethanil (RG ≤ 10 and RG > 70 , respectively), were 24.7 and 71.0 ($P < 0.001$) (Table 2), suggesting that isolate responses to the two fungicides were not independent. Indeed, myclobutanil and pyrimethanil sensitivities declined in parallel until pyrimethanil sensitivities reached RG values between 40 and 50 (Table 2).

This indication of partial cross sensitivity between the two independent fungicides was further supported by the converse analysis of mean pyrimethanil sensitivities among subpopulations defined by gradually declining myclobutanil sensitivities (Table 3). Again, isolate sensitivities to myclobutanil decreased in parallel to those of pyrimethanil up to RG values between 40 and 50 (Table 3), which reflect the mean sensitivities of *V. inaequalis*

populations to pyrimethanil in DMI-resistant orchards (Table 1).

Performance of AP fungicides in the management of apple scab. The partial correlation between pyrimethanil and myclobutanil sensitivities (Fig. 3, Tables 2 and 3) prompted us to analyze the relative contributions of AP fungicides to the management of scab in our fully DMI-resistant experimental orchard (Table 1). Cyprodinil had been tested on approximately 4 to 8% of the trees at this site for two seasons prior to the performance tests described below. Summaries of respective results have been communicated previously (52–56).

Relative contribution of cyprodinil in mixture with mancozeb. For the management of apple scab in the United States, only cyprodinil is presently registered, with pyrimethanil pending registration. Cyprodinil is labeled for use alone or in a tank mixture with other fungicides. Applications of cyprodinil alone require the maximum label rate of 260 g/ha and are restricted to the prebloom stage, whereas a rate of 160 to 260 g/ha is labeled for use in tank mixtures. Performance levels achieved with these recommended programs were evaluated from 1996 to 2000, with mancozeb chosen as the companion fungicide in tank-mixture treatments.

Due to heavy rain preventing fungicide applications during early spring of 1996, ascospore infections occurred prior to the initiation of the scab management program. It was, therefore, expected that control provided by postinfection fungicides, such as the APs (6,7,20), would be superior to that provided by purely protective fungicides, such as mancozeb. Indeed, a mixture of cyprodinil with mancozeb provided significant control of apple scab under these high-pressure conditions (Table 4).

However, when the two fungicides were applied singly at their respective mixture doses, the mancozeb component provided significantly more disease control on both fruits and terminal leaves than did cyprodinil, and each component provided equivalent control on cluster leaves. On

Table 2. Sensitivity to myclobutanil among subpopulations of *Venturia inaequalis* differing in their sensitivity to pyrimethanil

Pyrimethanil subpopulations ^x	Mean RG myclobutanil ^y	Comparisons of mean RG values to myclobutanil among subpopulations with declining pyrimethanil sensitivities (<i>P</i>) ^w							
		0–10	11–20	21–30	31–40	41–50	51–60	61–70	>70
0–10	24.7	–							
11–20	32.6	0.08	–						
21–30	38.7	hs ^z	0.02	–					
31–40	48.4	hs	hs	hs	–				
41–50	56.2	hs	hs	hs	0.01	–			
51–60	56.8	hs	hs	hs	0.02	0.90	–		
61–70	63.5	hs	hs	hs	hs	0.21	0.26	–	
>70	71.0	hs	hs	hs	hs	0.05	0.06	0.41	–

^w Comparisons of mean relative growth (RG) values (t test) to myclobutanil among pyrimethanil subpopulations.

^x Subpopulations of isolates within the indicated ranges of RG values at the discriminatory dose of 0.2 $\mu\text{g ml}^{-1}$.

^y Mean RG values at 0.1 $\mu\text{g ml}^{-1}$ for isolates in the pyrimethanil subpopulations.

^z Highly significant ($P < 0.001$).

both cluster leaves and fruit, the level of control provided by the mixture was extremely close to the theoretical value expected from additive effects (Table 4). Mean daily temperatures ranged from 3 to 19°C during the 35-day period over which AP fungicides were applied; mean temps were ≤6°C on 4 days and ≥14°C on 13 days. The two temperatures of 6 and 14°C reflect the approximate borders of conditions unfavorable or optimal for the development of apple scab (49).

