Orchard Factors Associated with Resistance and Cross Resistance to Sterol Demethylation Inhibitor Fungicides in Populations of *Venturia inaequalis* from Pennsylvania

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ABSTRACT

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Orchard management practices, such as destroying of overwintered inoculum and limiting the number of fungicide applications, are often recommended as tactics for slowing the development of resistance to sterol demethylation-inhibitor (DMI) fungicides in populations of *Venturia inaequalis*. However, there is little quantitative evidence relating the use of such practices to levels of resistance in orchards. The aim of this study was to evaluate the sensitivity of *V. inaequalis* isolates from Pennsylvania to DMI fungicides, and to identify orchard management factors related to the incidence of resistant isolates. In total, 644 single-spore *V. inaequalis* cultures obtained from 20 apple orchards in 2008 or 2009 were tested for sensitivity to myclobutanil, fenbuconazole, or difenoconazole. Growers provided management history of the sampled plots. Widespread shifts toward resistance to the three fungicides were noted, with mean effective concentration for 50% inhibition (EC_{50}) values of 2.136, 0.786, and

0.187 $\mu\text{g/ml}$ for myclobutanil, fenbuconazole, and difenoconazole, respectively. Cross resistance to the three fungicides was documented in high correlation (Spearman's $r > 0.6$) between mean EC_{50} values for 14 orchards. Based on a 0.5- $\mu\text{g/ml}$ threshold, 66 and 26% of isolates were resistant to myclobutanil and fenbuconazole, respectively, and 22% were cross resistant to the two fungicides. A significant between-year shift toward increased resistance was noted in two of three orchards surveyed in both years. Failure to use dormant copper sprays, older trees, larger orchards, orchards with ≤ 10 cultivars, and application of > 4 DMI sprays were positively correlated ($0.0001 < P < 0.05$) with the incidence of resistant isolates. Isolates from orchards with > 4 DMI sprays were four times as likely to be resistant to fenbuconazole (odds ratio = 4.57; $P = 0.015$). Isolates from orchards without dormant copper sprays were twice as likely to be cross-shifted toward resistance to all three fungicides (odds ratio = 1.76; $P = 0.048$). Results identify management practices that can reduce the risk of *V. inaequalis* developing resistance to DMI fungicides.

Additional keywords: generalized linear mixed models.

Apple scab, caused by *Venturia inaequalis* (Cooke) G. Winter, is one of the most economically damaging diseases of apple, the most important fruit crop in the northeastern United States. Although apple scab has been recognized for > 400 years, control is difficult and scab epidemics remain a problem for many of today's growers (5,23,24). Management of apple scab consists of two main strategies: use of cultural control methods and application of chemical fungicides. Chemical fungicides are the most effective and economical tools for scab management in commercial apple production (23). However, the reliance on chemical fungicides has its challenges, including human and environmental health concerns, as well as the development of resistance to the active ingredients in populations of *V. inaequalis*, all of which can result in deregistration.

Among the chemical fungicides, the sterol demethylation-inhibitor (DMI) fungicides serve as the backbone of apple disease management programs in the northeastern United States, where the focus is primarily on scab control. DMI fungicides are characterized by a single-site mode of action, and are classified as having a medium risk for resistance development according to

their Fungicide Resistance Action Committee code. Resistance to DMI fungicides in populations of *V. inaequalis* from commercial apple orchards was first reported in 1997 in populations from New York (20). Since then, *V. inaequalis* resistance to these fungicides has been documented in most major apple-growing areas in North America (10,14,17,20,25,31) but a thorough study in Pennsylvania, the fourth-leading state in apple production in the United States, has never been undertaken.

V. inaequalis resistance to DMI fungicides is suggested to develop quantitatively over a number of years (31). DMI fungicides achieve control through a single-site mode of action by inhibiting 14- α -demethylase, which is critical to the biosynthesis of ergosterol (30). Despite this single-site activity, several mechanisms have been suggested to confer resistance in fungal pathogens; these include the overexpression of *CYP51* (31), point mutations in *CYP51* (8,9,37), and the presence of cellular efflux mechanisms (7). The possibility of multiple mechanisms conferring resistance suggests that, even under similar environmental conditions, the status of resistance to DMI fungicides is likely to differ among populations from different orchards as a result of differential impacts of management practices on the various mechanisms. Moreover, because *V. inaequalis* has a localized spore-dispersal pattern (2), we hypothesize that migration events play a limited role in the development of fungicide resistance. Thus, orchard-specific management practices may be the key factors determining the level of resistance to DMI fungicides.

Within the apple industry, orchard management practices differ tremendously among enterprises depending on many factors, in-

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cluding economics, orchard history, geographical location, practical convenience, and a multitude of other conditions. Some of these orchard-specific factors, such as the type and number of cultivars, age and size of the trees, planting pattern of the orchards (e.g., blocks of single cultivars versus alternating rows of multiple cultivars), and fungicide spray programs and methods (e.g., alternate-row middle [ARM] as opposed to complete sprays, or limiting the number of DMI fungicide applications per season), as well as the targeted market for the crop (i.e., fresh or processing market), are thought to influence the rate at which the populations of the scab fungus shift toward resistance to the DMI fungicides; however, there is a paucity of data to support this hypothesis.

Cultural practices used for apple scab management entail preventative tactics designed to either reduce primary inoculum or create an unfavorable environment for *V. inaequalis* development, spore production, and spread (6,24). Such practices may include altering tree spacing and performing regular pruning to maintain air circulation in the canopy (6,12,16,24); shredding leaves or stimulating microbial breakdown of debris (12,33); and planting a mixture of cultivars, including those with resistance (3). In addition to their role as scab control methods, these practices, along with the use of copper or urea during dormant stages of tree growth, are also routinely recommended as tactics for slowing the development of resistance to fungicides in populations of *V. inaequalis* (29,34). However, whereas the effectiveness of these practices in the integrated control of apple scab has been confirmed in many studies (12,24,33), their impact on the development of resistance to DMI fungicides in populations of *V. inaequalis* has never been investigated. Evidence relating these cultural and orchard management practices to the levels of resistance to DMI fungicides in commercial apple orchards is needed in order to support their recommendation as tools for mitigating the risk of resistance development.

The overall goal of this study was to develop recommendations to manage the risk of resistance to DMI fungicides in populations of *V. inaequalis*. We hypothesized that the levels of resistance to DMI fungicides would be variable among orchards in Pennsylvania but strongly related to orchard-specific management practices. The specific objectives were to (i) document the incidence of resistance to the DMI fungicides myclobutanil, fenbuconazole, and difenoconazole in populations of *V. inaequalis* from Pennsylvania apple orchards and investigate the incidence of cross resistance to these fungicides; and (ii) identify orchard-specific cultural and management factors related to the incidence of resistance to the DMI fungicides.

