Sensitivity Distribution of *Venturia inaequalis* to the Sterol Demethylation Inhibitor Flusilazole: Baseline Sensitivity and Implications for Resistance Monitoring

Franzine D. Smith, Diana M. Parker, and Wolfram Köller

Research associate, research assistant, and assistant professor, respectively, Department of Plant Pathology, Cornell University, New York State Agricultural Experiment Station, Geneva 14456.

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ABSTRACT

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Sensitivities (ED_{50} values) of 300 monoconidial isolates of *Venturia inaequalis* to the sterol demethylation inhibitor (DMI) flusilazole were determined, based on the inhibitory effect on mycelial growth. Isolates were collected from three different orchards: in orchards 1 and 2, DMIs had never been used, whereas various DMIs had been tested for 12 yr in orchard 3. ED_{50} values for individual isolates were lognormally distributed, ranging from 0.0006 to 0.17, 0.0016 to 0.14, and 0.0007 to 0.065 $\mu g\ ml^{-1}$ in orchards 1, 2, and 3, respectively. Population means of the log transformed ED_{50} values were 0.0068, 0.01, and 0.076 $\mu g\ ml^{-1}$ for orchards 1, 2, and 3, respectively. Although the mean sensitivities were similar for all three sites, the mean ED_{50} value of orchard 2, in which DMI fungicides had never been used, was significantly higher than the mean of the two other orchards. Furthermore, the population of orchard 3, which was exposed to DMI fungicides for 12 yr, had not become more resistant to flusilazole compared to unexposed populations. Thus, differences in mean sensitivities of *V. inaequalis* populations are not necessarily related to the history of use of DMIs. Regardless of small differences among orchards, the variance of sensitivities determined for the three populations was homogenous, and all ED_{50} values could be combined in one distribution. Sample sizes necessary to detect differences in mean population sensitivities were determined based on the variation among all 300 isolates. A sample size of 50 was sufficient to detect a difference of 1.6 times the mean ED_{50} value. Sample sizes of >50 did not greatly improve the precision of the test, whereas with sample sizes of <15, the detectability of sensitivity differences among populations was decreased. The magnitude of growth inhibition at a single fungicide concentration close to the mean ED_{50} value of the baseline population was found to include a precise measure of flusilazole sensitivities and, thus, represented an appropriate alternative for the monitoring of population sensitivities.

Sterol demethylation inhibitors (DMIs) constitute a modern class of fungicides with a broad spectrum of fungicidal activity (15,18). Regardless of their diverse chemical structures, all DMIs have been identified as potent inhibitors of the C-14 demethylation of 24-methylenedihydrolanosterol, a precursor of fungal sterol biosynthesis (3,12,28). Despite this site-specific mode of action, the development of practical resistance has been less severe and widespread than initially predicted (4,5,8,13). This difference in development of practical resistance between the DMIs and some other site-specific fungicides such as the benzimidazoles or the phenylamides has been explained by the patterns of population responses caused by exposure to these fungicides (9,10,13,20,21). For the DMIs, the buildup of resistant subpopulations was shown to proceed in a quantitative and, thus, gradual way. This pattern contrasts the qualitative change of populations observed for some other site-specific fungicides (2,13,20,21).

The sensitivities of fungal phenotypes in wild-type populations not yet exposed to any DMI fungicide are continuous in character and range from highly sensitive isolates to others with considerably reduced sensitivities (2,13,20,21). According to the recommended terminology (6), a fraction of the phenotypes with reduced sensitivities can be considered resistant. Under the selection force of a DMI, this fraction will gradually increase in frequency, and the proportion of resistant phenotypes may reach a level at which satisfactory disease control at economically and legally permitted fungicide rates is no longer provided (2,13,20,21). To date, this threshold of control has been surpassed by powdery mildew of cereals and cucumber, and resistance-management strategies have been developed and implemented (2,17). Although DMI-resistant field isolates have been described for several other plant pathogens, disease control under field conditions has not yet been widely affected (2,13). For most of these pathogens, however, the mean and variance of DMI sensitivities in field populations and the rate at which selection for resistance is proceeding remain unknown (2).

