Baseline Sensitivities of *Venturia inaequalis* Populations to the Strobilurin Fungicide Kresoxim-methyl

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ABSTRACT

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The efficacies of the new strobilurin fungicide kresoxim-methyl for the protection of apple leaves from infection by baseline populations of *Venturia inaequalis* were uniform across five major apple growing regions in North America. The mean ED₅₀ value determined for 25 populations was 0.35 µg ml⁻¹, with values ranging from 0.11 µg ml⁻¹ to 0.75 µg ml⁻¹. The mean level of scab control achieved at the kresoxim-methyl dose of 4 µg ml⁻¹ was 93%. For one of the five orchards sampled in each region, kresoxim-methyl sensitivities of germinating conidia were determined. Sensitivities of 250 isolates were broadly distributed, with ED₅₀ values ranging from 0.003 µg ml⁻¹ to 0.14 µg ml⁻¹ and a mean of 0.02 µg ml⁻¹. This broad range of in vitro sensitivities was not reflected for the in vivo efficacy of kresoxim-methyl in the protection of apple leaves from scab infections. The discrepancy between in vivo and in vitro sensitivities implies that in vivo tests are more useful for the monitoring of kresoxim-methyl sensitivities of orchard populations. Because it can be expected that only isolates resistant under both test conditions will be prone to future selection, such isolates will contribute to increased frequencies of the least sensitive isolates described in this baseline study. Testing of in vitro isolate sensitivities will, therefore, provide an additional tool in the monitoring of kresoxim-methyl resistance development.

Kresoxim-methyl (BAS 490F), a new fungicide of the strobilurin type, has been introduced for the control of apple scab, caused by *Venturia inaequalis* (Cooke) G. Wint. (5,10). The physical mode of action of kresoxim-methyl was described as primarily protective, with after-infection control of apple scab provided by antispore activities (10). Like all strobilurins, kresoxim-methyl acts as a specific inhibitor of respiration by binding to the center Q_p of cytochrome *b* (1-4,23,25,29,32).

The site-specific mode of action of strobilurin fungicides imposes a potential risk of resistance development (12). In several *Saccharomyces cerevisiae* mutants, substitutions of amino acids within the strobilurin binding site of cytochrome *b* were found to decrease strobilurin sensitivities (7-9). A similar mechanism was reported for fungal organisms naturally tolerant to strobilurins, such as the strobilurin-producing basidiomycetes *Strobilurus tena-*

cellus and *Mycena galopoda*, and for the fission yeast *Schizosaccharomyces pombe* (18). Thus far, a similar mechanism of mutational strobilurin resistance or natural tolerance has not been reported for any plant-pathogenic fungus. For *V. inaequalis*, the amino acid sequence determined for the cytochrome *b* target site indicated full sensitivity to strobilurins (33).

In addition to target site mutations, strobilurin sensitivities can also be diminished by the induction of alternative respiration in response to the action of respiration inhibitors such as strobilurins (6). This alternative pathway, which is active in the presence of alternative oxidase (22), has been identified as the cause for relatively low strobilurin sensitivities of fungal mycelia (11,23-26,30). Although germinating spores of several plant-pathogenic fungi were found to be highly sensitive to strobilurins (10,26,29,34), exceptions have been identified. Interference of alternative respiration with strobilurin action was found to be active in spores of a *Septoria tritici* laboratory mutant (34) and a field isolate of *V. inaequalis* (26). Surprisingly, this particular mechanism of strobilurin resistance active in vitro had no adverse effect on the in vivo level of disease control (26,34). Strobilurin-resistant phenotypes of this type are thus unlikely to be selected under practical conditions of disease control.

The potential discrepancy between strobilurin sensitivities of spores germinating in the absence or presence of their hosts

(26,34) complicates the development of monitoring procedures. The present study was initiated to establish in vivo sensitivities of baseline populations of *V. inaequalis* to the strobilurin fungicide kresoxim-methyl, to test the sensitivities of a large number of clonal conidia germinating under in vitro conditions, and to determine the correlation of the two alternative measures of strobilurin sensitivities.

