First publ. in: Phytopathology 87 (1997), December, pp. 1272-1278

Acquisition of Resistance to Sterol Demethylation Inhibitors by Populations of *Venturia inaequalis*

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Accepted for publication 28 August 1997.

ABSTRACT

Acquisition of resistance to sterol demethylation inhibitors (DMIs) by populations of *Venturia inaequalis* was investigated using a microscopical method developed by C. Siebels and K. Mendgen. Microscopical analysis of conidiophore formation enabled the earlier detection of resistance and a clearer distinction between DMI-resistant and DMI-sensitive populations than other in vivo methods commonly used to analyze inhibitory effects of fungicides. In addition, because observations were made on the level of individuals, quantitative measures of the composition of conidial populations were obtained. The development of DMI sensitivity was followed over a period of 3 years in control apple orchards that had never been treated with fungicides and in orchards with DMI history. The 50% effective dose values determined by microscopical evaluation of conidiophore development for untreated populations revealed the baseline sensitivities of 0.3, 0.96, 0.09, 1.22, and 1.92 mg/liter for flusilazole, fenarimol, difenoconazole, tebuconazole, and pyrifenox, respectively. As compared with the baseline sensitivity, all populations with DMI history showed significant resistance to flusilazole. A strong nonlinear correlation (R = 0.96) was found between the resistance factors and the sum of all DMI treatments of the 3 years before taking the sample. According to this correlation, resistance can be expected in all apple orchards of the fruit-growing area along Lake Constance, Germany, in which more than two DMI treatments per season have been applied. Due to cross-resistance, the recently introduced DMI fungicides difenoconazole, tebuconazole, and pyrifenox did not allow the control of *V. inaequalis* populations resistant to flusilazole.

The success of modern, integrated disease control strategies strongly depends on the availability of efficient curative fungicides (2). Until 1995, sterol demethylation inhibitors (DMIs) were the only curative fungicides registered for use in apple orchards in Germany. Since DMI fungicides represent single-site inhibitors interfering with C-14 demethylation of 24-methylendihydrolanosterol in ergosterol biosynthesis (6,18), a high risk of development of resistance exists (18). In fact, resistance problems have been observed in several pathogens including powdery mildews (10,11,25), *Penicillium digitatum* (7), *Pyrenophora teres* (26), *Sclerotinia homoeocarpa* (9), and *Venturia inaequalis* (13). Isolates of *V. inaequalis* with reduced sensitivities to several DMI fungicides have been reported to occur worldwide (4,13,23,24,31,32). Resistance to DMI fungicides is under multigenic control, increasing gradually by additive action of the different resistance genes (6,15). Depending on the stringency of the selection pressure, sensitivity of *V. inaequalis* populations may, therefore, shift gradually from baseline sensitivity to a distinct level of resistance (1,8,20). The exclusive and frequent use of DMI fungicides may thus lead to complete control failure in the field (13,23).

The detection of insensitivities to DMI fungicides in isolates of *V. inaequalis* does not necessarily indicate DMI resistance. Significant variations of DMI sensitivity within populations that had never been treated with DMI fungicides have been described (3,27,30).

While no resistance was detected in an American orchard after 12 years of extensive DMI application (30), complete control failure occurred in a Canadian orchard with a comparable number of DMI treatments (13,16). This contradiction may be due to differences in infection pressure (16). In the fruit-growing area along Lake Constance in southern Germany, high infection pressure exists and acquisition of DMI resistance by populations of *V. inaequalis* is likely to occur.

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Siebels and Mendgen (28) have developed a microscopical in vivo method to evaluate the DMI sensitivity of the apple scab fungus on the population level. We used this method to study the time course of shifts in sensitivity to the DMI fungicide flusilazole in conidia and ascospores of different populations of *V. inaequalis* over a period of 3 years. Furthermore, the impact of the number of DMI treatments on the resistance factor and the sensitivity of *V. inaequalis* populations to DMI fungicides not yet registered for use in apple orchards in Germany were also investigated.

MATERIALS AND METHODS

Test plants. Potted apple trees (*Malus domestica*) of the cultivars Jonagold and Golden Delicious grafted on M 25 rootstocks at the Schweizerischen Forschungsanstalt für Obst und Weinbau, Wädenswil, Switzerland, were held in a greenhouse at 18 to 25°C and a 16-h light period. The trees were fertilized weekly with 0.1% Hakaphos red (N-P-K = 8:12:24) and 0.1% Hakaphos blue (N-P-K = 15:10:15) (Compo GmbH, Münster, Germany).

Fungicides. The concentrations of the fungicides used in the sensitivity tests were based on the doses recommended by the manufacturer for use in the field. The recommended doses for flusilazole (Benocap-20 WP; Du Pont de Nemours GmbH, Bad Homburg, Germany), fenarimol (Rubigan-12 EC; Spiess & Sohn, Kleinkarlbach, Germany), difenoconazole (Score-10 WG; Ciba Geigy AG, Basel, Switzerland), tebuconazole (Folicur-25 WP; Bayer AG, Leverkusen, Germany), and pyrifenox (Dorado-20 EC; Ciba Geigy AG, Frankfurt/M., Germany) were 25, 36, 25, 75, and 50 mg active ingredient/liter, respectively.