In 1997, treatments with fungicides were initiated 1 day prior to the first ascosporic infection period. A program consisting of two sprays of cyprodinil alone followed by four in combination with mancozeb provided significantly better disease control on cluster leaves than that provided by six applications of mancozeb alone employed at the mixture rate. However, the two programs provided equivalent control on fruit and terminal leaves (Table 5). When mancozeb was omitted from the

final four sprays of the cyprodinil program, control of fruit and terminal leaf scab was significantly less than that provided by mancozeb alone, although control of cluster leaf scab was equivalent (Table 5). Between the first and last treatment with cyprodinil (51 days), mean daily temperatures ranged from 4 to 17°C, with ≤6°C recorded at 5 days and ≥14°C at 9 days.

The experimental design was repeated in 1999, yielding very similar results. A cyprodinil program, initiated with two

Table 3. Sensitivity to pyrimethanil among subpopulations of *Venturia inaequalis* differing in their sensitivities to myclobutanil

Myclobutanil subpopulations ^x	Mean RG pyrimethanil ^y	Comparisons of mean RG values to pyrimethanil among subpopulations with declining myclobutanil sensitivities (<i>P</i>) ^w									
		0–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90	>90
0–10	13.5	–									
11–20	19.8	hs ^z	–								
21–30	28.9	hs	hs	–							
31–40	29.2	hs	hs	0.89	–						
41–50	33.2	hs	hs	0.07	0.06	–					
51–60	37.1	hs	hs	hs	hs	0.16	–				
61–70	39.2	hs	hs	hs	hs	0.01	0.45	–			
71–80	41.6	hs	hs	hs	hs	0.02	0.29	0.50	–		
81–90	40.8	hs	hs	hs	hs	0.03	0.39	0.69	0.80	–	
>90	49.3	hs	hs	hs	hs	hs	0.01	0.03	0.25	0.22	–

^w Comparisons of mean relative growth (RG) values (*t* test) to pyrimethanil among myclobutanil subpopulations.

^x Subpopulations of isolates within the indicated ranges of RG values at the discriminatory dose of 0.1 µg ml⁻¹.

^y Mean RG values at 0.2 µg ml⁻¹ for isolates in the myclobutanil subpopulations.

^z Highly significant (*P* < 0.001).

Table 4. Relative control of apple scab provided by cyprodinil and mancozeb in 1996

Treatment	Timing ^y	Rate (g a.i./ha)	Fruit scab incidence (%) ^u		Leaf scab incidence (%) ^u	
			16 July ^w	19 August ^w	16 July ^x	19 August ^y
Check			85.5 a	85.3 a	28.9 a	71.4 a
Cyprodinil		140				
+ mancozeb	1–6	2,500	8.0 b (91)	9.3 b (89)	4.3 c (85)	10.5 b (85)
Mancozeb	1–6	2,500	14.3 b (83)	16.8 b (80)	10.4 b (64)	6.6 b (91)
Cyprodinil	1–6	140	45.0 c (47)	41.3 c (52)	14.2 b (51)	32.0 c (55)
Additive ^z			(91)	(90)	(82)	(96)

^u Mean values from four replicate trees per treatment. Means within a column not followed by a common letter are significantly different (*P* ≤ 0.05) according to the Waller-Duncan *k*-ratio LSD rule. Values in parentheses denote percent control relative to the untreated check.

^y Fungicides were applied on: 1 = 2 May (early tight cluster); 2 = 8 May (early pink bud); 3 = 15 May (pink); 4 = 22 May (bloom); 5 = 29 May (petal fall); 6 = 5 June (first cover). Five additional applications of benomyl (210 g/ha) plus captan (1,700 g/ha) were applied on a 2-week schedule, starting 18 June.

^w Incidence on 100 fruits per tree.

^x Incidence on all leaves of 25 fruiting clusters per tree.

^y Incidence on all leaves of 10 terminal shoots per tree.

^z Percent control expected from theoretical additive effects.

Table 5. Relative control of apple scab provided by cyprodinil and mancozeb in 1997

Treatment	Timing ^w	Rate (g a.i./ha)	Fruit scab incidence (%) ^y		Leaf scab incidence (%) ^y	
			27 June ^x	18 August ^x	27 June ^y	19 August ^z
Check			91.8 a	99.1 a	63.1 a	99.8 a
Mancozeb	1–6	2,500	13.8 b (85)	28.3 b (71)	10.9 b (83)	66.2 b (34)
Cyprodinil	1,2	260				
Cyprodinil	3–6	160				
+ mancozeb		2,500	15.3 b (83)	22.5 b (77)	6.5 c (90)	45.2 b (55)
Cyprodinil	1,2	260				
Cyprodinil	3–6	160	48.3 c (47)	71.0 c (27)	18.7 b (70)	84.2 c (16)

^y Mean values from four replicate trees per treatment. Means within a column not followed by a common letter are significantly different (*P* ≤ 0.05) according to the Waller-Duncan *k*-ratio LSD rule. Values in parentheses denote percent control relative to the untreated check.