The spectrum of fungal pathogens controlled by some of the DMIs includes *Venturia inaequalis* (Cooke) G. Wint., the causal agent of apple scab (15,18,26). The presence of DMI-resistant phenotypes of *V. inaequalis* in field populations has been described in the past (24,27). Unsatisfactory control of apple scab in research orchards with prolonged histories of DMI use was correlated with reduced sensitivities of isolates of *V. inaequalis* collected from these sites (7,11). Based on these reports and the fact that the presence of fruit scab would greatly diminish the marketability of apples, a preventive antiresistance strategy for the use of DMIs against *V. inaequalis* was presented (17). This strategy recommended the restriction of DMIs to one early season application, followed by mixtures of DMIs with protectant fungicides that act toward *V. inaequalis*; this is similar to the general antiresistance strategy recommended for the benzimidazoles (5) and phenylamides (25).

The merits of the suggested mixture strategy are not easily evaluated because both experimental data and past experiences are lacking. Thus, future field-monitoring of fungal population responses will be required to correlate the levels of disease control achieved under various conditions with the sensitivity distributions of pathogen populations at particular sites. Future population changes will have to be compared with the status of unexposed wild-type populations. Unfortunately, extensive experimental data on the baseline sensitivities of *V. inaequalis* to DMI fungicides are not available. The present study was initiated to determine the range and frequency of sensitivities of three separate field populations of *V. inaequalis* to the representative DMI flusilazole,

to analyze the sensitivity data with regard to adequate sample sizes, and to develop simplified procedures for future monitoring efforts.

MATERIALS AND METHODS

Materials. Flusilazole (technical grade) as a solution in xylene (491 μg ml^{-1}) was provided by E. I. Du Pont de Nemours & Co., Wilmington, DE. Potato-dextrose agar (PDA) and Bacto-agar (Difco) were obtained from Difco Laboratories, Detroit, MI. Tetracycline, chloramphenicol, and streptomycin sulfate were purchased from Sigma Chemical Company, St. Louis, MO.

Isolation and propagation of *Venturia inaequalis*. One hundred infected leaves with distinct sporulating apple scab lesions were collected on 10, 16, 21 June 1988 from 10 unsprayed apple trees at three different sampling sites. According to the epidemiological data, the bulk of lesions present on leaves developed after the end of the primary infection season and, thus, originated from conidial infections. One lesion was cut from each leaf with a cork borer (0.5 cm diameter). The lesion was wet with water and streaked across water agar (4%) containing 50 μg ml^{-1} each of tetracycline, chloramphenicol, and streptomycin sulfate. Plates were incubated for at least 15 hr at 20 C, and four individual germinated conidia were transferred to a PDA plate. Colonies of *V. inaequalis* were incubated for 3–4 wk at 20 C; only one of the monoconidial colonies per lesion was used for sensitivity tests.

Histories of the sampling sites. Orchard sites were chosen according to their different fungicide histories and their geographical separation. Orchard 1 was a group of apple trees of unknown parentage at the Montezuma National Wildlife Refuge, Seneca County, NY. The apple trees had never been treated with fungicides, and the refuge is not surrounded by managed apple orchards. Thus, the isolates collected at this site represented an isolated wild-type population. Orchard 2 was an abandoned orchard of Baldwin apples in Wayne County, NY, a large apple-production area. The orchard had not been managed for 15 yr, and DMI fungicides were never used in this orchard. The population of *V. inaequalis* at this site represented a pathogen population not directly affected by the selection force of any DMI fungicide. The fungal population could have been influenced, however, by influx of propagules from the surrounding managed orchards. Within the region, the DMIs triforine and, for one season, fenarimol had been used in these orchards. Orchard 3 was a research orchard at the New York State Agricultural Experiment Station in Geneva; it included a mixed planting of McIntosh and Cortland apples. A large number of experimental DMI fungicides had been tested at this site, starting in 1976. Thus, the population of *V. inaequalis* in orchard 3 had been exposed to various DMI fungicides for 12 yr.