Inocula. Inocula were obtained from nine different sampling sites. The location, apple cultivar, and fungicide history of the orchards are listed in Table 1. Orchard 1 was located about 60 km

from the nearest conventionally managed apple orchard. All other orchards were situated in the fruit-growing area along Lake Constance. Orchard 2 was a test orchard of the University of Constance, where no pesticides are applied. Orchards 3, 4, 5, 6, 7, and 8 were commercial orchards treated in accordance with the guidelines of integrated production. Orchard 9 was part of a test orchard of the Amtliche Pflanzenschutzberatung Überlingen, where flusilazole was the only fungicide used from 1993 to 1995. A minimum of 100 naturally infected leaves with sporulating scab lesions scattered over the whole orchard were collected per sample and stored in plastic bags at –70°C; the conidia were used for sensitivity tests. To obtain ascospores, naturally infected leaves were collected in November and overwintered in wire mesh cases on the ground of the orchard. The overwintered leaves were examined for development of pseudothecia, beginning at the end of February of the following year. Apple leaves with pseudothecia containing 5 to 10% mature ascospores were collected on 6 March 1995, and 22 April 1996, and stored in plastic bags at –70°C. To obtain ascospore suspensions, the leaves were thawed and incubated in a dark moist chamber for 10 days at 20°C. Leaves containing mature, preconditioned pseudothecia were washed in deionized water for 10 min and placed upside down into a glass petri dish (14.5 cm diameter) filled with 30 ml of deionized water. The petri dish was illuminated from beneath with 1,800 lx for 1 h at 5°C to allow ascospore discharge into the water (29). Conidia or ascospores from one orchard collected during 1 day are referred to as a sample.

Conidia of many commercial orchards had a low germination rate. Thus, to obtain a sufficient number of fungal propagules, conidia from orchards 3, 4, 5, 6, 7, and 8 from 1994 and 1995 were used to inoculate nontreated apple trees. Inoculated plants were incubated at 18°C and 100% relative humidity in the dark for 24 h. Thereafter, plants were kept in the greenhouse. Leaves with sporulating scab lesions containing multiplied conidia were collected after 14 to 20 days and stored in plastic bags at –70°C. Importantly, the dose-response relationships in multiplied conidia did not statistically differ from those of the original samples ($P = 0.43$ for the sensitive sample [orchard 1] and $P = 0.76$ for the resistant sample [orchard 9]).

Sensitivity test. Ten leaves with multiplied conidia or 50 naturally infected leaves with sporulating scab lesions were thawed and shaken in 100 ml of deionized water to obtain conidial suspensions. Ascospore suspensions were produced as described above, using 20 leaves with mature pseudothecia. Conidial and ascospore suspensions were concentrated by centrifugation (1,500 × *g* for 10 min at 5°C) or diluted with deionized water to yield a final concentration of 10^5 spores ml^{-1} (spray inoculation) or 5×10^4 spores ml^{-1} (droplet inoculation). Only when at least 50 ml of a spore suspension was available, test plants were spray-inoculated with a glass atomizer until runoff. If the spore suspension was less than 50 ml, droplets were placed onto the two youngest unfolded leaves of each shoot of the test plants. The inoculated plants were incubated in the dark at 18°C and 100% relative humidity for 24 h. Different fungicide concentrations were applied by spraying the plants until runoff with a glass atomizer 28 h after inoculation using concentration ranges of 0.125 to 250 mg of flusilazole/liter, 0.36 to 360 mg of fenarimol/liter, 0.025 to 250 mg of difenoconazole/liter, 0.25 to 750 mg of tebuconazole/liter, and 0.5 to 500 mg of pyrifenox/liter. Three shoots were used per treatment. Control shoots were sprayed with deionized water.

Subsequently, plants were incubated at 20°C and 80% relative humidity during the 15-h light (19,000 lx) period and 14°C and 90% relative humidity during the 9-h dark period. Six days after inoculation, the two youngest inoculated leaves per shoot were fixed in 1% (vol/vol) glutaraldehyde for 20 min, bleached in acetic acid/ethanol (4:96% [vol/vol]) for 20 h, boiled in lactic acid/glycerol/ethanol/H_2O (8:8:66:18% [vol/vol]) for 10 min, and incubated for at least 20 h in this solution. Thereafter, the leaves were placed on glass slides, covered with a piece of cotton cloth, and fixed with a wire frame and paper clips. The glass slides with the leaves were autoclaved for 15 min in 0.5 M KOH (14), washed three times with deionized water, and boiled in the staining solution containing 0.02% (wt/vol) methyl blue in lactic acid/glycerol/ethanol/H_2O (8:8:66:18% [vol/vol]) for 10 min. The leaves were then washed again with deionized water, the wire frame and the cotton cloth were removed, and the leaves were mounted in glycerol/lactic acid/H_2O (50:25:25% [vol/vol]) and evaluated under a microscope at 200× magnification. One hundred infection sites were investigated on each leaf with respect to differentiation of conidiophores. An infection site was defined as a conidium or an ascospore differentiated up to the appressorium with a visible penetration pore. To calculate dose-response relationships, eight different leaves were analyzed per treatment.

This microscopical method was compared with two other methods frequently used to evaluate fungicide efficiencies, i.e., determination of the diseased leaf area and determination of the capacity of production of conidia. The diseased leaf area of the three youngest inoculated leaves was estimated 16 days after inoculation according to Dahmen and Staub (5). To measure conidia production, the three youngest inoculated leaves were shaken in 30 ml of deionized water 16 days after inoculation. The conidial concentration was determined with a haematocytometer and based on the fresh weight of the leaves (5). Fungicide treatments were performed as described above. Thirty-six leaves per fungicide treatment were analyzed to calculate dose-response relationships.