^w Fungicides were applied on: 1 = 16 April (0.5 cm green tissue); 2 = 29 April (open cluster); 3 = 7 May (pink); 4 = 14 May (bloom); 5 = 29 May (petal fall); 6 = 5 June (first cover). Five additional applications of benomyl (210 g/ha) plus captan (1,700 g/ha) were applied on a 2-week schedule, starting 19 June.

^x Incidence on 100 fruits per tree.

^y Incidence on all leaves of 25 fruiting clusters per tree.

^z Incidence on all leaves of 10 terminal shoots per tree.

applications alone, followed by five applications in mixture with mancozeb provided significant control of scab. Omitting mancozeb from the cyprodinil program yielded levels of fruit scab control inferior to mancozeb alone, while control of scab on both cluster and terminal leaves was equivalent (Table 6). In 1999, mean temperatures during the scab management program (57 days) ranged from 3 to 26°C, with ≤6°C recorded at 8 days and ≥14°C at 24 days.

In summary, the consistent results obtained during the 3 years of comparative performance tests revealed that the relative contributions of cyprodinil to scab management when used over the entire scab season according to the U.S. label were significantly lower than for a low-rate mancozeb in the control of fruit scab. In the control of terminal leaf scab assessed at the end of respective growing seasons, the relative contribution of cyprodinil was inferior (1996 and 1997) or equivalent (1999) to mancozeb. In the control of cluster leaf scab, performances of cyprodinil and mancozeb were equivalent.

Relative contributions of cyprodinil and pyrimethanil restricted to prebloom applications. In trials conducted in 1998 and 2000, applications of AP fungicides were restricted to the prebloom period, followed

by a block of alternative fungicides. In order to assess the relative contributions of APs to the management of scab, the two early treatments with APs were, in comparative trials, either omitted or substituted.

In 1998, fungicide sprays did not begin until 7 days after the first infection period, which occurred as the fruit buds were beginning to break from dormancy. A program consisting of two cyprodinil applications followed by four of trifloxystrobin (79 g/ha) provided significant control of apple scab, but omitting the initial two cyprodinil applications did not affect any aspect of its performance (Table 7). In contrast, applying trifloxystrobin even at a lower rate (53 g/ha) in all six sprays resulted in significantly less fruit scab 3 weeks after this part of the program was completed, indicating that conditions were suitable for disease development during the period the first two fungicide applications were made (Table 7).

In 2000, three prebloom applications of pyrimethanil were followed by four treatments with the protectant captan. With the exception of cluster leaf scab control, the performance of the program involving pyrimethanil was significantly lower than for the protectant captan applied alone over the entire course (Table 8). The re-

sults suggested that the relative contribution of pyrimethanil to the block-spray program was inferior to captan used alone.

For the two scab seasons of 1998 and 2000, mean daily temperatures between first fungicide application and bloom were as follows: In 1998 (30 days), temperatures ranged from 3 to 19°C, with 9 days at ≤6°C and 5 days at ≥14°C. In 2000 (32 days), temperatures ranged from 0 to 23°C, with 12 days at ≤6°C and 6 days at ≥14°C.

DISCUSSION

In contrast to the considerable information documented for resistance of *B. cinerea* populations to AP fungicides (5,11,12,16,17,32,33), few detailed studies have characterized responses of *V. inaequalis* populations to these fungicides. In the current report, we have, for the first time, documented pronounced differences in sensitivities to the AP pyrimethanil among baseline populations of this fungus. We furthermore have shown that mean sensitivities in six out of seven baseline orchard populations were significantly lower than that in a composite wild-type population, and we have established a statistical association between sensitivities of individual isolates to pyrimethanil and to the unrelated DMI fungicide, myclobu-

Table 6. Relative control of apple scab provided by cyprodinil and mancozeb in 1999