Sensitivity tests. The sensitivity of 100 monoconidial colonies per orchard to flusilazole was determined as follows. Two agar plugs (3 mm diameter) cut from each monoconidial colony were transferred to plates containing 20 ml of PDA with either no flusilazole or with flusilazole at concentrations of 0.0005, 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, or 0.1 μg ml^{-1}. The fungicide was dissolved in acetone before mixing with PDA that was cooled to 60 C. In all cases (including unamended control plates), the final acetone concentration was 0.1% (v/v). Inoculated plates were incubated for 4 wk at 20 C, and the mean colony diameter (minus the diameter of the inoculation plug) was determined and expressed as the percentage of the mean diameter for the untreated control. The ED_{50} values were calculated by regressing the relative growth (colony diameter on flusilazole-amended medium divided by the diameter on unamended medium $\times$ 100) against the log of the fungicide concentration.

Data analysis. Frequency distributions of log transformed ED_{50} values determined for individual orchard populations were analyzed according to the univariate procedure of SAS (Statistical Analysis System, SAS Institute Inc., Cary, NC). Because sample sizes exceeded 50, the Kolomorov D statistics rather than the Shapiro-Wilk test was applied. The Hartley test (16) was used to analyze the homogeneity of variance among ED_{50} values determined for the individual sampling sites. Based on the same set of data, the minimum sample size necessary to detect differences in mean sensitivities of individual populations was determined. According to the procedure described by Snedecor and Cochran (23) for sample sizes in comparative experiments, the ratio of mean ED_{50} values (μ_1 and μ_2) determined for any two populations of *V. inaequalis* were plotted against the sample number.

RESULTS

Sensitivity distributions of populations of *V. inaequalis*. Quantification of sensitivities of *V. inaequalis* to flusilazole was based on the inhibition of mycelial growth. These tests require incubation periods of several weeks before colony diameters can be accurately measured (7). Some fungicides might be too unstable over this long period of incubation, leading to sensitivity tests severely affected by a decreasing concentration of active material being incorporated into the agar. Growth rates of fungal isolates in this study were constant through 28 days on both unamended and flusilazole-amended media. This indicated that flusilazole was stable through this time period and that nutritional depletion of the medium had not yet become a factor. Therefore, colony diameters were measured after 4 wk of incubation.

Differences were apparent with regard to the flusilazole sensitivity distribution of populations of *V. inaequalis* among the three sampling sites (Table 1). However, the frequency distribution of ED_{50} values (Fig. 1) was lognormal at all three sites when analyzed according to Kolomorov D statistics ($P = 0.15$). When the means of the log transformed ED_{50} values of the orchards were compared, there was a significant difference between orchards 1 and 2 ($P = 0.0029$), and between orchards 2 and 3 ($P = 0.02$). However, there was no significant difference ($P = 0.05$) between orchards 1 and 3, which represented an undisturbed wild-type population and a population with a prolonged exposure to DMIs, respectively.

There was homogeneity of variance among the ED_{50} values of all three populations when analyzed at the 1% level. Consequently, a frequency distribution of all three populations that represented a less localized baseline distribution could be constructed (Fig. 1). Sensitivities in this lognormal ($P = 0.01$) distribution of combined data ranged from 0.0006 to 0.17 μg ml^{-1} and, thus, were separated by a factor of 280 (Table 1). Taking the mean population sensitivity of 0.008 μg ml^{-1} as the 'normal' sensitivity of *V. inaequalis* to flusilazole, the resistance factor (the highest ED_{50} divided by the mean ED_{50}) was 21 within the population of 300 baseline isolates.