MATERIALS AND METHODS

Materials. Kresoxim-methyl (technical) and a commercial kresoxim-methyl formulation (50% water-dispersible granule) were obtained from BASF Corporation (Research Triangle Park, NC). Potato dextrose agar (PDA) was from Difco Laboratories (Detroit, MI). Disposable petri dishes were from Fisher Scientific (Pittsburgh, PA). All other chemicals were from Sigma Chemical Company (St. Louis, MO).

Sampling procedure. Populations of *V. inaequalis* were collected from orchards located in the major apple growing regions of North America. Regions were located in Nova Scotia representing the Atlantic Northeast, in western New York and in Michigan representing the Great Lakes region, in North Carolina representing the Atlantic Southeast, and in Oregon representing the Pacific Northwest. Five orchards were sampled in each of the five locations. The sampling sites included apple trees at the Montezuma Wildlife Refuge in Seneca County, New York (NY1), which has been described before as a wild-type population of *V. inaequalis* never exposed to fungicides (15,17,31). All other orchards were dispersed throughout each location and were selected arbitrarily (Table 1). The orchards were sampled during June and July 1997.

From each site, 50 to 60 diseased leaves were collected from several trees arbitrarily selected and distributed throughout the orchard. The leaves sampled outside of New York were contained in paper bags and sent by overnight mail to the New York State Agricultural Experiment Station for further analysis. From each leaf, one sporulating lesion was cut with a cork borer; the lesions chosen were of similar sizes and in similar stages of sporulation. The sample size of 50 lesions per orchard was chosen, because approximately 95% of phenotypes of a particular population are represented (21), and because the precision

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of detecting sensitivity differences among populations is not substantially increased with sample sizes >50 (31). All leaf disks derived from a particular sampling site were placed into 50 ml of water. Conidia were removed by vigorous shaking, and leaf material was separated by filtration through glass wool. The aqueous suspensions of conidia were stored at -20°C until further analysis.

In vivo assay of population sensitivities to kresoxim-methyl. Protective activities of kresoxim-methyl for the control of apple scab were tested on leaves of McIntosh seedlings as described by Rich and Richards (28). Seedlings were grown in a growth chamber at 20°C with a 14-h light/10-h dark cycle at 80% relative humidity (RH) in the light and 92% in the dark. Leaves of 5- to 6-week-old seedlings were used for the experiments. Both sides of susceptible leaves were sprayed with various concentrations of a commercial kresoxim-methyl formulation (50% water-dispersible granule) to runoff with a spray-paint gun. Kresoxim-methyl concentrations were 0, 0.125, 0.25, 0.5, 1, 2, 4, and 8 µg ml⁻¹. All spray solutions contained Tween 20 at a concentration of 0.05%.

Both sides of treated leaves were inoculated 24 h after fungicide applications by uniformly misting both leaf surfaces with conidial suspensions (2 × 10⁵ conidia per ml). Conidia used in the experiments were either mixed populations derived from sporulating lesions collected at a particular sampling site or mixtures of conidia prepared from four isolates representing distinct classes of in vitro sensitivities. Respective conidia were prepared as described below and were mixed in equal proportions prior to the inoculation of apple leaves.

All inoculated seedlings were incubated for 48 h at 95 to 100% RH and 20°C in a mist chamber before they were returned to the growth chamber conditions described above. Disease severity was determined 2 weeks after inoculation by visually assessing the percentages of leaf areas infected. A total of at least eight leaves was evaluated per treatment. Mean percentages of infected leaf areas were 65 (±11) for untreated leaves.

In vitro assay of isolate sensitivities to kresoxim-methyl. One orchard in each of the five locations sampled was selected to determine kresoxim-methyl sensitivities of germinating conidia derived from monoonidial isolates. One of the orchards was NY1, representing a wild-type population; the other orchards were arbitrarily selected. With the exception of NC4, none of the orchards had been exposed to fungicides prior to sampling (Table 1); the NC4 orchard had been treated with fenarimol.