MATERIALS AND METHODS

Orchard survey and sample collection. In all, six orchards were surveyed in June to July 2008 and 14 orchards were sampled from late May to mid-July 2009. Most of the orchards surveyed were in or around Adams County, in a region that accounts for $\approx 70\%$ of the total acreage under apple in Pennsylvania. Leaves and immature fruit with scab symptoms were collected from the cultivars 'Fuji,' 'Golden Delicious,' 'Delicious,' 'Rome Beauty,' and 'York Imperial.' In 2008, samples were taken from suspected problem areas of the blocks from at least four trees whereas, in 2009, samples were obtained from five trees of each cultivar sampled in a 'W' pattern across blocks of 0.81 to 2.02 ha in size. Samples were kept in brown paper bags in a cold room at 4°C until further processing. During the isolation process, efforts were made to ensure that the isolate cohorts equally represented each of the sampling points from a block and cultivar; a master list was prepared to keep track of the origin of each isolate. *V. inaequalis* cultures of two isolates with baseline sensitivity and two isolates confirmed as resistant to DMI fungicides from Dr. Kerik Cox (Department of Plant Pathology, Cornell University) were used as positive and negative controls, respectively, with each batch of

media made. A nonexposed, baseline population was not locally available.

Grower survey on management strategies. Growers completed a written questionnaire on orchard-specific characteristics and apple scab management tactics used in the sampled blocks. Data was obtained on the overall size of the orchard, the number of cultivars grown, the age of the sampled trees, the cultivar and rootstock of the sampled trees, and the planting pattern (or arrangement) of the trees within the sampled plot (i.e., whether trees were grown in a monoculture block or in row-by-row mixtures). Growers were also surveyed on the history of DMI fungicide use in the sampled plots, whether applications were made as full sprays or ARM sprays, whether the grower used copper on dormant trees, and whether the leaf litter was managed in any way in the fall and winter months (e.g., subjected to leaf shredding with a mower or other equipment).

***V. inaequalis* isolation.** All isolations were carried out on quarter-strength potato dextrose agar (1/4 PDA) prepared by suspending 5 g of PDA and 5 g of agar in 500 ml of deionized water and autoclaving for 15 min at 120°C. Using a scalpel, a single sporulating lesion was excised from a leaf or fruit and streaked, spore-side down, onto 100-mm-diameter petri plates of 1/4 PDA medium amended with either streptomycin at 100 parts per million (ppm) or tetracycline at 50 ppm. Plates were incubated at 25°C with ≈ 12 h of light in the laboratory. Then, 24 to 48 h later, the plates were viewed under a Nikon or Olympus dissecting microscope at 100 $\times$ magnification. A germinating conidium was chosen and aseptically transferred to a fresh plate containing 1/4 PDA to obtain a single-spore culture. This procedure was repeated for 74 samples from three orchards in 2008 and 579 samples from 2009.

Screening for resistance to DMI fungicides. Isolates were screened for resistance to myclobutanil, fenbuconazole, and difenoconazole using the agar plug assay described by Smith et al. (32) and Köller et al. (20), with minor modifications as described below. Technical-grade myclobutanil, fenbuconazole, and difenoconazole were obtained from the manufacturers (Dow Agrochemicals [myclobutanil and fenbuconazole], and Syngenta [difenoconazole]). Fungicides were suspended in 70% ethanol and filter-sterilized with a 0.2- μ m polyethersulfone filter (VWR International). Media (1/4 PDA) were amended with myclobutanil at 0, 0.05, 0.125, 0.5, 1.0, or 2.5 μ g/ml or with fenbuconazole or difenoconazole at 0, 0.05, 0.125, 0.25, 0.5, or 1.25 μ g/ml. Fungicide was added to media maintained at 55°C, which was then stirred for 60 s to ensure uniform distribution before dispensing into 60-mm-diameter plates.

Cultures were transferred onto fungicide-amended plates after ≈ 2 months of growth on nonamended 1/4 PDA, when colonies were ≈ 3 cm in diameter. Plug transfer was accomplished by using a 0.32-cm-diameter cork borer to remove medium out of the center of the fungicide-amended plates, to create core holes for holding the mycelial plugs. A 0.32-cm-diameter mycelial plug was then removed from the advancing edge of an isolate culture and placed in the core hole. Three replicate plates of each fungicide concentration were prepared for each isolate tested. In total, 644, 545, and 539 isolates were individually assayed on at least three concentrations of myclobutanil, fenbuconazole, and difenoconazole, respectively. Of these, 563 isolates on myclobutanil, 395 on fenbuconazole, and 385 on difenoconazole were assayed in five or more concentrations to estimate the mean effective concentration necessary to cause 50% inhibition (EC₅₀) values. By the time the study was completed, 498 of the 644 total isolates obtained had separately been tested on all three fungicides, with the remaining isolates being assayed on one or more of the fungicides. In all assays, $>70\%$ of the isolates obtained from each orchard were evaluated.

After plating, isolates were incubated in the dark at 23°C in either an incubator or plastic containers kept in drawer cabinets in

a laboratory, with closely monitored room temperature set at 25°C. Ten days were allowed to pass before commencing weekly measurements of colony diameter. Each fungal colony was measured across the same two perpendicular diameters each week, for a total of five measurements. An average diameter was calculated from the two measurements for each replicate of each fungicide concentration after the fifth week of measurement.

Statistical analysis. *Estimates of orchard mean EC₅₀ values.* For each isolate plated on a given fungicide, the relative growth (RG) value was calculated by dividing the average diameter of the colony on the respective fungicide concentration after 45 days of growth by the average diameter of the colony of the same isolate and age grown on nonamended 1/4 PDA plate and multiplying by

100 (20). The mean EC₅₀ was then computed for each within-orchard population with the PROBIT procedure of SAS (SAS version 9.2; SAS Institute Inc.). RG values were regressed against the log of each concentration of the fungicide and the EC₅₀ value and its corresponding 95% confidence limits were determined from the regression, as described by Hsiang et al. (13). The effects of cultivar on EC₅₀ values were also evaluated as a term in the probit regression model, and orchards where cultivars had significantly different ($P < 0.05$) EC₅₀ values were noted. Mean EC₅₀ values were computed only for 14 of the 20 orchards surveyed.

Tests for shifts toward increased resistance. To test for shifts toward resistance to the DMI fungicides, EC₅₀ values and distributions of RG values on myclobutanil at 0.5 µg/ml for isolates from three orchards sampled in both 2008 and 2009 were compared using a two-sample *t* test and the Kolmogorov-Smirnov (K-S) statistic, respectively. The mean EC₅₀ values were analyzed with and without a natural logarithm transformation.

Modeling orchard management factors associated with resistance to DMI fungicides. To assess the effects of orchard management and cultural practices on sensitivity of *V. inaequalis* populations to DMI fungicides, growth responses of individual isolates on plates amended with the active ingredient at 0.5 µg/ml were evaluated for each of the three fungicides. Two response variables were analyzed for each fungicide: (i) the RG value for each isolate at the 0.5 µg/ml threshold and (ii) a binary variable in which isolates with RG values ≥75 were classified as resistant and assigned a value of 1 while those with RG values <75 were designated as sensitive and assigned a 0. For a given population, an RG value determined at a specific concentration of a DMI fungicide is a continuous variable indicating the continuum of sensitivity to the fungicide because individual isolates will grow at a rate dependent on their intrinsic sensitivity (Fig. 1). RG values and the resistance threshold used here are comparable with the methods used by Köller et al. (20). To allow for comparison with previously published EC₅₀ values (21,38), isolates tested on fenbuconazole and difenoconazole were also evaluated at the 0.25 and 0.125 µg/ml concentrations, respectively. Isolates with RG values ≥75 at these concentrations were classified as “shifted” toward resistance to these products to obtain a binary variable similar to that described for resistance.