Requirements for monitoring of population shifts. Based on all ED_{50} values determined for flusilazole in this study, the minimum sample size necessary to detect differences in mean sensitivities of any two populations of *V. inaequalis* was determined for three different significance levels (0.1, 0.05, and 0.01) (Fig 2). A sample size of 50 was adequate to detect a 1.6-fold and higher difference between mean sensitivities of any two populations of *V. inaequalis*. An increase in sample size from 50 to 100 would not greatly increase the precision of the detectable differences. On the other hand, at a significance level of 0.05,

TABLE 1. Sensitivity distribution of *Venturia inaequalis* to the sterol demethylation inhibitor fungicide flusilazole

Sampling site	ED_{50} (μg ml^{-1})		Variation in population	
	Range	Mean[a]	Coefficient of variation	Resistance factor[b]
Orchard 1	0.0006–0.170	0.0068	7.03	25
Orchard 2	0.0016–0.140	0.0102	6.17	14
Orchard 3	0.0007–0.065	0.0076	9.24	9
Combined	0.0006–0.170	0.0080	7.64	21

[a]Means of log transformed ED_{50} values were determined for 100 monoconidial isolates per sampling site.
[b]Highest ED_{50} divided by the mean ED_{50}.

a sample size of 10 would only allow detection of mean differences of >3. The differences between orchards 1 and 2 and orchards 2 and 3 were less pronounced according to the sample-size requirement. At a significance level of 0.05, a sample size of 100 would have been appropriate to detect a mean ratio of 1.5. The ratio was 1.5 between orchards 1 and 2, and 1.34 between orchards 2 and 3. Thus, the two mean ratios are close to the theoretical detectability of population differences. These differences in mean ED_{50} values reflect, most likely, natural variation in mean DMI sensitivity of populations of *V. inaequalis* residing in isolated orchards.

Determination of ED_{50} values for 50 individual isolates is too cumbersome to be useful for routine monitoring of population shifts toward resistance. Consequently, the set of data collected during our flusilazole-sensitivity testing was used to explore options for a simplified monitoring procedure. Our goal was to test isolates at only one fungicide concentration instead of the eight required for the determination of ED_{50} values, and to maintain an adequate precision of population sensitivities with a sample size of not more than 50 monoconidial isolates. The relative growth of the individual isolates at one discriminatory flusilazole concentration close to the mean baseline ED_{50} proved to be an appropriate alternative measure for sensitivities. The relative growth of each set of 100 isolates at 0.01 μg ml^{-1} of flusilazole was normally distributed (Fig. 3). Highly sensitive isolates were almost completely inhibited at this discriminatory dose (low relative growth), whereas the inhibitory effect on resistant isolates remained small (high relative growth).

The mean relative growth proved to be the most appropriate measure for site comparisons. When the sensitivities of the populations from the three orchards were compared with this mean value, the ranking of orchards and the relationship among orchards was similar to the comparison with mean ED_{50} values (Table 1). The mean relative growth at 0.01 μg ml^{-1} of flusilazole was 41.4, 47.3, and 44.5 for orchards 1, 2, and 3, respectively. Thus, the ranking of sensitivities expressed by the mean relative growth was not changed. There was no significant difference in mean relative growth when orchards 1 and 3 ($P = 0.18$) and orchards 2 and 3 ($P = 0.24$) were compared, but there was a significant difference between orchards 1 and 2 ($P = 0.014$). The detectability of differences based on mean sensitivities is thus similar to the analysis of ED_{50} values according to the sample-size requirement (Fig. 2). It departs from the Student's t test analysis of ED_{50} values, which indicated significant differences between orchards 2 and 3.