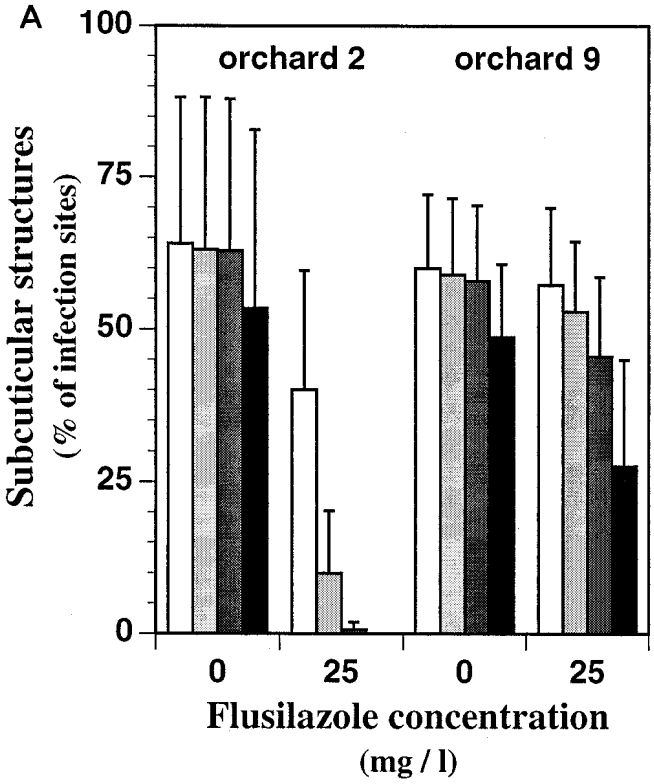
Data analysis. Different dose-response relationships were statistically compared with a factorial analysis using ranks (21) with a macro written by W. Nagl, Universität Konstanz, under SAS (Statistical Analysis System; SAS Institute, Inc., Cary, NC) to evaluate actual differences. The averages of the efficiencies were logit-transformed (logit efficiency = ln(efficiency/1 – efficiency) (22). Effi-

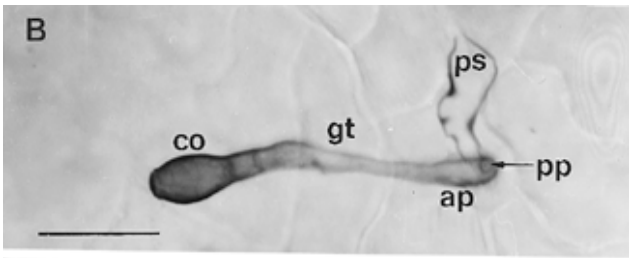
TABLE 1. Origin and fungicide history of the *Venturia inaequalis* populations used

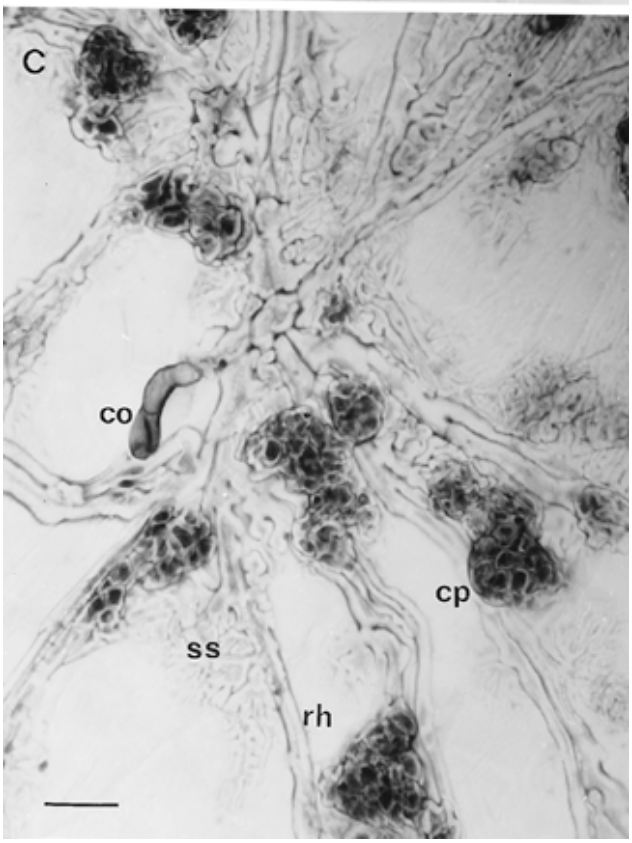
				Fungicide treatments[b]							
				1992		1993		1994		1995	
Orchard	Location	Cultivar[a]	Year of planting	DMI	Total	DMI	Total	DMI	Total	DMI	Total
1	Öschingen	GD	1980	0	0	0	0	0	0	0	0
2	Konstanz	GD	1991	0	0	0	0	0	0	0	0
3	Kippenhausen	JG	1991	2	15	1	15	6	21	1	21
4	Lindau	JG	1981	3	16	4	15	8	15	0	17
5	Bodman	JG	1987	4	12	2	9	1	15	0	17
6	Oberdorf	JG	1980	7	15	4	15	8	20	0	24
7	Kleintobel	GD	1969	5	18	6	16	1	16	4	18
8	Litzelstetten	GD	1980	5	13	5	13	3	13	3	13
9	Überlingen	GD	1990	5	19	12	12	11	11	14	14

[a] Samples were taken from the cultivars Golden Delicious (GD) or Jonagold (JG).