The RG of an isolate *i* from an orchard subjected to a set of *m* management practices (i.e., factors) can be modeled as follows:

$$y_i = \mu + \alpha_1 x_{1i} + \alpha_2 x_{2i} + \dots + \alpha_m x_{mi} + e_i \quad (1)$$

where y_i is the response (RG) of isolate *i* and, μ is the overall population mean, α s are fixed but unknown parameters quantifying the effects of management factors ($x_1, x_2, \dots, x_m$) on the isolate. The e_i represents the random variation on the mean response of each isolate to the management effects and is assumed to follow an independent normal distribution with mean 0 and a variance σ^2 (i.e., $e_i \sim N[0, \sigma^2]$); therefore, the variance of the individual isolate is $\text{var}(y_i) = \sigma^2$. For *n* isolates subject to *m* management factors, there are *n* responses $y_1, y_2, \dots, y_n$, each with its own relationship with explanatory variables identical to equation 1, except for the subscripts to identify individual isolates. The x s can take the form of continuous variables or, as in our case, categorical variables representing orchard management practices. The model representing the responses of *n* isolates can be rewritten in matrix notation as follows:

$$\mathbf{y} = \mathbf{X}\boldsymbol{\alpha} + \mathbf{e} \quad (2)$$

The bold notation indicates that $\mathbf{y}$ is a vector of observed responses, $\mathbf{X}$ is a design matrix of x s, $\boldsymbol{\alpha}$ is a vector of unknown parameters to be estimated, and $\mathbf{e}$ is the vector of independent and normally distributed random errors.

Fig. 1. Distributions of relative growth (RG) values of *Venturia inaequalis* isolates from Pennsylvania apple orchards on **A**, myclobutanil; **B**, fenbuconazole; or **C**, difenoconazole at 0.5 µg/ml, based on samples collected in 2008 and 2009. Bars represent percent isolates grouped according to RG values based on sample sizes of 644, 545, and 539 for myclobutanil, fenbuconazole, and difenoconazole, respectively. Dotted vertical lines indicate the average RG value of two confirmed baseline isolates (A, $n = 31$; B, $n = 27$; and C, $n = 25$) and dot-dash vertical lines indicate the average RG value of two isolates with confirmed shifted sensitivity (A, $n = 33$; B, $n = 27$; and C, $n = 25$). Solid vertical lines indicate a threshold value (RG = 75); isolates with RG above this threshold were determined to be resistant to the respective fungicide.

Two other assumptions were made about the populations of *V. inaequalis* evaluated in this study; namely, that (i) the 20 orchards surveyed represent a random sample of all orchards within Pennsylvania and (ii) the isolates obtained during the survey represent a random sample of the entire *V. inaequalis* population within an orchard. As a consequence, it is expected that variation occurs in RG values among isolates from different orchards and that the responses of isolates from the same orchard are correlated. The effects of this between-orchard variation on the RG of a given isolate (*i*) can be modeled with a mixed-model approach (4,22) by expanding equation 1:

$$y_{ij} = \mu + \alpha_1 x_{1ij} + \alpha_2 x_{2ij} + \dots + \alpha_m x_{mij} + b_j + e_i \quad (3)$$

$$e_i \sim N(0, \sigma^2_e)$$

$$b_j \sim N(0, \sigma^2_b)$$

where b_j represents the random effects of the *j*th orchard, while μ , α_i , and e_i are as defined in equation 1. The orchards are assumed to be drawn from a random sample with a normal distribution with a mean = 0 and a variance σ^2_b . For *n* isolates, *m* management factors, and *p* random effects, a more general form of equation 3 that allows for the joint modeling of the effects of various orchard management factors can be specified as:

$$y = X\alpha + Z\beta + e \quad (4)$$

where y , $X\alpha$, and e are as defined in equation 2, β is a vector of parameters represent the random effects, and Z is a design matrix for the random variables. That is, $\alpha = (\mu, \alpha_1, \alpha_2, \dots, \alpha_m)$, $\beta = (\beta_1, \beta_2, \dots, \beta_p)$, and $e = (e_1, e_2, \dots, e_n)$. Each of the random effects in model contributes to the overall variation in the response of the *i*th isolate through a variance component parameter that quantifies the random variation due to that effect only. Thus, the variance in the response of an individual isolate based on the random-effects model with orchards as the only random effects was $\text{var}(y_i) = \sigma^2 + \sigma^2_b$.

The model in equation 4 was used to analyze data on RG values from 20 orchards (a continuous variable with a normal distribution) and to obtain the solutions for parameters describing the fixed effects representing the orchard management factors. Only "orchard" was specified as a random effect in the model because, in preliminary analysis, including a random-effects parameter defining isolates nested within an orchard did not result in significantly better models. Orchard management factors were analyzed as fixed effects and separate models were fit for data on each fungicide. Models were fitted with the restricted maximum likelihood method using the GLIMMIX procedure of SAS (version 9.2; SAS Institute Inc.). The best model was selected based on several criteria, including the Akaike information criterion (1) and the negative log likelihood, or deviance (26). For a given set of fitted models, the model resulting in the minimum value of these statistics corresponds to the best-fitting model. The models were also evaluated for goodness of fit by examining plots of Studentized residuals. The effects of various orchard management practices were evaluated based on the type III analysis of fixed effects, with the denominator degrees of freedom adjusted for bias with a Kenward and Roger's method (15). The *lsmeans* statement of GLIMMIX was used to obtain estimates and standard errors and contrasts for the fixed effects along with their 95% confidence intervals (95% CI).

The mixed models used to analyze RG data were generalized to accommodate the binary variable describing the resistance status based on a specified threshold (0.5 µg/ml for resistance or 0.125 and 0.25 µg/ml for shifted sensitivity to difenoconazole and fenbuconazole, respectively). This was done by specifying the binary distribution and a logit link function in the model statement of the GLIMMIX procedure. The resulting model was

$$\text{logit}(p_{ij}) = \mu + \alpha_1 x_{1ij} + \alpha_2 x_{2ij} + \dots + \alpha_m x_{mij} + b_j \quad (5)$$

with $b_j \sim N(0, \sigma^2_b)$, where p_{ij} is the probability that the response of isolate *i* has a value of 1 (i.e., $y_i = 1$), and the other terms are as defined in equation 3. The parameterization in matrix notation is similar to that for model in equation 4. Note that, for reasons beyond the scope of this article, no error term is specified in this model. Mathematical and statistical details relating to the generalized linear mixed models can be found in McCullough and Neider (26), Littell et al. (22), and Brown and Prescott (4). The effects of any of the terms in the model can be interpreted in terms of odds ratios *r*, computed by taking exponential of the parameter estimates (e.g., the odds ratio of the first explanatory variable is given by $r_1 = \exp(\alpha_1)$). Models were then fitted with the residual pseudo-likelihood method, and the effects of orchard management factors evaluated based on the *F* statistic from the type III tests of fixed effects, with degrees of freedom adjusted for bias with the Kenward and Roger's method. Model selection and evaluation for goodness of fit was as described above for the mixed models for variables with normal distribution. The *lsmeans* statement of GLIMMIX was used to compute point estimates of odds ratio contrasts of fixed effects and their 95% CI. For comparison, the same models but without specifying the random effects (i.e., a fixed-effects model identical to the random-effects model) were evaluated on the binary data.