A sample-size analysis based on variation in relative growth of all 300 isolates revealed that a sample size of 50 was large enough to detect differences in mean relative growth of 4.6 ($\alpha = 0.05$). The actual difference between orchards 1 and 2 was 5.9. Thus, the mean relative growth determined with 50 individual isolates was adequate to detect the difference observed when the population sensitivities were evaluated according to the sample-size requirements shown in Figure 2. Overall, the comparison of the two procedures used to investigate differences among populations indicated that both methods are suitable to detect sensitivity variations that represent a 1.6-fold and higher difference in mean ED_{50} values. This difference appears to include the theoretical limit of detectability when sensitivity tests are based on a sample size of 50–100 isolates.

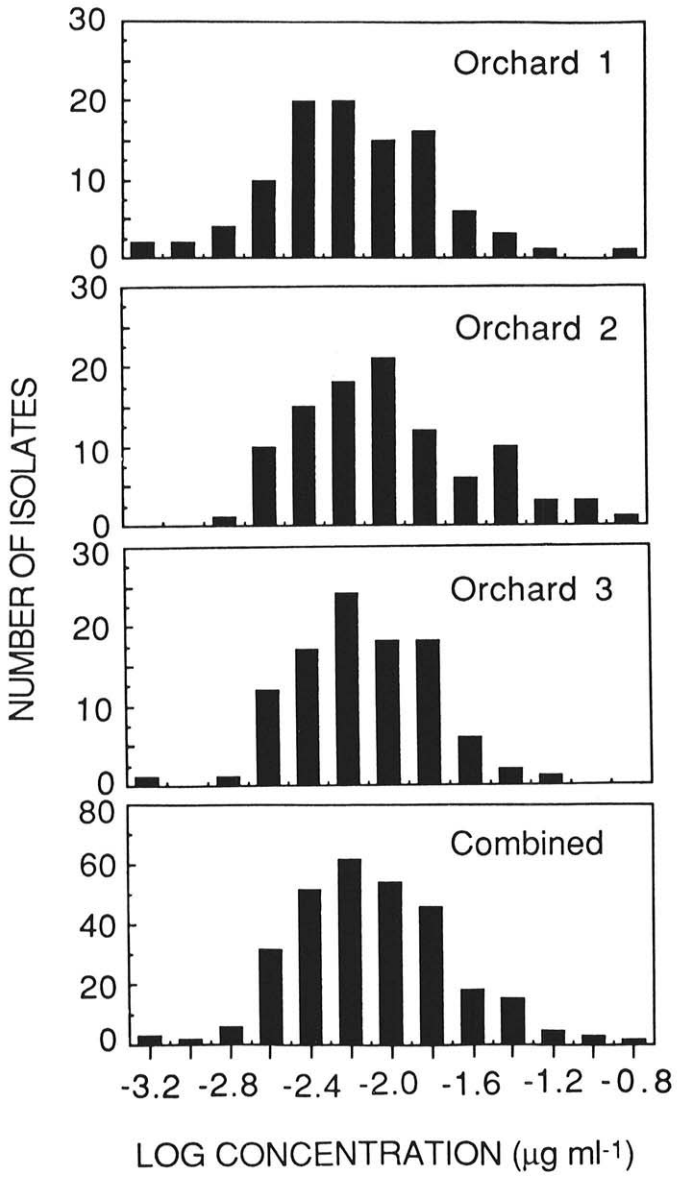

Fig. 1. Frequency distributions of ED_{50} values to flusilazole determined for monoconidial isolates of *Venturia inaequalis* sampled from three apple orchards in New York; and the regional distribution (combined) derived from the pooled data. A total of 100 isolates per orchard was tested.