The mixtures of conidia derived from 50 sporulating lesions per sampling site and also used in the infection studies described above were allowed to germinate on water

agar amended with antibiotics as described previously (31). Germination rates were >85% for all conidial suspensions. For each site, 60 germinating conidia were arbitrarily selected and transferred to PDA. Conidia were prepared from mycelial colonies of 50 isolates per site as described by Parker et al. (27).

Kresoxim-methyl was dissolved in acetone. Sensitivities of germinating conidia (10⁵ conidia per ml) were tested in water containing acetone only or kresoxim-methyl at concentrations of 0.01 or 0.1 µg ml⁻¹; the final acetone concentration was 0.1% in all tests. The discriminatory dose of 0.01 µg ml⁻¹ reflected typical ED₅₀ values reported previously (10,26,29); the 10-fold higher dose was chosen to determine the frequency of isolates resisting high doses of the fungicide. For 16 isolates arbitrarily chosen from groups of isolates representative of the entire range of sensitivities, ED₅₀ values were determined. In these tests, kresoxim-methyl concentrations were 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 µg ml⁻¹.

In all germination tests, conidial suspensions (30 µl) were placed on the polystyrene surface of petri dishes and covered with a cover glass (2.2 × 2.2 cm). A polystyrene surface was chosen because the physical properties are very similar to cuticular leaf surfaces (20). Petri dishes were sealed with Parafilm, and conidia germination was assessed for 100 conidia at each inhibitor concentration after incubation for 24 h at 20°C. A conidium was rated as germinated if a normally developing germ tube had at least the length of a conidium

or if an appressorium had formed at the tip of a germ tube. Isolate sensitivities were expressed as relative germination (RG), which was defined as percent conidia germination in the presence of the fungicide per percent germination in the absence of the fungicide × 100. Germination rates in the absence of inhibitor were >70% for all isolates tested. Tests at the discriminatory dose of 0.01 µg ml⁻¹ were performed twice for 16 isolates in order to determine the precision of the RG test used.

Data analysis. ED₅₀ values were calculated by regressing percentages of control (in vivo tests) or RG values (in vitro tests) against the log of inhibitor concentrations. Mean sensitivities determined in infection studies were compared by ANOVA. Because frequency distributions of RG values were asymmetric, mean RG values were compared by Kolmogorov-Smirnov analysis (16,17). The homogeneity of variance among RG values of different populations was determined as described before (31). Statistical tests were supported by SYSTAT, Version 5.2 (Systat, Inc., Evanston, IL).

RESULTS

Control of *V. inaequalis* populations with kresoxim-methyl. The in vivo efficacies of kresoxim-methyl in the protection of apple leaves from scab infections were quantified with populations of *V. inaequalis* reconstructed from 25 baseline orchards. Mean ED₅₀ values for the five orchard populations tested for each of the five regions (Table 1) ranged from 0.32 µg ml⁻¹ in New York to 0.38 µg ml⁻¹ in North

Table 1. Origin of *Venturia inaequalis* populations tested for kresoxim-methyl sensitivities

Location	Orchard	History
Nova Scotia	NS1	Commercial orchard ^x
	NS2 ^y	Commercial orchard ^x
	NS3	Commercial orchard ^x
	NS4	Commercial orchard ^x
	NS5	Commercial orchard ^x
New York	NY1 ^y	Wild-type at a remote location
	NY2	Non-managed trees
	NY3	Commercial orchard
	NY4	Abandoned orchard (4 years)
	NY5	Commercial orchard
Michigan	MI1	Commercial orchard
	MI2 ^y	Experimental orchard ^{x,z}
	MI3	Experimental orchard ^{x,z}
	MI4	Commercial orchard
	MI5	Commercial orchard
North Carolina	NC1	Abandoned orchard (5 years)
	NC2	Commercial orchard
	NC3	Commercial orchard
	NC4 ^y	Commercial orchard
	NC5	Commercial orchard
Oregon	OR1	Non-managed trees
	OR2 ^y	Experimental orchard ^{x,z}
	OR3	Abandoned orchard (4 years)
	OR4	Commercial orchard
	OR5	Commercial orchard

^x Apple trees were not treated with fungicides prior to sampling.

