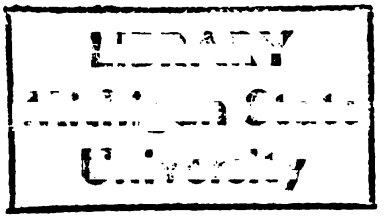

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GENETICS OF BENOMYL RESISTANCE AND REDUCED SENSITIVITY TO STEROL-INHIBITING FUNGICIDES IN VENTURIA INAEQUALIS (COOKE) WINT.

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Victor Frederick Stanis

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Alan L. Jones

Major professor

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GENETICS OF BENOMYL RESISTANCE AND REDUCED SENSITIVITY TO STEROL-INHIBITING FUNGICIDES IN *VENTURIA INAEQUALIS* (COOKE) WINT.

By

Victor Frederick Stanis

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ABSTRACT

GENETICS OF BENOMYL RESISTANCE AND REDUCED SENSITIVITY TO STEROL-INHIBITING FUNGICIDES IN *VENTURIA INAEQUALIS* (COOKE) WINT.

By

Victor Frederick Stanis

Resistance to benomyl and reduced sensitivity to sterol-inhibiting fungicides were studied in field isolates of *Venturia inaequalis*. Four levels of benomyl resistance were identified among 59 benomyl-resistant isolates of *V. inaequalis* from Australia, Chile, West Germany, Italy, New Zealand, and five states in the United States. Genetic analysis of 23 resistant isolates corroborates reports that in *V. inaequalis* different levels of benomyl resistance are controlled by alleles of a single gene. No linkage was detected between the gene for benomyl resistance and four genetic markers for auxotrophy or three colony color markers.

Isolates of *V. inaequalis* exhibiting reduced sensitivity to sterol-inhibiting fungicides were obtained from an orchard in West Germany. With the sterol inhibitors BAS 454 06 F (1-(2,4-dichlorophenyl)-2-(1H-1,2,4-trizol-1-yl)), bitertanol, CGA 71818 (1-[2-(2,4-dichlorophenyl)-pentyl]-1H-1,2,4-triazole), DPX H6573 (bis(4-fluorophenyl)methyl(1H-1,2,4-triazol-1-yl methyl)silane), etaconazole, fenarimol, Ro 15-1297, and triflumizole, minimal inhibitory concentrations preventing colony formation by individual conidia were 4 to 8 times higher for these less sensitive isolates than for other similarly tested isolates from West Germany and the United States. Levels of sensitivity did not change

in culture. Genetic analysis of nine isolates with reduced sensitivity indicates that in V. inaequalis reduced sensitivity to sterol-inhibiting fungicides is controlled by a single gene.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GENERAL INTRODUCTION AND LITERATURE REVIEW

The first of several fungicides based on the benzimidazole structure was benomyl, introduced into agriculture in 1967. Benomyl became the fungicide of choice for control of a number of plant pathogens. For control of apple scab, caused by Venturia inaequalis (Cooke) Wint., benomyl admirably filled the void created by declining use of dodine after reports of dodine resistance in a number of apple growing regions (18,26). Perhaps foremost among the favorable features of benomyl was its systemic activity which made possible a degree of after infection control previously unknown for agricultural fungicides. Benomyl also fit well into newly evolving programs of integrated pest management which depend on a diversity of options in fungicide application.

Benomyl was used extensively for scab control with excellent results. The success was, however, short-lived. In Australia, after only three years of use, benomyl was found ineffectual for scab control (33). Similar experiences with the failure of benomyl and other benzimidazoles to control apple scab were reported worldwide: Japan (6), West Germany (28), Michigan (8), New Zealand (27), Poland (16), South Africa (19), Maine (15), and France (17). The pattern was invariably the same -- three to four years after scab control with benzimidazoles was initiated, V. inaequalis possessed resistance to these fungicides.