[b] The number of sterol demethylation inhibitor (DMI) applications and the total number of fungicide treatments (total) in the respective year are given. Applications with triadimenol were not counted as DMI treatments because of the inefficiency of the compound against *V. inaequalis*.







ciencies of 100% had to be modified before logit transformation. Modified efficiency = 1 – 1/2*n*, in which *n* was the number of all infection sites in this treatment (22). A linear regression with the Regression program of "Statistica for Macintosh" (StatSoft, Inc., Tulsa, OK) between the logit-transformed efficiencies and the logarithm of the fungicide concentration revealed the ED_{50} value (fungicide concentration that gave 50% efficiency) of the sample. The resistance factor was calculated by dividing the ED_{50} value of the sample by the ED_{50} value of the baseline sensitivity. Nonlinear regression analyses were performed with the aid of the Nonlinear program of "Statistica for Macintosh" (StatSoft, Inc.).

RESULTS

Comparison of infection structure differentiation in DMI-sensitive and DMI-resistant populations. The development of subcuticular structures from conidia of sensitive and resistant populations of *V. inaequalis* on water-treated control leaves and on leaves

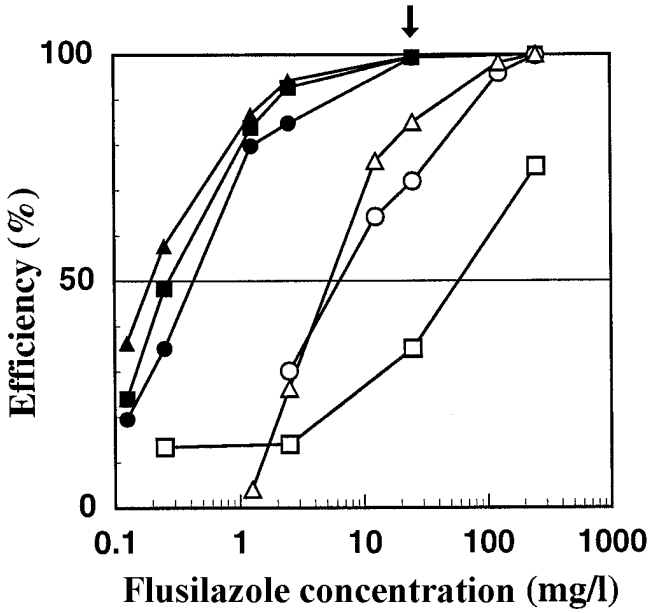


Fig. 2. Comparison of three different methods to determine sterol demethylation inhibitor (DMI) efficiency. Fungicide treatments were performed 28 h after inoculation with conidia of *Venturia inaequalis*. The efficiency of flusilazole was determined by microscopical analysis of conidiophore formation (squares) 6 days after inoculation by estimating the percentage of diseased leaf area (circles) or by counting conidia produced (triangles) 15 days after inoculation. Filled symbols represent flusilazole efficiencies determined with sensitive *V. inaequalis* populations, open symbols represent the respective values for a resistant population. The arrow indicates flusilazole concentration recommended in the field.

Fig. 1. Development of *Venturia inaequalis* on apple leaves treated with the sterol demethylation inhibitor (DMI) fungicide flusilazole. A sensitive conidial population from orchard 2 and a resistant population from orchard 9 were compared. Leaves were treated with water (0) or with 25 mg of flusilazole/liter 28 h after inoculation, and fungal structures were evaluated 6 days after inoculation. **A,** Formation of fungal structures on control and fungicide-treated leaves. Eight leaves were evaluated per treatment. □ = primary subcuticular stroma, ▒ = runner hyphae, ▓ = secondary subcuticular stroma, and ■ = conidiophores. Error bars indicate standard deviation. **B,** DMI-sensitive conidium (co) from orchard 2 with germ tube (gt), appressorium (ap), and penetration hypha visible as the penetration pore (pp). After penetration of the cuticle, the subcuticular primary stroma (ps) is formed in the presence of 25 mg of flusilazole/liter. **C,** DMI-resistant conidium from orchard 9 in addition to the structures seen in B, differentiated runner hyphae (rh), and the secondary stroma (ss) with conidiophores (cp) on a leaf treated with 25 mg of flusilazole/liter. Bars are 20 µm.

treated with flusilazole was compared 6 days after inoculation (Fig. 1). Sensitive and resistant fungal structures were indistinguishable on control leaves (Fig. 1A), but significant differences were seen after the application of 25 mg of flusilazole/liter. While differentiation of the primary stroma was only slightly reduced, formation of the secondary stroma and of conidiophores was strongly inhibited in DMI-sensitive populations (Fig. 1A and B). In contrast, the resistant population was able to form conidiophores on flusilazole-treated leaves with high efficiency (Fig. 1A and C).