Frequency distribution histograms of the RG values of isolates, evaluated at 0.5 µg/ml, were plotted in SigmaPlot software (SigmaPlot for Windows Version 10; Systat Software Inc.). To test the hypothesis that isolates were cross resistant to the three fungicides, RG rates of the isolates with each fungicide at 0.5 µg/ml were subjected to Spearman's rank correlation analysis. The hypothetical relationship depicting the resistance status among the 498 individual isolates screened on all three fungicides was illustrated with a Venn diagram (35).

RESULTS

Estimates of EC₅₀ values for myclobutanil, fenbuconazole, and difenoconazole. Of the 20 orchards surveyed over the 2 years, mean EC₅₀ values were computed for 14 orchards. Overall, EC₅₀ values were higher for myclobutanil than for fenbuconazole or difenoconazole. Orchard mean EC₅₀ values were 1.120 to 3.534 µg/ml with a mean of 2.136 ± 0.116 µg/ml (mean ± standard error) for myclobutanil, 0.240 to 1.123 µg/ml and a mean of 0.786 ± 0.048 µg/ml for fenbuconazole, and 0.041 to 0.277 µg/ml and a mean of 0.187 ± 0.009 µg/ml for difenoconazole (Table 1). Interestingly, the highest mean EC₅₀ values were observed for isolates obtained from cultivars grown for the processing market, although these cultivars were different for each fungicide. Among the orchards, isolates from the Golden Delicious from orchard BC-1 had the highest mean EC₅₀ on myclobutanil (3.534 µg/ml) (Table 1). Isolates from Rome Beauty in AC-10 had the highest mean EC₅₀ value on fenbuconazole, 1.188 µg/ml, and isolates from York Imperial in the AC-5 population had the highest difenoconazole mean EC₅₀ value at 0.277 µg/ml (Table 1). There were highly significant correlations among EC₅₀ values for the different fungicides based on Spearman's rank correlation tests. The correlation coefficient between EC₅₀ values for myclobutanil and fenbuconazole was 0.670 ($t = 6.049$; $P < 0.001$) and 0.605 for myclobutanil and difenoconazole ($t = 5.102$; $P < 0.001$). The correlation between EC₅₀ values for fenbuconazole and difenoconazole was 0.650 ($t = 5.743$; $P < 0.001$).

Resistance to myclobutanil, fenbuconazole, and difenoconazole. A frequency distribution histogram demonstrated that the distribution of RG values on myclobutanil at 0.5 µg/ml was approximately normal, with a mean of 80.3 and a median of 83.1 (Fig. 1A). In comparison, the mean RG value for replicates of the two baseline isolates with myclobutanil at 0.5 µg/ml was 21.7 ($n = 33$) (Fig. 1A). In all, ≈66% of the 644 isolates tested on myclobutanil had RG values ≥75, the threshold RG for desig-

nating an isolate as resistant (Fig. 1A). For fenbuconazole, $\approx 26\%$ of the 545 isolates tested were resistant, and the distribution of RG values on this product at 0.5 $\mu\text{g/ml}$ also approximated a normal distribution (Fig. 1B). The mean and median RG of the population tested on fenbuconazole at 0.5 $\mu\text{g/ml}$ were 57.1 and 61.5, respectively, compared with the baseline replicates' mean RG value of 6.8 ($n = 27$). The distribution of the RG values on difenoconazole at 0.5 $\mu\text{g/ml}$ was skewed to the right, typical of a lognormal distribution (28), with a mean RG of 14.5 and median RG of 12.3 ($n = 539$) (Fig. 1C). Only 2 of the 539 isolates (i.e., 0.37%) analyzed were resistant to difenoconazole at 0.5 $\mu\text{g/ml}$, with the majority of isolates having an $\text{RG} \leq 25$ (Fig. 1C).

When evaluated at the lower concentrations, 47% of isolates were shifted toward resistance on fenbuconazole at 0.25 $\mu\text{g/ml}$, while 9% were shifted toward resistance on difenoconazole at 0.125 $\mu\text{g/ml}$ (Fig. 2). The mean and median RG values on fenbuconazole at 0.25 $\mu\text{g/ml}$ were 68.4 and 72.5, respectively (Fig. 2A). The mean and median RG values on difenoconazole at

0.125 $\mu\text{g/ml}$ were 39.5 and 36.7, respectively (Fig. 2B). In comparison, average RG values of the confirmed baseline isolates on fenbuconazole at 0.25 $\mu\text{g/ml}$ and difenoconazole at 0.125 $\mu\text{g/ml}$ were 14.8 and 3.5, respectively.

Incidence of cross resistance to DMI fungicides among isolates. In total, 498 isolates were tested on each of the three DMI fungicides. Of these, $\approx 22\%$ of isolates were resistant to both myclobutanil and fenbuconazole, whereas only two isolates (0.40%) were resistant to all three active ingredients based on a 0.5 $\mu\text{g/ml}$ threshold for each fungicide (Fig. 3). Isolates with cross resistance to myclobutanil and fenbuconazole had incidences $>50\%$ in five of the populations (AC-3, AC-4, AC-7, AC-8, and BC-1) (data not shown) but the incidence of cross resistant isolates varied among orchards. For example, whereas none of the isolates from orchard FC-1 were cross resistant to myclobutanil and fenbuconazole, 65% of isolates from orchard AC-8 were cross resistant to these fungicides (data not shown). As noted for EC_{50} values, orchard mean RG values for isolates tested sepa-

TABLE 1. Mean effective concentration for 50% inhibition (EC_{50}) values and corresponding limits of the 95% confidence interval (95% CI, $\mu\text{g/ml}$) for *Venturia inaequalis* isolates from 14 Pennsylvania apple orchards tested on the sterol demethylation-inhibitor fungicides myclobutanil, fenbuconazole, and difenoconazole, based on samples collected in 2009^a