DISCUSSION

The sensitivity distribution of the large population of *V. inaequalis* described in this study was based on ED_{50} values derived from the inhibition of mycelial growth. This method has been used before (7,27) and is most suitable for DMIs. Mycelial growth is related to the formation and proliferation of subcuticular stromata, the developmental stage most effectively inhibited by DMI fungicides under practical conditions. In contrast, spore germination, appressoria formation, and cuticle penetration are not affected by DMI fungicides (18). Furthermore, dose-response

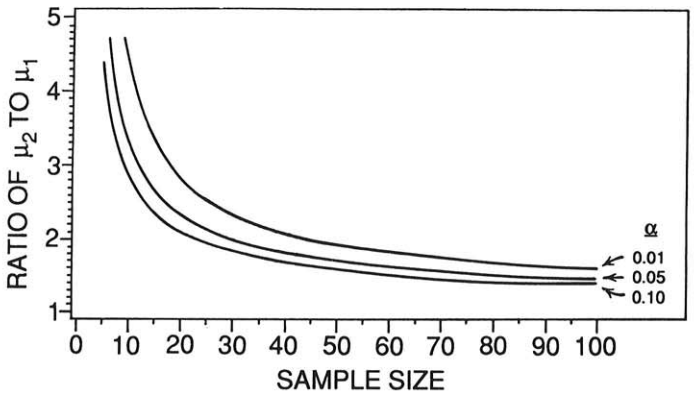

Fig. 2. Sample sizes necessary to detect differences between mean ED_{50} values of populations of *Venturia inaequalis* for flusilazole. μ_1 and μ_2 are the mean ED_{50} values of any two populations of *V. inaequalis*. Sample sizes were determined at three significance levels (α) and based on 90% confidence limits.

curves are linear around 50% of growth inhibition after log transformation, and ED values are considered more precise than minimum inhibitory concentrations (1), which have been determined by Stanis and Jones (24) and Hildebrand et al (11) in studies on sensitivities of *V. inaequalis* to DMIs.

The sensitivity distribution of two populations of *V. inaequalis* not yet exposed to a DMI was lognormal in character and, thus, similar to distributions described for other pathogens (13,21). Surprisingly, the lognormal distribution and the mean sensitivity determined for the research orchard (orchard 3) with 12 yr of DMI history was not different from a totally isolated wild-type population (orchard 1). Thus, a population shift toward resistance to DMI similar to that reported by others (7,11) was not found at this particular research orchard. It should be emphasized, however, that the conditions at this site were very different from those found in commercial orchards. DMIs have not been the exclusive materials used for apple scab control. Instead, mixtures of DMIs with protectant compounds such as captan or mancozeb have been used with increased frequency, and nontreated control trees with a high incidence of apple scab have always been present. Ascospores produced in leaf litter from these control trees may have constituted the bulk of the primary spring inoculum each season, and a 'shifted' population originating from DMI-treated trees would have been continuously diluted by the preponderance of primary inoculum produced by a population not under selection for DMI resistance. For these reasons, it cannot be concluded that population shifts toward resistance are unlikely to occur in commercial orchards.