Comparison of different methods to evaluate fungicide sensitivity. In Figure 2, flusilazole sensitivities of a DMI-resistant population and DMI-sensitive control populations were compared using (i) microscopical analysis of conidiophores formed, (ii) the diseased area of the leaf, and (iii) the number of conidia released. The three methods used were statistically indistinguishable when baseline sensitivities were determined. With all three methods, fungicide efficiencies with untreated populations differed significantly ($P < 0.0005$) from those of the population with DMI history. However, while measurement of diseased area and numbers of conidia formed gave indistinguishable efficiency curves for the resistant population, the microscopical analysis of conidiophores yielded a curve differing significantly from the former ($P < 0.01$). With recommended field concentrations of 25 mg of flusilazole/liter, differences were much more distinct using microscopical analyses as compared with the other two methods tested (Fig. 2). As compared with the other two methods used here, microscopical analyses of conidiophore development indicated occurrence of resistance earlier and distinctions between DMI-resistant and DMI-sensitive populations were clearer. Furthermore, because observations were made on the level of individuals, the microscopical method could give quantitative measures of the share of resistant spores. Therefore, microscopy was used in all further experiments.

Sensitivity of *V. inaequalis* populations to flusilazole. Samples from the control orchards 1 (conidia of 1993, 1994, and 1995 and ascospores of 1995) and 2 (conidia of 1994 and 95) were tested for sensitivity to flusilazole. The sensitivity tests yielded ED_{50} values between 0.21 and 0.59 mg/liter. Since no significant difference ($P = 0.44$) between the dose-response relationships of the six samples was found, values were combined to determine the baseline sensitivity of *V. inaequalis* populations to flusilazole (Fig. 3). The combined efficiencies were logit-transformed, and the linear regression of the transformed efficiencies (Fig. 3, insert) revealed an ED_{50} of 0.3 mg of flusilazole/liter as the baseline sensitivity.

The course of sensitivity to flusilazole was followed over a period of 3 years in three orchards differing in DMI history (Fig. 4). Orchard 3 was planted in 1991 and treated with DMI twice in 1992 and once in 1993. No significant resistance to flusilazole was found in conidia collected in 1993. In 1994, however, scab incidence was high in orchard 3 and six DMI applications were performed. Since resistance increased significantly (Fig. 4), scab control was unsatisfactory. DMI was applied only once in 1995, but resistance did not markedly decrease.

In orchard 4, three, four, and five DMI treatments were carried out in 1992, 1993, and 1994, respectively, and, consequently, conidia collected in 1994 showed pronounced resistance to flusilazole (Fig. 4). Ascospores were obtained from naturally infected leaves and tested for DMI sensitivity. The sexually formed ascospores of 1995 had a level of resistance similar to that of conidia collected in 1994 (Fig. 4), indicating that resistance was maintained in *V. inaequalis* after sexual reproduction. Although, in 1995, no DMI fungicides were applied in orchard 4, resistance did not decrease significantly in conidia of 1995 or in ascospores of 1996 (Fig. 4).

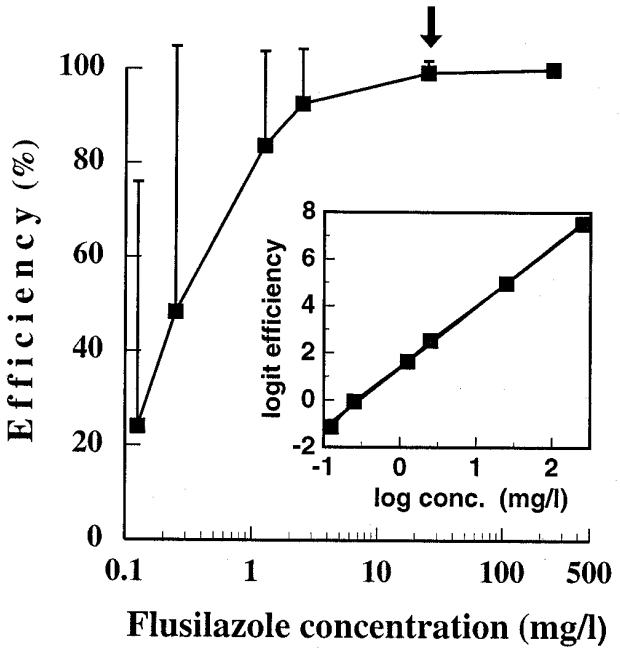


Fig. 3. Baseline sensitivity of *Venturia inaequalis* populations to flusilazole. Forty-eight leaves were evaluated for each fungicide concentration to yield dose-response relationships of sterol demethylation inhibitor (DMI)-sensitive populations. The arrow marks the flusilazole concentration recommended in the field. Error bars give the standard deviation between different leaves. The insert shows the regression line of the logit-transformed baseline sensitivity ($R = 0.999$).