Orchard, cultivars ^b	Myclobutanil			Fenbuconazole			Difenoconazole		
	<i>n</i>	EC_{50}	95% CI	<i>n</i>	EC_{50}	95% CI	<i>n</i>	EC_{50}	95% CI
AC-4									
FJ, GD, RD, RM, YK	64	2.470	1.964–3.045	54	1.001	0.742–1.313	56	0.209	0.141–0.277
AC-1									
FJ	9	2.498	1.949–3.583	8	0.698	0.505–1.200	8	0.147	0.076–0.213
GD	10	1.687	1.371–2.150	9	0.240	0.133–0.380	9	0.073	0.011–0.120
YK	13	2.050	1.704–2.561	13	0.818	0.584–1.600	13	0.103	0.061–0.140
AC-2									
GD	7	1.938	1.501–2.681	7	0.883	0.611–1.838	7	0.155	0.084–0.224
RD	13	1.466	1.195–1.851	10	0.366	0.285–0.511	7	0.110	0.043–0.168
RM	10	2.071	1.619–2.897	6	0.452	0.329–0.809	6	0.201	0.122–0.288
AC-3									
GD, RD, RM, YK	47	2.355	1.919–2.923	35	1.103	0.829–1.456	35	0.107	0.035–0.177
BC-1									
GD	9	3.534	2.497–7.205	9	1.060	0.765–1.969	10	0.180	0.119–0.245
RD	9	1.870	1.444–2.635	6	0.473	0.318–0.712	7	0.102	0.028–0.160
RM	10	2.374	1.883–3.306	10	0.888	0.620–1.821	10	0.233	0.169–0.309
AC-5									
FJ, GD, RD, RM, YK	51	2.892	2.313–3.597	50	0.974	0.754–1.233	49	0.277	0.212–0.346
BC-2									
FJ	8	1.338	1.020–1.829	6	0.359	0.253–0.615	8	0.060	-0.013–0.112
GD	9	1.328	1.049–1.718	9	0.416	0.322–0.578	9	0.041	-0.017–0.082
RD	10	2.389	1.850–3.523	9	0.698	0.524–1.098	9	0.065	-0.012–0.119
RM	9	1.120	0.854–1.488	9	0.514	0.342–0.808	9	0.055	-0.015–0.104
AC-6									
RD (2)	13	1.734	1.306–2.306	12	0.946	0.674–1.450	12	0.163	0.096–0.229
AC-7									
FJ	7	1.853	1.424–2.604	7	0.715	0.509–1.094	7	0.120	0.041–0.185
GD	10	2.613	2.040–3.765	9	0.973	0.741–1.461	8	0.175	0.106–0.244
RD	5	1.640	1.169–2.528	5	0.927	0.621–1.816	6	0.196	0.114–0.288
RM	7	3.286	2.305–6.806	4	1.096	0.767–2.154	3	0.161	0.092–0.247
AC-8									
FJ, GD, RD	45	2.098	1.823–2.436	17	1.123	0.858–1.639	17	0.170	0.109–0.231
YC-1									
GD, RM	24	1.948	1.618–2.378	15	0.813	0.652–1.027	15	0.133	0.078–0.185
AC-9									
GD	11	1.969	1.613–2.495	5	1.032	0.720–1.989	4	0.204	0.116–0.312
GD	20	1.462	1.244–1.735	8	0.501	0.346–0.722	10	0.099	0.032–0.152
YK	11	2.026	1.586–2.812	9	0.808	0.598–1.260	8	0.175	0.114–0.237
FC-1									
GD, RD, RM, YK	28	1.382	0.965–1.845	26	0.660	0.437–0.907	25	0.222	0.149–0.299
AC-10									
GD, RD, RM, YK	40	1.916	1.560–2.344	32	0.814	0.597–1.097	31	0.142	0.079–0.205
Total	563	...	...	395	...	...	385	...	...
Mean $\pm$ SE		2.136 $\pm$ 0.116			0.786 $\pm$ 0.048			0.187 $\pm$ 0.009	

^a Mean EC_{50} values were calculated with the probit analysis based on relative growth rates of three replicate plates of each isolate on quarter-strength potato dextrose agar medium amended with a range of concentrations of each fungicide. Values in bold indicate orchards and fungicides for which cultivars have different mean EC_{50} values ($P < 0.05$); n = number of isolates from each orchard that were screened.

^b Cultivars from which diseased tissue for *V. inaequalis* isolation was collected: FJ = Fuji, GD = Golden Delicious, RD = (Red) Delicious, RM = Rome Beauty, and YK = York Imperial. SE = standard error.

rately on each of the three active ingredients at 0.5 µg/ml were also highly correlated. The Spearman's rank correlation coefficient was $r = 0.631$ ($t = 3.82$; $P = 0.001$) for the association between mean RG values of isolates on myclobutanil and fenbuconazole, and 0.672 ($t = 5.81$; $P < 0.001$) for the correlation between the mean RG values for fenbuconazole and difenoconazole.

Evidence for between-year population shift toward increased resistance. Orchards sampled in both 2008 and 2009 were compared based on the mean EC_{50} values and the median RG values of their *V. inaequalis* populations of myclobutanil at 0.5 µg/ml (Table 2). A significant difference ($P = 0.005$) in the estimates of mean EC_{50} values for isolates from the two sample years was noted only for values from one of the three orchards (AC-4) but only after subjecting the EC_{50} data to a natural log transformation before the t test. However, when the EC_{50} values were analyzed with the nonparametric K-S test, significantly higher values in 2009 were noted for populations from orchard AC-1 ($P = 0.047$) and AC-4 ($P = 0.007$) (data not shown). This outcome was confirmed with analysis based on RG values, whereby the mean and median RG values increased significantly ($P = 0.029$ for AC-1 and $P = 0.005$ for AC-4 based on the K-S test) (Table 2) in the two orchards, even though the use of DMI fungicides was discontinued in AC-1. For example, the median RG value for isolates from orchard AC-1 increased from 73.58 in 2008 to 89.22 in 2009 while the mean RG for populations AC-4 increased from 60.39 to 83.28 over the same period (Table 2).

Orchard management practices associated with *V. inaequalis* resistance to DMI fungicides. Not using copper on dormant trees or debris, larger orchards, and >4 DMI applications in 2009

were correlated with a high incidence of *V. inaequalis* isolates classified as resistant to myclobutanil at 0.5 µg/ml (Table 3). This association was significant regardless of whether data were analyzed with a random- or fixed-effects model; however, wider CIs were obtained for estimates of the random-effects model (Table 3). Isolates from orchards not using copper were almost twice as likely to be resistant to myclobutanil (odds ratio = 1.90; 95% CI = 1.03 to 3.48) while those from orchards with >4 DMI applications were more than three times as likely to be resistant (odds ratio = 3.27; 95% CI = 1.26 to 8.47) (Table 3). In a random-effects model analysis of continuous RG data, only use of dormant copper and number of DMI applications but not orchard size were significantly related to RG values. Isolates from orchards using dormant copper had a mean RG value of 75.3, which was significantly ($P = 0.019$) smaller than 84.7 for isolates from orchards that did not use that practice (Fig. 4A). Mean RG values were 73.5 and 86.4 for orchards with ≤4 and >4 DMI applications, respectively ($P = 0.046$) (Fig. 4A).

Orchard size, the use of dormant copper, age of trees, number of cultivars grown in an orchard, and the number of DMI fungicide sprays in 2009 were the factors significantly associated with the incidence of isolates shifted toward resistance to fenbuconazole evaluated at 0.25 µg/ml (Table 3). As with data on myclobutanil, there were only minor differences between the random- and fixed-effects models in the estimates of the odds ratios but estimates from the random-effects model had much wider CIs (Table 3). The results of this categorical data analysis were confirmed when RG values of the isolates evaluated on fenbuconazole at 0.5 µg/ml were analyzed in a random-effects model. Except for age of trees within an orchard, use of copper sprays on dormant trees, limiting the number of DMI applications to ≤4, planting ≤10 cultivars, and younger trees were significantly related to reduced *V. inaequalis* RG rates on media amended with fenbuconazole at 0.5 µg/ml ($0.008 \leq P \leq 0.039$) (Fig. 4B and C).