Treatment	Timing ^w	Rate (g a.i./ha)	Fruit scab incidence (%) ^v		Leaf scab incidence (%) ^v	
			26 June ^x	17 August ^x	26 June ^y	1 August ^z
Check			37.5 a	68.5 a	42.8 a	81.4 a
Mancozeb	1–7	2,500	10.0 b (73)	12.3 b (82)	33.4 ab (22)	43.0 b (34)
Cyprodinil	1,2	260				
Cyprodinil	3–7	160				
+ mancozeb		2,500	8.3 b (78)	5.5 b (92)	13.5 b (68)	46.2 b (43)
Cyprodinil	1,2	260				
Cyprodinil	3–7	160	30.0 c (20)	30.3 c (46)	22.8 b (46)	49.7 b (39)

^v Mean values from four replicate trees per treatment. Means within a column not followed by a common letter are significantly different ($P \leq 0.05$) according to the Waller-Duncan *k*-ratio LSD rule. Values in parentheses denote percent control relative to the untreated check.

^w Fungicides were applied on: 1 = 12 April (0.5 cm green tissue); 2 = 23 April (early tight cluster); 3 = 5 May (pink); 4 = 13 May (bloom); 5 = 20 May (petal fall); 6 = 27 May (first cover); 7 = 7 June (second cover). Four additional applications of benomyl (210 g/ha) plus captan (1,700 g/ha) were applied on a 2-week schedule, starting 18 June.

^x Incidence on 100 fruits per tree.

^y Incidence on all leaves of 25 fruiting clusters per tree.

^z Incidence on all leaves of 10 terminal shoots per tree.

Table 7. Relative control of apple scab by cyprodinil and trifloxystrobin in 1998

Treatment	Timing ^w	Rate (g a.i./ha)	Fruit scab incidence (%) ^v		Leaf scab incidence (%) ^v	
			15 June ^x	11 August ^x	15 June ^y	11 August ^z
Check			86.0 a	100 a	77.0 a	87.8 a
Cyprodinil	1,2	260				
Trifloxystrobin	3–6	79	20.7 c (76)	30.5 c (70)	19.7 c (74)	20.5 c (78)
Trifloxystrobin	3–6	79	23.5 c (73)	38.8 c (61)	22.9 c (70)	24.3 c (73)
Trifloxystrobin	1–6	53	9.5 d (89)	17.0 d (83)	16.0 c (79)	28.8 c (68)

^v Mean values from four replicate trees per treatment. Means within a column not followed by a common letter are significantly different ($P \leq 0.05$) according to the Waller-Duncan *k*-ratio LSD rule. Values in parentheses denote percent control relative to the untreated check.

^w Fungicides were applied on: 1 = 6 April (0.5 cm green tissue); 2 = 14 April (early tight cluster); 3 = 24 April (pink); 4 = 5 May (late bloom); 5 = 13 May (petal fall); 6 = 26 May (first cover). Four additional applications of benomyl (210 g/ha) plus captan (1,700 g/ha) were applied on a 2-week schedule, starting 5 June, with the exception of the continuous trifloxystrobin program, where the program was continued throughout the entire season.

^x Incidence on 100 fruits per tree.

^y Incidence on all leaves of 25 fruiting clusters per tree.

^z Incidence on all leaves of 10 terminal shoots per tree.

tanil. These results have both theoretical and practical implications.

Küng et al. (30), who analyzed “bulk samples” of *V. inaequalis* isolates obtained from European orchards, found no significant correlation between sensitivities to the AP fungicide cyprodinil and the DMI difenconazole. Analogously, when we compared the mean sensitivities to pyrimethanil and myclobutanil for our nine sample populations, we also found no statistically significant correlation. However, when we compared sensitivities to the two fungicides for each of the 469 individual isolates that comprised the total of isolates tested, a highly significant correlation emerged. This finding, which suggests the presence of a mechanism responsible for lowering sensitivities to two unrelated fungicides simultaneously, is consistent with our previous report that *V. inaequalis* phenotypes originally selected for resistance to dodine were apparently predisposed to selection for resistance to DMI fungicides (28), and with the correlation of sensitivities of *Uncinula necator* isolates to the QoI azoxystrobin and the DMI myclobutanil (57).

Although highly speculative at present, a gene conferring a low level of resistance to both pyrimethanil and myclobutanil might encode an ATP-binding cassette (ABC) transporter responsible for decreased intracellular concentrations of multiple fungicides, and overexpression of such genes has been reported to confer low-level resistance to multiple antifungal inhibitors (10,50). For example, the transcription level of a particular ABC transporter gene of *B. cinerea* was strongly induced in response to treatments with different fungicides, including the AP cyprodinil and different DMIs (51). While the high-level resistance to APs described for *B. cinerea* was not affiliated with decreased AP uptake, low-level resistance described for the same pathogen was consistent with an ABC transporter-mediated restriction of fungicide uptake (5). Homologues of genes encoding ABC transporters have also been described for *V. inaequalis* (46).