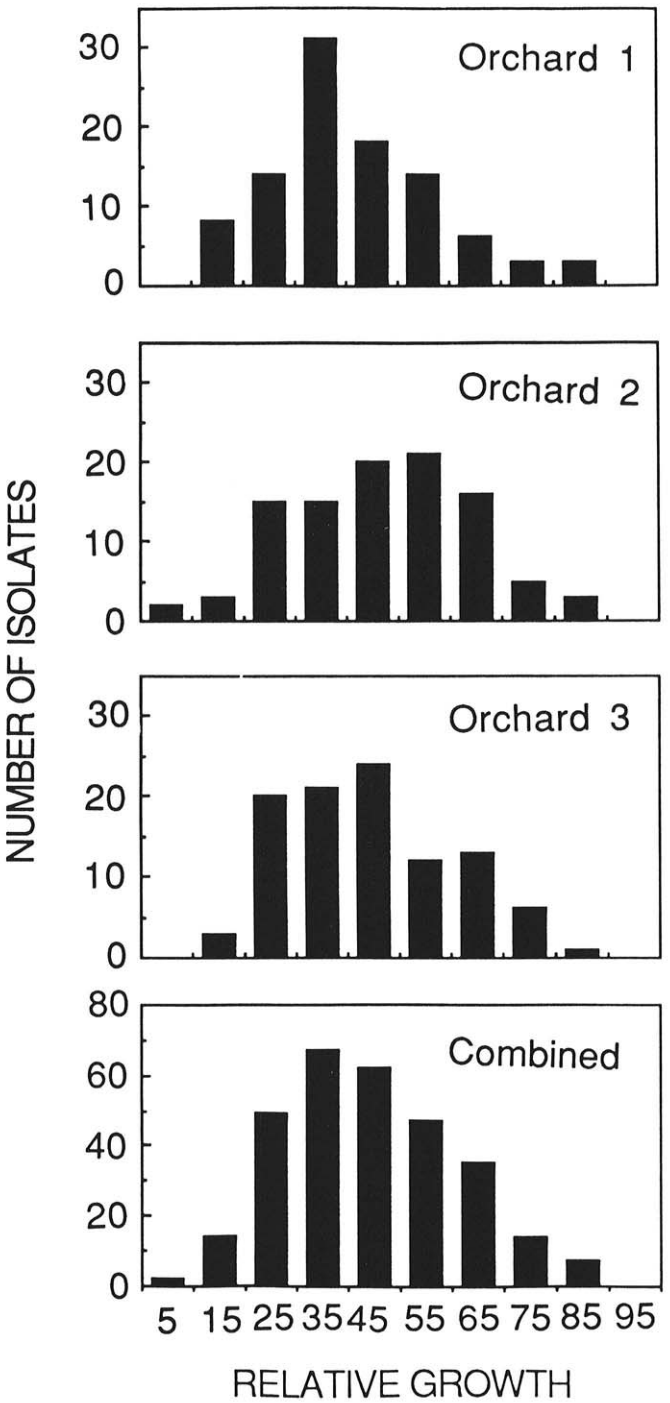

Fig. 3. Frequency distribution based on relative growth of individual isolates of *Venturia inaequalis* on flusilazole-amended medium (0.01 μg ml^{-1}). Relative growth equals the colony diameter on amended medium per colony diameter on unamended medium × 100. Relative growth was determined for 97, 100, and 98 isolates of *V. inaequalis* collected from orchards 1, 2, and 3, respectively.

The sensitivities to flusilazole determined for wild-type populations of *V. inaequalis* were variable, and the two extremes of the lognormal distribution were separated by a factor of 280. This remarkable variation exemplifies a problem with regard to the term 'resistance.' The recommended use of this term is for fungal isolates with sensitivities significantly less than the original wild-type population. The level of resistance should be expressed as the ratio of ED_{50} (resistant)/ED_{50} (sensitive), with a representative of the original wild-type population as a sensitive reference (6). In the case of a continuous and wide sensitivity distribution of isolates from a wild-type population, this definition can cause confusion. Clearly, a phenotype that represents the mean population sensitivity is resistant when compared to a phenotype grouped in the highly sensitive part of the lognormal distribution. For continuous sensitivity distributions and quantitative shifts of populations toward resistance, it appears mandatory to define the sensitive reference isolate as a representative of the mean sensitivity found in wild-type populations. This reference isolate should originate from baseline monitoring similar to that described in this study. Unfortunately, this requirement is rarely met. With the criterion proposed above, the highest resistance factor residing in a population of 300 wild-type isolates of *V. inaequalis* was 21 for flusilazole (Table 1).