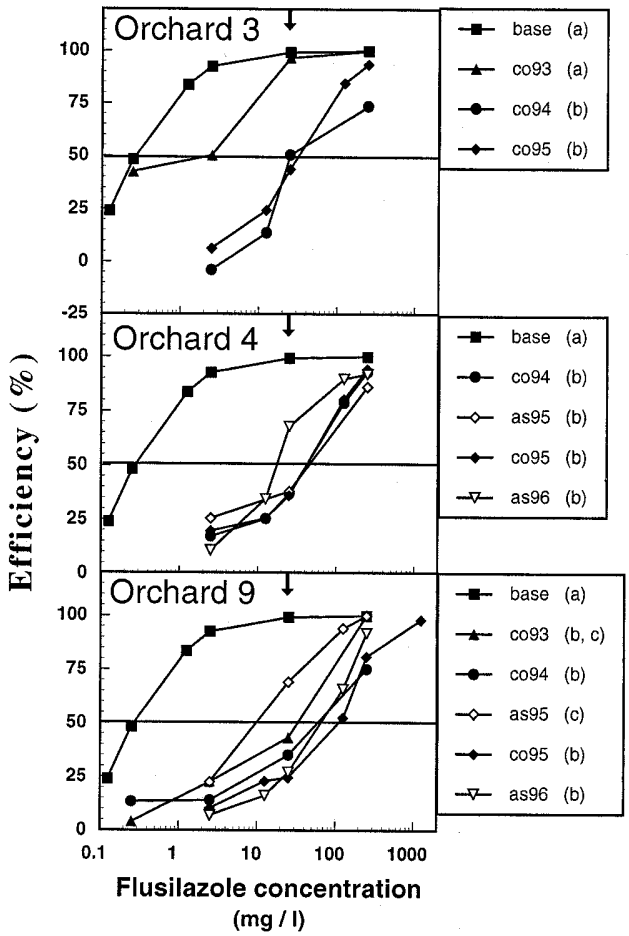


Fig. 4. Flusilazole sensitivity of *Venturia inaequalis* populations from orchards 3, 4, and 9. Dose-response relationships of conidia (co) and ascospores (as) from different years are shown in comparison to the baseline sensitivity (base). Eight leaves were evaluated per treatment. Different letters (a, b, and c) in the legend indicate significant differences ($P < 0.05$) as determined with a factorial analysis using ranks. The arrow marks the flusilazole concentration recommended in the field.

In orchard 9, only flusilazole was used for scab control from 1993 to 1995. Within 3 years (from 1993 to 1995), the resistance factor in conidia increased from 35 to 161 (Table 2). However, ascospores of 1995 were more sensitive than conidia of 1994. Comparison of ascospores of 1996 with conidia of 1995 again indicated that resistance was maintained during sexual reproduction (Fig. 4).

To investigate whether the number of fungicide applications and the degree of resistance correlated, conidia from four additional orchards (orchards 5, 6, 7, and 8) differing in numbers of DMI treatments were tested for sensitivity to flusilazole in 1995. The samples listed in Table 2 showed varying levels of resistance as indicated by ED_{50} value and resistance factor. To test whether the different levels of resistance correlated with the total numbers of fungicide treatments or with the number of applications performed in the sampling year before sampling, nonlinear regression analyses were conducted. A total of 22 samples collected from nine orchards tested for sensitivity to flusilazole (Table 2) fitted best ($R = 0.96$) to the nonlinear function $\log RF = (a \times DMI_3)/(b + DMI_3)$, in which RF is the resistance factor and DMI_3 is the sum of all DMI treatments in the year the sample was taken (until the sampling date) plus the DMI treatments of the two preceding years. The factors *a* and *b* were determined as 2.20 and 2.64, respectively. The regression curve thus approached a maximum level, given by factor *a* of the function. Therefore, a maximum resistance factor of 158 could be expected. This function allowed calculation of the expected resistance factors for nontested populations based on the known number of DMI treatments of the last 3 years.

TABLE 2. Flusilazole resistance in *Venturia inaequalis* populations[a]

Sample[b]	DMI_1[c]	DMI_3[d]	Efficiency (%)[e]	ED_{50}[f]	RF[g]	*P*[h]
Baseline[i]	0	0	99	0.3	…	…
Orchard 3						
Co 1993	1	3	95	1.4	5	0.55
Co 1994	6	10	50	55.1	186	1.5×10^{-11}
Co 1995	1	9	44	28.4	96	4.4×10^{-12}
Orchard 4						
Co 1994	5	12	37	26.4	89	4.4×10^{-12}
As 1995	8	15	39	22.4	76	2.6×10^{-10}
Co 1995	0	12	36	24.0	81	3.3×10^{-11}
As 1996	0	12	68	18.4	62	1.7×10^{-13}
Orchard 5						
Co 1995	0	3	87	6.6	22	6.0×10^{-7}
Orchard 6						
Co 1995	0	12	75	11.4	38	4.2×10^{-7}
Orchard 7						
Co 1995	4	11	69	10.5	35	1.7×10^{-7}
Orchard 8						
Co 1995	3	11	42	19.5	66	1.2×10^{-10}
Orchard 9						
Co 1993	12	20	43	10.4	35	2.4×10^{-6}
Co 1994	7	24	35	29.4	131	6.2×10^{-15}
As 1995	11	28	69	9.4	32	3.4×10^{-6}
Co 1995	7	30	25	47.8	161	2.4×10^{-14}
As 1996	14	37	27	44.0	149	2.0×10^{-14}

[a] To determine flusilazole resistance, apple leaves were treated with different fungicide concentrations 28 h after inoculation with conidia or ascospores from orchards with different sterol demethylation inhibitor (DMI) history. Eight leaves per treatment were evaluated by microscopical in vivo analysis of conidiophore formation 6 days after inoculation. Dose-response curves were calculated to characterize the samples.
[b] Orchard number, spore type (conidia [Co] or ascospores [As]), and the year the sample was taken.
[c] Number of DMI treatments in the year the sample was taken before the sampling date. Treatments with triadimenol were disregarded.
[d] Number of DMI treatments in the year the sample was taken before the sampling date and in the two preceding years. Treatments with triadimenol were disregarded.
[e] The efficiency of 25 mg of flusilazole/liter.
[f] Fungicide dosage in milligrams/liter needed for 50% efficiency.
[g] Resistance factor
[h] *P* value after comparison of the efficiencies of 2.5, 25, and 250 mg of flusilazole/liter to the baseline with a factorial analysis using ranks.
[i] Dose-response relationships of six samples from orchards 1 and 2 were combined to yield the baseline sensitivity.