The resistance factors conferred by genes encoding ABC transporters are in

general small in comparison with mechanisms governing high levels of resistance (10,50). However, they can be expected to have impact on the performance of independent classes of fungicides, if population responses follow the course of quantitative (multigenic) resistance (21,23). As documented for the DMI fungicides, which serve as an example for the development of quantitative resistance (23,24), several unrelated genes conferring a relatively small level of resistance each will collectively have a pronounced impact on the sensitivities of genotypes expressing most or all of the resistance genes in question. When exposed to the fungicide, respective genotypes will be selected over time until the status of practical resistance is reached (24,29).

If one of the several genes responsible for DMI resistance lowers simultaneously the sensitivity to other fungicides, DMI-resistant populations of *V. inaequalis* would, consequently, be also less sensitive to a new fungicide to which they had no previous exposure. In such cases, the level of scab control achieved with a new class of fungicides will likely depend on its recommended dose (23,24,27). Doses of AP fungicides effective in DMI-sensitive populations might be less effective in populations in which genes conferring multiple resistance have already been selected to a high frequency, regardless of a relatively small factor of resistance conferred.

For example, it was documented that *V. inaequalis* phenotypes least sensitive to DMIs and selected toward practical DMI resistance were approximately 8 to 10 times less sensitive than typical baseline isolates (29), demonstrating that relatively small factors of resistance can be of practical importance. The mean sensitivity to the APs pyrimethanil and cyprodinil (as determined by characteristic ED₅₀ values) was approximately sixfold lower in our experimental orchard than in the wild-type population, and was reflective of sensitivities in two of the five commercial orchards never treated with AP fungicides. Although such shifts are not of the magnitude reported from orchards in Europe exposed to

prolonged cyprodinil use (i.e., >30-fold relative to baseline populations) (30), they may nevertheless be practically relevant.

Use strategies for the management of apple scab with APs were developed primarily in northern Europe (6,7,30). In an early report of trials conducted in Belgium, pyrimethanil was rated as providing generally superior control relative to nonspecific protectants such as captan and mancozeb (6). Subsequently, it was noted (7) that postinfection activities of the APs are particularly pronounced when temperatures are low (near 6°C) during and after an apple scab infection period. However, it also was noted that these fungicides provide better control of scab on leaves than on fruit. These various observations led to the recommendation that APs be used only until the end of bloom, in mixture with a protectant fungicide to provide additional protection of the fruit (7).

In our DMI-resistant experimental orchard located in the Great Lakes region of the United States, AP fungicides provided relatively modest benefits when we tested them in various programs according to the general guidelines. The relative contributions of both cyprodinil and pyrimethanil to control of cluster leaf scab were generally equivalent to that of the mancozeb or captan component in such programs, although mancozeb was usually applied at only half of the rate recommended when using this fungicide alone. In contrast, the APs provided significantly less control of fruit scab than did the protectants, and control of scab on the terminal leaves was consistently lower in treatments where only the APs were applied early in the season versus the complementary treatment receiving only the protectant.

Because most terminal leaves were formed after the treatment programs (and ascospore discharge) had ended, and all trees were subjected to an identical spray regime thereafter, these results suggest that the APs may have provided less reduction of secondary inoculum than did the protectants. Although this was not reflected by the ratings of cluster scab incidence conducted at the start of summer, it is possible that at least some of the control of early

Table 8. Relative control of apple scab provided by pyrimethanil and captan in 2000

Fungicide	Timing ^w	Rate (g a.i./ha)	Fruit scab incidence (%) ^y		Leaf scab incidence (%) ^y	
			19 June ^x	21 August ^x	19 June ^y	21 August ^z
Check			91.3 a	99.8 a	90.1 a	100 a
Pyrimethanil	1–3	825				
Captan	3–7	3,400	19.3 b (79)	16.3 b (84)	5.3 b (94)	50.5 b (50)
Captan	1–7	3,400	7.0 c (92)	9.3 c (91)	2.5 b (97)	29.4 c (71)

^v Mean values from four replicate trees per treatment. Means within a column not followed by a common letter are significantly different ($P \leq 0.05$) according to the Waller-Duncan k -ratio LSD rule. Values in parentheses denote percent control relative to the untreated check.