There were too few isolates deemed resistant to difenoconazole to permit an assessment of the effects of orchard management factors with the categorical data analysis techniques. However, a random-effects model showed that isolates from larger orchards ($P = 0.0005$), and those with >4 DMI applications in 2009 ($P = 0.0179$), had significantly larger RG values than isolates from smaller orchards and those with fewer DMI applications, respectively. For example, the mean RG value for isolates from orchards >25 acres grown on 1/4 PDA plates amended with difenoconazole

Fig. 2. Distributions of relative growth (RG) values of *Venturia inaequalis* isolates from Pennsylvania apple orchards on **A**, fenbuconazole at 0.25 µg/ml and **B**, difenoconazole at 0.125 µg/ml based on samples collected in 2008 and 2009. Bars represent percent isolates in each RG category based on a sample of 542 isolates for fenbuconazole and 539 isolates for difenoconazole. Dot-dash vertical lines indicate the average RG value of two isolates with confirmed shifted sensitivity (A, $n = 27$ and B, $n = 25$). Solid vertical lines indicate a threshold value (RG = 75); isolates with RG value above this threshold determined to be shifted toward resistance to the fungicide.

Fig. 3. Incidence of cross resistance among *Venturia inaequalis* isolates from Pennsylvania apple orchards tested on myclobutanil, fenbuconazole, and difenoconazole at 0.5 µg/ml based on samples collected in 2008 and 2009. Values are number of isolates in each category ($n = 498$).

at 0.125 µg/ml was 47.9 compared with 27.4 for isolates from orchards that were ≤25 acres in size (data not shown).

Management practices associated with cross-shifted isolates. Cross-shifted isolates had RG values ≥75 when screened on myclobutanil at 0.5 µg/ml, fenbuconazole at 0.25 µg/ml, and difenoconazole at 0.125 µg/ml. Dormant copper applications, younger trees, and smaller orchards were all associated with a reduced incidence of cross-shifted isolates (Table 4). For example, the incidence of cross-shifted isolates in orchards with ≤25 acres was 17.7% compared with 33.3% for orchards >25 acres in size ($\chi^2 = 10.67$, $P = 0.001$) (Fig. 4), whereas the odds of an isolate from an orchard not using dormant copper being cross-shifted were almost twice that for an isolate from an orchard practicing dormant copper application (odds ratio = 1.76; 95% CI = 1.01 to 3.09; $P = 0.048$) (Table 4). Moreover, a random-effects model confirmed that the cross-shifted isolates had significantly higher mean RG rates on all three fungicides and were also associated with higher orchard mean EC₅₀ values (data not shown).

DISCUSSION

Estimates of the mean concentration of an active ingredient that is effective at inhibiting 50% of the growth of the sampled population (EC₅₀ values) provide a universally comparable statistic for

differentiating levels of sensitivity to toxins among diverse populations. In the literature, EC₅₀ values for *V. inaequalis* populations from orchards with a history of DMI fungicide use range from 0.09 µg/ml for fenbuconazole to 5.213 µg/ml for myclobutanil (11,21). At the time of sample collection in this study, mean EC₅₀ values for *V. inaequalis* populations in Pennsylvania apple orchards were within or above these estimates, at 1.120 to 3.534 µg/ml for myclobutanil, 0.240 to 1.123 µg/ml for fenbuconazole, and 0.041 to 0.277 µg/ml for difenoconazole, which confirms widespread resistance or shifts toward resistance to these fungicides (Table 1). One study from the United Kingdom suggests that satisfactory scab control may only become a problem at orchard EC₅₀ values >2.0 µg/ml for myclobutanil (11). Of the 14 95% CIs of the EC₅₀ values from Pennsylvania orchards sampled in this study, 12 exceed that level (Table 1) and confirm grower observations of scab control failure for myclobutanil, which prompted this study.

RG values determined at discriminatory doses are also a straightforward way to determine sensitivity to fungicides (20), and this study used a concentration of myclobutanil at 0.5 µg/ml as the threshold for resistance. This threshold, which is more conservative than that of Köller et al. (20), was selected to allow comparison of our results with more recent studies (10,25), and because lower concentrations of myclobutanil did not inhibit

TABLE 2. Mean effective concentration for 50% inhibition (EC₅₀) values and median relative growth (RG) values for *Venturia inaequalis* isolates obtained from three Pennsylvania orchards sampled in 2008 and 2009 and tested on myclobutanil

Orchard	2008						2009							
	<i>n</i> ^a	DMI ^b	EC ₅₀ (µg/ml) ^c		RG ^d		<i>n</i>	DMI	EC ₅₀ (µg/ml)		RG		Probability ^e	
			Mean ^f	95% CI ^g	Median	Mean			Mean	95% CI	Median	Mean	<i>t</i> test ^h	K-S ⁱ
AC-1	19	Yes	2.39	1.93–3.99	73.58	74.89	20	No	2.34	1.99–2.70	89.22	85.45	0.220	0.029
AC-4	35	Yes	2.09	1.63–3.20	71.03	60.39	19	Yes	2.44	2.33–3.97	77.08	83.28	0.005	0.005
AC-8	20	Yes	3.70	2.55–4.85	90.08	80.13	31	Yes	3.68	2.46–4.90	88.64	88.66	0.979	0.195

^a Number of isolates from cultivars sampled both years.

^b Use of sterol demethylation-inhibitor (DMI) fungicides in orchard during the year the samples were collected.

^c EC₅₀ estimates.

^d RG with myclobutanil at 0.5 µg/ml.

^e Probability for equality of estimates.

^f Mean EC₅₀.

^g CI = confidence interval (µg/ml).

^h *P* value from a two-sample Student's *t* test comparing the orchard mean EC₅₀ values from both years. Data were subjected to a natural logarithm transformation before the *t* test.

ⁱ *P* value from a nonparametric Kolmogorov-Smirnov (K-S) test comparing the distribution of RG values from both years.

TABLE 3. Relationship between orchard management factors and the incidence of *Venturia inaequalis* resistance to sterol demethylation-inhibitor (DMI) fungicides in Pennsylvania apple orchards based on samples collected in 2008 and 2009

Response variable, factor compared ^a	Random-effects model ^b				Fixed-effects model ^c			
	Odds ratio	95% CI ^d	<i>F</i> value	<i>P</i> value	Odds ratio	95% CI	<i>F</i> value	<i>P</i> value
Resistant to myclobutanil ^e								
Copper vs. no copper	1.90	1.03–3.48	4.30	0.0386	2.10	1.50–2.95	18.34	<0.0001
Small vs. large orchards	0.38	0.15–0.98	4.03	0.0450	0.36	0.21–0.59	15.78	<0.0001
≤4 vs. >4 DMI applications in 2009	3.27	1.26–8.47	5.96	0.0149	3.06	1.50–2.95	19.34	<0.0001
Shifted sensitivity to fenbuconazole ^f								
Copper vs. no copper	2.02	1.05–3.91	5.75	0.0382	1.92	1.25–2.95	10.81	0.0029
Small vs. large orchards	0.16	0.06–0.46	16.24	0.0041	0.15	0.08–0.29	32.97	<0.0001
≤4 vs. >4 DMI applications in 2009	4.57	1.48–9.16	9.84	0.0150	4.44	2.31–8.55	24.06	<0.0001
Old vs. young trees	1.72	1.09–2.72	5.60	0.0200	1.83	1.24–2.71	11.71	0.0040
≥10 vs. >10 cultivars	0.41	0.18–0.91	5.90	0.0324	0.43	0.24–0.76	10.34	0.0025

^a Response = incidence of isolates. Factors were always analyzed as fixed effects in both the random- and fixed-effects models. Interaction terms between orchard and these factors could not be estimated because each orchard had only one of two levels for each of these factors. Orchards were grouped based on (i) whether they received least one application of a copper-containing product on dormant trees or leaf litter in the fall or spring preceding the survey, (ii) size (≤25 acres or >25 acres), (iii) age of trees (≤20 years; or >20 years), and (iv) number of cultivars planted.