Sensitivity of *V. inaequalis* populations to fenarimol. Conidia of *V. inaequalis* dating from 1993 and 1994 were collected from orchard 1 and tested for sensitivity to fenarimol. There was no significant difference ($P = 0.54$) between the dose-response relationships of the two samples, and they were combined for determination of the fenarimol baseline sensitivity. After logit transformation of the efficiencies, the linear regression revealed an ED_{50} of 0.96 mg of fenarimol/liter as the baseline sensitivity. Three samples from orchards with DMI history and differing levels of sensitivity to flusilazole (conidia from orchard 3 collected in 1993 and conidia from orchard 9 collected in 1993 and 1994) were tested for sensitivity to fenarimol. The resistance factors were between two and six times higher than those for flusilazole. Even conidia dated 1993 from orchard 3, which were sensitive to flusilazole, showed significant resistance to fenarimol (Table 3). With the recommended dose of 36 mg of fenarimol/liter, efficiencies below 90% were observed for the three samples (Table 3).

Cross-resistance to different DMI fungicides. While pyrifenox has been registered for use in German apple orchards since 1995, the DMI fungicides difenoconazole and tebuconazole are not certified for use in apple orchards in Germany. Therefore, these fungicides are excellent tools to test occurrence of cross-resistance. The baseline sensitivities for difenoconazole, tebuconazole, and pyrifenox were determined with conidia from orchard 1 of 1994. The ED_{50} values are given in Table 4. The highest sensitivity was attributed to difenoconazole. The sensitivity of conidia collected in 1994 from orchard 9, where only flusilazole had been applied, was significantly reduced with respect to the baselines for all DMI fungicides tested (Table 4). Importantly, the DMI fungicides difenoconazole, tebuconazole, and pyrifenox had not previously been applied in orchard 9. These data indicated the existence of cross-resistance among populations of *V. inaequalis* to flusilazole and the new DMIs tested. Treatment of conidia of 1994 from orchard 9 with the commercially recommended doses revealed that all three DMI fungicides had efficiencies below 90% (Table 4). The application of a 10-fold higher concentration of difenoconazole resulted in an efficiency of 99%. However, a 10-fold increased concentration of tebuconazole or pyrifenox resulted in efficiencies of only 91 and 60%, respectively.

DISCUSSION

Commercial apple orchards of the fruit-growing area along Lake Constance have been treated with an average of 15 fungicide applications per season (12). Most of these treatments have been performed to control infections by the apple scab fungus *V. inaequalis*.

TABLE 3. Resistance of *Venturia inaequalis* populations to fenarimol[a]

Sample[b]	Efficiency (%)[c]	ED_{50}[d]	Resistance factor	*P* value[e]
Baseline[f]	100	0.96		
Orchard 3				
Co 1993	72	29.5	31	9.9×10^{-4}
Orchard 9				
Co 1993	9	144	151	4.5×10^{-5}
Co 1994	14	236.4	247	8.4×10^{-9}

[a] To determine fenarimol resistance, apple leaves were treated with different fungicide concentrations 28 h after inoculation with conidia from orchards with different sterol demethylation inhibitor (DMI) history. Eight leaves per treatment were evaluated by microscopical in vivo analysis of conidiophore formation 6 days after inoculation. Dose-response curves were calculated to characterize the samples.
[b] Orchard and the year the sample (conidia, Co) was taken.
[c] The efficiency of 36 mg/liter of fenarimol.
[d] Fungicide dosage in milligrams/liter needed for 50% efficiency.
[e] *P* value after comparison of dose-response relationships of the sample with the baseline sensitivity (factorial analysis using ranks).
[f] The baseline sensitivity for fenarimol includes two samples from orchard 1.

Great efforts have been made to reduce the number of fungicide treatments. This has been accomplished by integrated disease control strategies, combining indirect measures such as summer pruning and orchard sanitation with curative fungicide treatments after infection periods (2). Infection periods are defined based on climatic data, ascospore release, and presence of susceptible tissue. Such strategies have allowed adequate scab control with three to four fungicide applications (2). However, the success of such strategies strongly depends on the availability of efficient curative fungicides such as DMIs.

To investigate the effect of frequencies of flusilazole treatments on DMI resistance, six samples of two *V. inaequalis* populations never treated with fungicides were used to evaluate the baseline sensitivity. The finding of slight differences in sensitivity between *V. inaequalis* populations is in good agreement with other reports (3,19,27,30). Pronounced differences in baseline sensitivities can be found, depending on the method used. In vivo assays involving the host lead to higher ED_{50} values than in vitro assays performed on agar plates. Comparing the baseline sensitivities to flusilazole, fenarimol, and pyrifenox determined by the microscopical in vivo assay (this work) with values determined by in vitro assays (17) showed 24- to 64-fold higher baseline sensitivities in in vivo tests, depending on the fungicide used.