^y Orchards tested for kresoxim-methyl sensitivities of conidia germinating in vitro.

^z Strobilurin fungicides had not been tested in blocks of trees sampled.

Carolina (Table 2). These differences were not significant ($P > 0.6$). The mean ED_{50} determined for all 25 baseline populations was $0.35 (\pm 0.16) \mu\text{g ml}^{-1}$, with values ranging from $0.11 \mu\text{g ml}^{-1}$ to $0.75 \mu\text{g ml}^{-1}$ (Table 2).

Very similar to the ED_{50} values, the mean level of control achieved at a kresoxim-methyl dose of $4 \mu\text{g ml}^{-1}$ was uniform across the regions (Table 2), and differences detected for orchard populations among regions were not significant ($P > 0.4$). The mean level of control at this dose was $93 (\pm 7)\%$ for the 25 populations tested.

Sensitivities of germinating *V. inaequalis* conidia to kresoxim-methyl. To determine the in vitro sensitivities of germinating conidia to kresoxim-methyl, conidia were prepared from 50 monoconidial isolates collected at one of the orchards in each of the five locations sampled (Table 1). At the discriminatory dose of $0.01 \mu\text{g ml}^{-1}$, mean RG values were 63, 65, 68, 72, and 73 for the populations NY1, OR2, MI2, NS2, and NC4, respectively. Mean RG values were not significantly different among the populations NY1, OR2, and MI2 ($P > 0.1$; combined

mean RG = 65). Means were also not different for populations NC4 and NS2 ($P = 0.76$; combined mean RG = 72). However, differences of mean RG values were highly significant ($P < 0.001$) when data for the similar and thus combined populations were compared.

The sensitivity distributions of these two subpopulations are shown in Figure 1. Differences were apparent for lower frequencies of isolates with RG values < 60 . However, the frequencies of least sensitive isolates (RG > 90) were very similar for both populations (Fig. 1). There was homogeneity of variance among RG values of the two populations at the 1% level, and all data were therefore combined to a baseline sensitivity distribution (Fig. 2). RG values were broadly distributed and ranged from 17 to 95, with a mean value of 68. The mean standard error of repeated RG tests at the discriminatory dose of $0.01 \mu\text{g ml}^{-1}$ was $7 (\pm 3)$. ED_{50} values determined for representative isolates ranged from $0.0035 \mu\text{g ml}^{-1}$ (RG = 23) to $0.17 \mu\text{g ml}^{-1}$ (RG = 89). The mean RG values of 68 corresponded to an ED_{50} value of $0.02 \mu\text{g ml}^{-1}$. Log ED_{50} values and the means of RG values determined at the dose of $0.01 \mu\text{g ml}^{-1}$ were correlated for RG values < 85 ($r = 0.93$).

At the discriminatory dose of $0.1 \mu\text{g ml}^{-1}$ of kresoxim-methyl, 53% of the 250 isolates tested were fully prevented from germination (RG = 0); RG values > 50 ($ED_{50} > 0.1 \mu\text{g ml}^{-1}$) were determined for 1.6% of the isolates (Fig. 2).

Correlation of in vivo and in vitro sensitivities to kresoxim-methyl. The impact of the broad kresoxim-methyl sensitivity distribution obtained under in vitro conditions (Fig. 2) on the control of apple scab was investigated with three groups of isolates representing the most sensitive, the typical, and the least sensitive part of the sensitivity distribution obtained in in vitro tests.

The mean in vitro ED_{50} values determined for the three groups of conidia ranged from $0.0045 \mu\text{g ml}^{-1}$ to $0.091 \mu\text{g ml}^{-1}$ and thus were separated by a factor of 20 (Table 3). This difference was not reflected in the in vivo ED_{50} values obtained for the control of apple scab. Here, the two extremes were separated by a factor of 2.6 (Table 3). Group 1 was most sensitive under both in vitro and in vivo conditions, but the sixfold difference of in vitro sensitivities between group 2 and group 3 of isolates was not reflected in respective in vivo efficacies (Table 3). Moreover, the in vivo sensitivities determined for the three groups of representative isolates were within the range of sensitivities determined for the 25 populations reconstructed from orchards across North America (Table 2).