^w Fungicides were applied on: 1 = 7 April (0.5 cm green tissue); 2 = 19 April (early tight cluster); 3 = 1 May (pink); 4 = 8 May (bloom); 5 = 15 May (petal fall); 6 = 25 May (first cover); 7 = 7 June (second cover). Four additional applications of benomyl (210 g/ha) plus captan (1,700 g/ha) were applied on a 2-week schedule, starting 22 June.

^x Incidence on 100 fruits per tree.

^y Incidence on all leaves of 25 fruiting clusters per tree.

^z Incidence on all leaves of 10 terminal shoots per tree.

cluster leaf infection was transitory, i.e., these infections were merely suppressed rather than halted. Similar results concerning the relative contributions of AP fungicides and mancozeb have been reported recently from a DMI-resistant site in Michigan (19), although these workers also showed the superiority of APs for early postinfection control on cluster leaves (18).

Two factors might explain the discrepancy between AP performance described here and studies conducted in Europe. AP fungicides were found most effective at long periods with temperatures around 6°C (7), reflecting the transition from unfavorable to favorable conditions for scab development (49). Such climatic conditions are not common during the relevant portion of the year in the Great Lakes region of the United States. Of the total of 205 days spanning the periods between applications of APs in the 5 years of orchard trials described here, only 38 days had median daily temperatures ≤6°C. The duration of these periods never exceeded four consecutive days, and they were counterbalanced by longer periods (57 days) of temperatures ≥14°C comprising optimal conditions for scab development (49). Consequently, the climatic conditions conducive to good control of apple scab by APs are less common in the Great Lakes region of the United States or climatically similar regions than they are in the northern part of Europe. For example, comparatively weak performance of pyrimethanil in scab control has been reported recently for experimental orchards in Italy (4).

It must be mentioned that evidence for a relationship between low temperatures and improved control of apple scab by AP fungicides is largely circumstantial. It has been suggested that low temperatures might prolong the effective period of curative activity for these compounds by slowing the development of the pathogen (7) and/or by limiting their volatility at higher temperatures and thereby increasing their period of residual protective activity (6).

As a second parameter with possible effects on AP performances, the isolate sensitivity of the *V. inaequalis* population in our experimental orchard was significantly lower than that for wild-type populations. Although the sensitivity difference was relatively small, frequencies of isolates least sensitive to APs were increased approximately 10-fold at the experimental site, as typical for quantitative population responses to fungicides (21,23). The departure from wild-type sensitivities at the experimental orchard site may have resulted from previous testing of AP fungicides, but these fungicides had been applied to just a small fraction of the orchard over only two seasons prior to the first of the consistent trials presented in this study. Therefore, and with the additional consideration that very similar AP sensitivities

were detected in two of five commercial orchards never exposed to these materials, we believe that it is far more likely that the observed shift in AP sensitivities resulted from a selection of the *V. inaequalis* population in response to prolonged applications of DMI fungicides leading to practical DMI resistance. Thus, the impact of DMI resistance at the particular test site cannot be excluded as a major parameter affecting AP performances.

Because the AP sensitivity of the *V. inaequalis* population in our experimental orchard mimicked the population sensitivities in several commercial orchards never treated with APs, we conclude that our results are likely to reflect the relative contributions of AP fungicides to apple scab management in many commercial orchards in the Great Lakes region. At the recommended use rates of AP fungicides, we expect their contributions will be inferior (fruit scab) or largely equivalent (leaf scab) to the control provided by protectants such as mancozeb and captan. Consequently, their primary value in this region, or those with similar climates, is likely to reside in postinfection applications for the control of early cluster leaf infections in rotation with other specific fungicides, where the use of the nonspecific protectants is limited by considerations inherent to integrated pest management programs, such as the suppressive effects of mancozeb on predatory mite populations (3,14) or the incompatibility of captan with mineral oil treatments.

In summary, the results described in this study integrate a detailed analysis of *V. inaequalis* baseline sensitivities to AP fungicides with their intrinsic contributions to the management of apple scab. We conclude that AP use strategies developed under the climatic conditions of northern Europe do not apply fully to scab management under the climatic conditions of the Great Lakes or similar regions, and that mechanisms conferring low-level resistance to multiple fungicides including the DMIs may affect the relative performance of APs.

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