^b Orchards were analyzed as a random variable in a model fitted to the binary incidence data with the GLIMMIX procedure in SAS.

^c Orchard was analyzed as fixed effects in the model but not specified as a random variable in the GLIMMIX procedure.

^d CI = confidence interval.

^e Isolates were considered resistant if they had relative growth (RG) ≥75 on quarter-strength potato dextrose (1/4 PDA) agar amended with myclobutanil at 0.5 µg/ml.

^f Isolates were considered shifted towards resistance if they had RG ≥75 on 1/4 PDA amended with fenbuconazole at 0.25 µg/ml.

Fig. 4. Effects of dormant copper applications, number of sterol demethylation-inhibitor (DMI) sprays, orchard size, and number of cultivars planted in an orchard on growth responses of *Venturia inaequalis* isolates collected in 2008 and 2009 from apple orchards in Pennsylvania. Values are means and standard errors of relative growth values on plates amended with **A**, myclobutanil or **B and C**, fenbuconazole at 0.5 µg/ml based on estimates obtained with a model in which orchards and isolates were analyzed as random effects.

growth for the majority of the isolates in our study. Nevertheless, even with a 0.5 µg/ml concentration and an RG value of 75 as threshold, 66% of 644 isolates screened were resistant to myclobutanil, and 26% of 545 isolates were resistant to fenbuconazole (Fig. 1). When evaluated at lower concentrations of fenbuconazole and difenoconazole (0.25 and 0.125 µg/ml, respectively), 47 and 9% of isolates, respectively, were shifted toward resistance to these fungicides (Fig. 2). The combined results on the estimates of EC₅₀ values and tests based on specific discriminatory doses indicate high levels of resistance to myclobutanil and fenbuconazole, as well as strong shifts toward resistance to difenoconazole in populations of *V. inaequalis* from Pennsylvania apple orchards. Resistance to DMI fungicides has been documented in most of the apple-producing states in the eastern United States, including New York and Michigan (20) and Virginia (25), but this is the first report from Pennsylvania.

The observation that isolates were more likely to be resistant to myclobutanil than to fenbuconazole or difenoconazole (Table 1; Fig. 1) may be attributed to several factors. Myclobutanil has been used for control of apple scab in the United States for >20 years, whereas fenbuconazole and difenoconazole had only been used in commercial apple orchards for 3 and 1 years, respectively, at the time of this survey (18,27); thus, it would be expected that populations would be most adapted to myclobutanil. The breadth of resistance to fenbuconazole is surprising, given that it has been marketed for control of apple scab for a much shorter duration than myclobutanil. This may be accounted for by the strong cross resistance to these fungicides among *V. inaequalis* populations (Fig. 3), whereby individuals exposed to myclobutanil are already preselected for resistance to fenbuconazole compared with individuals from populations with no exposure to DMI fungicides (19). Nevertheless, other factors, such as introduction of different inactive ingredients, changes in other products that are tank-mixed with DMI fungicides, the overall solubility of the fungicides, and individual orchard spray schedules cannot be ignored and require further investigation.

Of the three orchards sampled in both 2008 and 2009, significant shifts toward increased resistance were noted in two of the orchards as higher median RG or mean EC₅₀ values in 2009 (Table 2). Indeed, when the EC₅₀ values were subjected to a natural log transformation before the *t* test, or analyzed with the nonparametric K-S test, the results were congruent with those of tests based on RG values. These results, therefore, indicate reduced sensitivity to myclobutanil in the *V. inaequalis* populations from the second year of the survey for these two orchards, and provide evidence for season-to-season shifts toward increased resistance to DMI fungicides in *V. inaequalis* populations. Increased resistance in orchard AC-1, even after DMI fungicides were withdrawn, suggests that the resistant population is ecologically fit, or had cross resistance to the fungicides used in place of the DMIs in 2009. Little is known about the rate of development of resistance to DMI fungicides in commercial apple orchards, or about the inheritance of resistance to DMI fungicides in populations of *V. inaequalis*. Knowledge of seasonal or long-

TABLE 4. Orchard management factors related to the incidence of *Venturia inaequalis* isolates with cross-shifted resistance to myclobutanil, fenbuconazole, and difenoconazole based on samples obtained in 2008 and 2009 from Pennsylvania apple orchards^a

Factor ^b	Num. df ^c	Den. df ^c	<i>F</i>	<i>P</i> > <i>F</i>	Odds ratio	95% CI ^d
Orchard size	1	6.29	8.99	0.0227	0.38	0.174–0.830
Tree age	1	30.15	8.13	0.0078	1.85	1.191–2.880
Use of copper	1	5.76	6.25	0.0482	1.76	1.006–3.089

^a Isolates were considered cross-shifted towards resistance if they had a relative growth value (RG) ≥ 75 on separate plates amended with myclobutanil at 0.5 µg/ml, fenbuconazole at 0.25 µg/ml, and difenoconazole at 0.125 µg/ml. In total, 203 of the 630 isolates tested were cross shifted on all three fungicides.

^b Based on survey response from growers whereby orchard blocks were classified based on (i) size (≤25 acres or >25 acres), (ii) age of trees (≤20 years or >20 years), and (iii) whether they received least one application of a copper-containing product on dormant trees or leaf litter in the fall or spring preceding the survey.

^c Num. df = numerator degrees of freedom and Den. df = denominator degrees of freedom adjusted for bias with the Kenward-Roger's method (15).

^d CI = confidence interval.

term changes in the population structure of *V. inaequalis*, therefore, would be beneficial in developing strategies for resistance management.

In total, $\approx 22\%$ of isolates tested were cross resistant to both myclobutanil and fenbuconazole and two isolates were cross resistant to all three fungicides evaluated at the 0.5 $\mu\text{g}/\text{ml}$ concentration (Fig. 3). Cross resistance to DMI fungicides has been documented in populations of *V. inaequalis* from the United Kingdom; however, this result was based on conidial populations, and individual isolates were not tested on each fungicide (38). Additionally, these researchers found that the incidence of cross resistance was dependent on the particular source orchard (38). In our study, isolate origin was correlated with the incidence of cross resistance to myclobutanil and fenbuconazole only ($\chi^2 = 45.82$; $P < 0.001$) (data not shown). By introducing a third active ingredient, difenoconazole, our data suggest at least two mechanisms conferring resistance to DMI fungicides: one determining resistance to myclobutanil and fenbuconazole and an additional mechanism, either rarely occurring or acquired with a cost to mutant individuals, that confers resistance to all three fungicides. These results, along with the widely held theory that *V. inaequalis* resistance to DMI fungicides is quantitatively inherited (14,20), support the hypothesis that shifts toward resistance to active ingredients within the DMI fungicide class may be conferred by different genetic changes. We speculate that the two isolates with cross resistance to all three DMI fungicides in this study possess multiple resistance mechanisms; however, confirmation of this would require a thorough genetics characterization of these isolates.