DISCUSSION

The study describes the sensitivity of *V. inaequalis* baseline populations to kres-

Table 2. Control of *Venturia inaequalis* populations on apple leaves protected with kresoxim-methyl

Orchards ^z	ED_{50} ($\mu\text{g ml}^{-1}$)		Control (%) at $4 \mu\text{g ml}^{-1}$	
	Population	Mean	Population	Mean
NS5	0.30		96	
NS1	0.31		98	
NS3	0.32		97	
NS4	0.34		96	
NS2	0.55	0.36	75	92
NY1	0.11		91	
NY4	0.22		96	
NY3	0.34		88	
NY5	0.37		98	
NY2	0.55	0.32	89	92
MI1	0.24		92	
MI5	0.27		100	
MI4	0.28		94	
MI3	0.44		89	
MI2	0.48	0.34	100	95
NC2	0.21		100	
NC3	0.30		95	
NC5	0.31		96	
NC1	0.33		98	
NC4	0.75	0.38	80	94
OR1	0.15		98	
OR4	0.24		100	
OR3	0.26		100	
OR2	0.46		87	
OR5	0.70	0.36	81	93

^z Orchards are described in Table 1.

Fig. 1. Frequency distributions of sensitivities of *Venturia inaequalis* isolates to kresoxim-methyl at a discriminatory dose of $0.01 \mu\text{g ml}^{-1}$. Distributions of populations NY1, OR2, and MI2 were combined (closed bars; $n = 150$), and populations NS2 and NC4 were combined (hatched bars; $n = 100$).

oxim-methyl and represents the first detailed analysis of population sensitivities to strobilurin fungicides. In *in vivo* assays, the efficacies of kresoxim-methyl in the protection of apple leaves were uniform across the major apple growing regions in North America, ranging from $ED_{50} = 0.11 - 0.75 \mu\text{g ml}^{-1}$. The variance of sensitivities reflects a composite of true differences among orchards, the variability inherent to populations reconstructed from 50 sporulating lesions, and the precision of the *in vivo* assay. The contribution of each of these parameters was not investigated in this study. Within the context of future monitoring programs, however, the sample size of populations tested allows the prediction (21) that 90% of baseline populations will fall into the ED_{50} range determined in this study if sensitivities are to be tested in routine monitoring programs (e.g., single sensitivity tests with single reconstructed populations). The *in vivo* ED_{50} value of $0.6 \mu\text{g ml}^{-1}$ reported for a single population of *V. inaequalis* sampled from an orchard in Germany and tested under similar conditions (19) indicates that our data are of broader relevance.

The sensitivities of populations of *V. inaequalis* to benomyl, DMI fungicides, and dodine have been quantified by testing *in vitro* sensitivities of 40 to 100 single isolates per population at a single discriminatory fungicide dose (13,14,16,17,31). This monitoring approach based on isolate sensitivities yielded consistent data over several seasons, allowed quantification of the frequencies of least sensitive isolates, and was successfully used in quantifying orchard differences within the limits of statistical tests (16,17). In this study, similar *in vitro* tests of isolate sensitivities to kresoxim-methyl were focused on the response of germinating conidia as the developmental stage most sensitive to stro-

bilurin fungicides (10,26,29). Baseline sensitivities of germinating conidia determined for 250 baseline isolates were unimodal and broadly distributed. The precision of the RG test and the correlation of ED_{50} and RG values established for representative isolates allow the prediction that the contribution of experimental errors to the broad variation of isolate sensitivities was relatively small. Based on the sample size used, 98% of baseline isolates (21) will display *in vitro* sensitivities within the range of sensitivities determined in this study.