Significant resistance to flusilazole was found in almost all samples from orchards with DMI history. The resistance was highly correlated with the number of DMI treatments during the 3 years before taking the sample. This correlation describes the selection performed by fungicide application. With the exception of the powdery mildew-specific fungicide triadimenol that has no effect on apple scab, all DMI fungicides (flusilazole, penconazole, fenarimol, and myclobutanil) used in the orchards were assumed to apply comparable selection pressure, especially since more than 60% of all DMI treatments in the commercial orchards tested were performed with flusilazole. This assumption, however, may not be perfectly fulfilled, due to varying intrinsic activities of the different DMIs. There is evidence that high disease pressure may be an important driving force of resistance selection (16). Based on this assumption, the correlation between resistance factor and the number of DMI treatments can only be expected if samples are collected from an area with similar disease pressure. In our study, this is the case, since the fruit-growing area along Lake Constance has been shown to be homogenous with respect to *V. inaequalis* disease pressure in the primary season. Because the test orchards used were distributed over the entire fruit-growing area along Lake Constance, the mathematical model correlating resistance and the number of DMI applications during the last 3 years could well be applied to other orchards of this area. The equation allows the prediction that DMI resistance would occur as soon as 3 years after introduction of the fungicide, and this prediction has been verified in orchard 3. The short time required to build up significant resistance levels may be due to the fact that DMI fungicides have been used in this area since 1985. In contrast to our findings, it took 7 years of fungicide application to induce resistance in a Canadian orchard (13). This clearly indicates that the equation presented here cannot be applied to other fruit-growing regions.

The equation allows the calculation of a maximal resistance factor approaching 158. The assumption that, in *V. inaequalis*, the fungicide dosage applied in the field represents the resistance-stabilizing factor is consistent with the results presented here. In a population characterized by a resistance factor of 161 (orchard 9, conidia of 1995), 75% of the conidia gave rise to conidiophores on leaves treated with 25 mg of flusilazole/liter (Fig. 4, Table 2). Thus, increasing the fungicide dosage would lead to increasing selection pressure that, in turn, would likely cause shifts towards higher levels of resistance. Likewise, the introduction of new DMI fungicides with higher intrinsic activity will also increase the selection pressure and induce higher levels of resistance.

Different DMI fungicides vary greatly with regard to intrinsic activities (16). The ranking of intrinsic activities against *V. inaequalis*, as indicated by baseline sensitivities, was difenoconazole > flusilazole > fenarimol > tebuconazole > pyrifenox. The finding that flusilazole has a higher intrinsic activity than fenarimol is in agreement with earlier reports (28,30,32), and as a result, higher resistance factors were found for fenarimol as compared with flusilazole (32). This could explain the control failure occurring in the field with fenarimol in populations in which flusilazole was fully effective (23). Likewise, in our studies, conidia from orchard 3 dating from 1993 showed significant resistance to fenarimol, whereas they were sensitive to flusilazole. Thus, new DMIs with higher intrinsic activity may have lower resistance factors, and populations resistant to flusilazole could possibly be controlled with such fungicides. Of the DMIs examined, only difenoconazole had a higher intrinsic activity than flusilazole. Although one could expect high efficiency of this fungicide, sufficient control of apple scab was not achieved in our experiments. Cross-resistance was also detected for tebuconazole and pyrifenox, both of which had not been used in this region. We would thus expect that an introduction of these three fungicides in orchards with *V. inaequalis* populations resistant to flusilazole would not result in satisfactory scab control.

TABLE 4. Cross-resistance to different sterol demethylation inhibitor (DMI) fungicides in *Venturia inaequalis* populations[a]

Sample[b]	Flusilazole	Difenoconazole	Tebuconazole	Pyrifenox
Baseline[c]				
Efficiency (%)[d]	99.3	100	100	100
ED_{50}[e]	0.30	0.09	1.22	1.92
Orchard 9 Co 1994				
Efficiency (%)	35	82	31	35
ED_{50}	39.0	5.4	195.0	112.2
Resistance factor	132	59	160	58
P value[f]	6.2×10^{-15}	2.7×10^{-7}	4.7×10^{-7}	9.6×10^{-9}

[a] An apple scab population resistant to flusilazole was used to determine sensitivity to different DMI fungicides that had not previously been applied. Apple leaves were treated with different concentrations of difenoconazole, tebuconazole, and pyrifenox 28 h after inoculation with *V. inaequalis* conidia. Eight leaves per treatment were evaluated by microscopical in vivo analysis of conidiophore formation 6 days after inoculation. Dose-response curves were calculated to characterize the samples.

[b] Orchard and the year the sample (conidia, Co) was taken.

[c] The baseline sensitivity for flusilazole includes six samples from orchard 1 and 2. For difenoconazole, tebuconazole, and pyrifenox, the baseline sensitivity includes one sample from orchard 1.

[d] The efficiency of the fungicide dosage recommended for the field (25, 25, 75, and 50 mg/liter for flusilazole, difenoconazole, tebuconazole, and pyrifenox).

[e] Fungicide dosage in milligrams/liter needed for 50% efficiency.

[f] *P* value after comparison of dose-response relationships of the sample with the baseline sensitivity (factorial analysis using ranks).

ACKNOWLEDGMENTS

This work was supported by the Ministry of Agriculture of Baden-Württemberg, Germany, and by the Fonds der chemischen Industrie. We thank E. Lange and P. Triloff for help in selecting the apple orchards and W. Nagl for help with the statistical analyses.

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