Management factors contributing to the development of resistance to DMI fungicides were identified in this study through a survey distributed to growers. Therefore, these data are observational and care should be taken not to infer a cause-and-effect relationship from the results. Nevertheless, sufficient evidence for association between certain management factors and incidence of resistant strains was deduced even after accounting for the wide variability between orchards (resulting, for example, from correlations in responses among isolates from the same orchard) with the random-effects model (equation 5). The most notable effect of using the random-effects model was wider confidence limits

compared with those of the fixed-effects model (Table 3). This is not surprising because every additional random term contributes a variance component to the overall variance on which the confidence limits are based (4,22). The advantage of using the random-effects model over the fixed-effects model is that the inference about the associations can be extended to orchards other than those sampled (4).

Among all isolates tested on myclobutanil, fenbuconazole, and difenoconazole, a greater proportion of resistant isolates originated from larger orchards (more than 25 acres) than from smaller orchards (Fig. 4; Tables 3 and 4). The effect of orchard size on the resistance status of *V. inaequalis* isolates was not surprising because appropriate spray intervals or coverage can be more difficult to achieve for larger orchard operations than for smaller orchards. For example, field applicators may be covering the orchard at a faster pace because there is more area to be sprayed, or applications may be interrupted by weather events, due to the simple fact that spraying a larger orchard takes more time.

The age of trees in an orchard was a significant factor in the status of resistance to fenbuconazole (Table 3), which was also expected. Most likely, *V. inaequalis* populations in older orchards have undergone more selection pressure due to a longer duration of exposure to DMI fungicides, as has been suggested in other studies (21). This result is in contrast to an earlier study that found no difference between the EC_{50} values of *V. inaequalis* populations from an orchard that had been using DMI fungicides for 12 years and from an orchard that had never been treated with DMI fungicides (32). A more recent study, however, has shown a positive linear relationship between RG values of *V. inaequalis* isolates and the number of DMI fungicide treatments applied to the orchard (11). It is estimated that a grower may have 30 to 40 DMI applications before a shift toward resistance to the fungicides occurs in *V. inaequalis* populations (11,18), and most orchards in this study had been treated with DMI fungicides for at least 10 years, with several applications per year (J. W. Travis, *personal communication*).

The overall diversity of the cultivars grown in an orchard had an influence on the resistance status of the *V. inaequalis* population to fenbuconazole (Table 3). Host diversity in an orchard may provide another challenge for *V. inaequalis*, in addition to that imposed by the use of DMI fungicides, thereby slowing the shift toward resistance. The number of cultivars grown in an orchard could also be an indication of the market the particular grower is focusing on; fresh-market growers may be more likely to plant a wide diversity of cultivars than growers focusing on processing-apple sales. Target markets and associated profit margins affect the approach to managing apple orchards. For example, processing-apple markets have a higher tolerance for apple scab, but processing crops also garner lower prices. This may lead to the use of less efficacious but less costly fungicides initially and, once scab incidence nears the economic threshold for processing apples, the more costly DMI fungicides may be used as a sort of "last resort" to save the crop. Such practices may account for the significantly higher mean EC_{50} values noted for processing cultivars (Table 1). Differential cultivar susceptibility to *V. inaequalis* has been documented (3,5,24). It may be possible that strains adapted to certain cultivars are more fit overall and can withstand fungicides better than strains from other cultivars. Additional research would be required to determine if this were the case.

The positive association between the number of DMI applications per year and the incidence of resistance to fenbuconazole (Table 3; Fig. 4) is intuitive, because isolates from orchards with more DMI applications per season would be subjected to more selection events. Indeed, our data corroborate those of a recent study, in which a positive linear relationship was identified between the number of DMI applications per year and the incidence of shifts toward resistance to myclobutanil (11). Product labels for

Fig. 5. Effect of orchard size, age of trees, and use of dormant copper on the incidence of *Venturia inaequalis* isolates obtained in 2008 or 2009 from Pennsylvania apple orchards and characterized as sensitive or cross-shifted toward resistance to myclobutanil, fenbuconazole, and difenoconazole. Isolates were considered cross-shifted toward resistance if they had relative growth value ≥ 75 on separate plates amended with myclobutanil at 0.5 $\mu\text{g}/\text{ml}$, fenbuconazole at 0.25 $\mu\text{g}/\text{ml}$, and difenoconazole at 0.125 $\mu\text{g}/\text{ml}$. For each orchard management practice, the incidence of isolates in each fungicide sensitivity category was significantly different ($P < 0.05$) based on generalized linear mixed model with orchards analyzed as random effects.

DMI fungicides explicitly limit the number of applications per season to four, and that recommendation has been emphasized to growers (29).

The relationship between the use of dormant copper application and resistance to DMI fungicides (Fig. 4; Tables 3 and 4) is the most noteworthy and statistically strong result in terms of DMI fungicide resistance management. Isolates from orchards that were not using copper were almost two times more likely to be cross shifted toward resistance than isolates from orchards treated with copper (Fig. 5; Table 4). A possible explanation for this relationship is that the effects of copper may be due to an overall reduction in mating populations of *V. inaequalis* that produce ascospores. An alternative explanation is that dormant copper sprays have a sanitation effect against overwintering conidial inoculum (23,24). Though little is known about the heritability of resistance to DMI fungicides in *V. inaequalis*, keeping the overall season-to-season populations low and reducing the number of the more genetically diverse ascospores would slow the development of predominantly resistant populations. Higher levels of initial inoculum imply a higher likelihood of resistant propagules establishing new infections. Additionally, higher overall inoculum levels may warrant higher rates of fungicides and shorter intervals between sprays, which would increase selection pressure on the *V. inaequalis* population (18). These hypotheses require additional investigation. In the meantime, existing equipment and personnel can be used for copper application, making this an inexpensive choice for fungicide resistance management. This recommendation should be offered with caution, however, because copper toxicity in orchard soils is a concern for orchards with prolonged use of copper (36).

After testing a large collection of *V. inaequalis* isolates on myclobutanil, fenbuconazole, and difenoconazole, we conclude that myclobutanil is no longer an effective compound for control of apple scab in most Pennsylvania orchards, and that the effectiveness of fenbuconazole and difenoconazole will quickly be lost. The use of copper fungicides on trees or litter during the dormant season, the age of apple trees in an orchard, the number of cultivars grown in the orchard, the number of DMI fungicide applications, and the size of the orchard were the factors influencing the likelihood of resistance to DMI fungicides in the orchards surveyed. Our data indicate that, in addition to using products with various modes of action, the application of copper fungicides during dormant sprays and limiting the number of DMI fungicide applications per season will reduce the rate at which resistance to DMI fungicides develops in commercial apple orchards. By implementing these management strategies, growers around the world can prolong the efficacy of DMI fungicides on *V. inaequalis* populations in their orchards.

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