Comparison of mean RG values among populations revealed significant differences in baseline sensitivities. Similar baseline variations have been reported previously for DMI fungicides (16,31). In this case, respective differences among baseline populations were very small in comparison to the population responses leading to practical DMI resistance (16). A similarly pronounced pattern of sensitivity changes during the course of resistance development has been identified for dodine (17). It appears likely that future changes of population sensitivities will be equally pronounced for kresoxim-methyl. Consequently, differences in *in vitro* sensitivities to kresoxim-methyl similar to the baseline variation reported here should not be considered as evidence for shifts of populations toward kresoxim-methyl resistance.

For DMI fungicides, *in vivo* and *in vitro* sensitivities of isolates were correlated (16), and *in vitro* test data were thus a suitable measure of population sensitivities. Preliminary results obtained with two field isolates of *V. inaequalis* (26) and a laboratory mutant of *S. tritici* (34) indicated that the two measures of isolate sensitivities were not correlated for the strobilurin fungicides. This lack of correlation was confirmed in the present study. The

discrepancy between isolate sensitivities determined under *in vivo* and *in vitro* conditions implies that the monitoring of *V. inaequalis* kresoxim-methyl sensitivities might require *in vivo* sensitivity tests. Unfortunately, such tests are laborious, more prone to test variabilities, and not easily standardized. *In vivo* tests are also difficult to conduct if large numbers of clonal isolates per population are to be tested in order to determine the fraction of baseline isolates selected during the course of resistance development (16,17).

As shown in previous studies (26,34), the discrepancy between *in vivo* and *in vitro* data observed for strobilurins is due to antagonistic effects of alternative respiration active under *in vitro* test conditions but of negligible importance in the infection of hosts treated with strobilurins. This discrepancy would imply that the frequency of baseline isolates quantified as least sensitive according to our *in vitro* test results is an overestimation of isolates prone to selection under practical conditions. It can be expected, however, that selectable isolates of *V. inaequalis* resisting high levels of kresoxim-methyl doses under both test conditions will contribute to the pool of least sensitive baseline isolates. In particular at the higher kresoxim-methyl dose of $0.1 \mu\text{g ml}^{-1}$ tested in this study, only 1.6% of baseline isolates displayed RG values >50 . Frequencies of such isolates can be expected to increase if they are insufficiently controlled under practical conditions and thus are selected over time. Consequently, the *in vitro* test data described in this study can be expected to facilitate and complement future monitoring programs.

In summary, two monitoring procedures with diagnostic value in the detection of population shifts toward practical strobilurin resistance were described in this study. The *in vivo* baseline sensitivity to kresoxim-methyl was determined with reconstructed populations derived from conidia of 50 distinct scab lesions. *In vitro* tests with germinating isolate conidia were conducted with 50 isolates per site, and both the sensitivities and baseline frequencies of least sensitive isolates were determined. At present, neither natural pheno-

Fig. 2. Frequency distributions of sensitivities of *Venturia inaequalis* isolates to kresoxim-methyl ($n = 250$). Distributions represent discriminatory doses of $0.01 \mu\text{g ml}^{-1}$ (closed bars) and $0.1 \mu\text{g ml}^{-1}$ (hatched bars).

Table 3. Comparison of *in vitro* and *in vivo* sensitivities of representative *Venturia inaequalis* isolates to kresoxim-methyl

Group	Germination ^y ED_{50} ($\mu\text{g ml}^{-1}$)	Control of scab ^z ED_{50} ($\mu\text{g ml}^{-1}$)
1	0.0045 (0.001)	0.13 (0.01)
2	0.016 (0.007)	0.26 (0.03)
3	0.091 (0.040)	0.34 (0.03)

^y Mean *in vitro* ED_{50} values determined for four isolates used in respective *in vivo* tests. Standard deviations are given in parentheses.

^z Values are the means determined in two independent *in vivo* tests. Standard errors are given in parentheses.

types nor laboratory mutants of pathogens resisting high doses of kresoxim-methyl under in vivo conditions have been identified, and the sensitivities of such resistant mutants relative to the sensitivities of baseline isolates described here remain unknown. Consequently, it cannot be predicted at present whether isolates selected under practical conditions will become part of a unimodal sensitivity distribution similar to the DMI fungicides (16) or dodine (17) or part of a distinctly different subpopulation such as that identified for benomyl (12,13).

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