The sample size necessary to detect significant differences in sensitivities of pathogen populations to DMIs has rarely been addressed and sample sizes used in previous studies have varied. For example, sensitivity distributions of *Erysiphe graminis* f. sp. *tritici* to triadimenol were based on sample sizes ranging from 97 to 11 isolates (19). Shifts of a population of *V. inaequalis* toward resistance to bitertanol were monitored for several years with sample sizes ranging from 4 to 16 (11). A sample size of 50 monoconidial isolates was found most adequate based on the sensitivities of *V. inaequalis* to flusilazole presented in this study. A larger size had little impact on precision, whereas precision was rapidly lost with sample sizes smaller than 50. Because the variance of ED_{50} values found in populations is one of the parameters that determines sample size (23), the data are only valid for flusilazole. A variance greater than that for flusilazole would increase the sample size required to detect the same differences of mean sensitivities. In contrast, a smaller variance would decrease the sample size. However, drastic departures from the sample size requirements determined for flusilazole are unlikely to be found for other DMIs. The variance in sensitivities is closely related to the resistance factor inherent to a particular DMI (14). For *Ustilago avenae* (14) and *V. inaequalis* (11,24,27), these resistance factors were not greatly different for the DMIs used for apple scab control; drastically different variances are unlikely to occur. The variability of sensitivities might also change as soon as population sensitivities shift due to selection by the fungicides, and sample size requirements will not be static. Evidence from *Sphaerotheca fuliginea* indicates, however, that the variance of sensitivities decreased while populations shifted toward DMI resistance (21). Thus, sample sizes derived from baseline data should be sufficiently high to warrant continued precision under these changing conditions.

In research orchards, unsatisfactory control of apple scab with the DMIs bitertanol, fenarimol, and penconazole has been correlated with population shifts toward resistance (7,11). By using a sample size of 12 and ED_{50} values as sensitivity tests, Fiaccadori et al (7) related mean sensitivity shifts by factors of 3, 1.8, and 4.8 for bitertanol, fenarimol, and penconazole, respectively, with unsatisfactory control of scab. Based on the sample size requirements presented for flusilazole, the number of isolates would have been sufficient to detect these differences, except with fenarimol. Hildebrand et al (11) correlated control failures observed for bitertanol on a mean ratio of 5–7, based on a sample size of 5–10 and on MIC values. Considering that differences based on MIC or ED_{95} are usually greater than those determined with ED_{50} values (7), these data again would indicate that a shift of mean ED_{50} values by a factor of three to four would be sufficient to considerably decrease the field performance of a DMI. Although, as discussed above, the sample sizes required to detect shifts of this magnitude have only been determined for flusilazole in this study, the mean sensitivities of the two wild-type populations present in orchard 1 and orchard 2, both without a DMI history, were significantly different. The mean sensitivity ratio of 1.5, with orchard 2 bearing the more 'resistant' population, indicated distinct populations with regard to their sensitivity to flusilazole. In practical terms, the higher level of DMI resistance in some orchards could result in the development of practical resistance sooner at some sites than at others. This would depend, however, on the yet largely unknown magnitude of population shifts before the threshold levels of disease control are surpassed.

As a prerequisite for resistance monitoring, the sensitivity of individual isolates should be determined with a range of concentrations (1). Analysis of our data confirmed this need for precise sensitivity data in the monitoring of quantitative population responses. It also emphasized the need for sample sizes large enough to determine sensitivity shifts of populations. Unfortunately, monitoring procedures based on these premises are extremely labor-intensive and are not easily managed, and alternative monitoring tools such as molecular probes will not become available until the mechanism of fungal resistance to DMIs has been fully elucidated (12,22). The extensive set of ED_{50} data collected during our baseline evaluation of *V. inaequalis*, however, enabled us to evaluate and propose an alternative. The relative growth of individual isolates on only one fungicide concentration close to the mean ED_{50} value of a baseline population and the mean relative growth as a measure for population sensitivities proved to include a precise but simplified tool to determine and compare the sensitivity of populations of *V. inaequalis* to flusilazole. Because the extent of population shifts over time remains largely unknown, it might be necessary to test a second discriminatory dose close to the highest level of resistance found in baseline populations. This higher dose would warrant the detectability of population shifts beyond the highest levels of resistance detected in baseline populations. The establishment of baseline mean ED_{50} values and, thus, discriminatory doses would be required before the procedure could be applied to a wider range of DMI fungicides. The acquisition of more discriminatory doses and the evaluation of the monitoring procedure under practical conditions are part of our ongoing work on DMI resistance of *V. inaequalis*.

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