Studies of a number of fungi, both laboratory-induced mutants (1,2,5,29,30) and field isolates (7,9,10,13,22,23), have substantiated the view that benzimidazole resistance originates through genetic changes in the fungal cell. Initial investigations into the genetics of benzimidazole resistance were done in the laboratory with non-pathogens. This was a result of the advantages of using

fungi which are well-understood and especially amenable to genetic study. Specific inquiries deemed important were: the relative ease with which benzimidazole resistance can be induced, often proposed as predictive of emergence of resistance in the field; the number of genes responsible for resistance; the levels of resistance which result from different genes or their interaciton. When other genetic markers were available, mapping or the placement of genes for resistance in known linkage groups was attempted. Thus, in 1971 Hastie and Georgopoulos (5) obtained nine benomyl-resistant mutants of Aspergillus nidulans after UV-irradiation of conidia. Five of the mutants were highly resistant, while the remaining four were less resistant. In terms of response to benomyl relative to the wild-type, this amounted to about a 14-fold increase resistance for the high resistance group and a 3-fold increase for the low resistance group. Two mutants, one from each of the two levels of resistance, were used in crosses with other strains carrying genetic markers. From combined meiotic and mitotic analysis, it was concluded that the two levels of resistance were determined by two unlinked genes, respectively, which were located on known linkage groups relative to other markers. Recombinants having both genes for resistance failed to show additive effects of an increased level of resistance. This work was confirmed by van Tuyl (30) who found the same two genes for high and low resistance in UV-mutants of A. nidulans and additionally discovered yet a third gene conferring low resistance. Each of the three genes conferring benomyl resistance was mapped on a different linkage group. Van Tuyl's findings with regard to possible additive effects of combining more than one gene for resistance in the same isolate were, however, somewhat more complicated than those of Hastie and Georgopoulos. Recombinants containing both genes for low resistance had only a slight increase in resistance over the low resistance level. However, crosses with a negatively cross-resistant

mutant, showing resistance to another benzimidazole, thiabendazole, and extra-sensitivity to benomyl, and either of the low resistance isolated produced recombinants having an intermediate resistance to benomyl and a somewhat higher resistance to thiabendazole.

Borck and Braymer (2) found different levels of benomyl resistance in UV-induced mutants of Neurospora crassa, but clear-cut categories were difficult to discern. Rather, a range in levels of resistance up to almost 100 times the level toxic to the wild-type was found among the 14 mutants tested. Genetic mapping of 15 resistant mutants showed that only a single gene for resistance was involved, and it was suggested that modifier genes played a role in determining varying degrees of resistance. From the data presented, neither the existence of multiple alleles, each conferring a different level of resistance, nor the possibility of cytoplasmic influence on the single gene for resistance could be discounted. Tests for benomyl resistance among heterokaryons of resistant and sensitive isolates showed resistance to be dominant and at a level comparable to that found in the resistant member.

The first investigations into the genetics of benzimidazole resistance of a plant pathogen was carried out by Jones and Ehret in 1976 (7). Benomyl-resistant isolates of Venturia inaequalis were obtained from Michigan orchards in which scab control with benomyl had failed. When tested on benomyl-amended media, high and low levels of resistance could be distinguished among these isolates. Although the genetic basis of this observation was not pursued, the monogenic inheritance of benomyl resistance in V. inaequalis was demonstrated by the 1:1 ratio of resistant and sensitive progeny produced in each of four crosses between resistant and sensitive isolates.

Only a single high level of resistance was found among nine carbendazim-resistant Israeli isolates of Venturia pirina, the cause of pear scab (21). From an

analysis of crosses involving five of the resistant isolates, it ws concluded that resistance is controlled by a single gene, since crosses between resistant and sensitive isolates yielded resistant and sensitive progeny in a 1:1 ratio, while crosses between different resistant isolates yielded only resistant progeny. Resistant progeny always showed the same level of resistance irrespective of whether one or both parents were resistant. This uniformity in response ruled out any contribution by modifying genes or cytoplasmic components. Cross-resistance to thiabendazole was also found. It was shown that the trait of carbendazim resistance was not linked to mating-type, but lacking an array of genetic markers as is possible for such fungi as A. nidulans or N. crassa, the subject was not investigated further.

Kiebacher and Hoffmann (11) analyzed a total of five crosses using V. inaequalis isolates from West Germany; three crosses of benomyl-resistant and sensitive isolates resulted in 1:1 ratios of resistant and sensitive progeny, while a cross of two sensitive isolates produced only sensitive progeny, and a cross of two benomyl-resistant isolates produced only sensitive progeny, and a cross of two benomyl-resistant isolates produced only resistant progeny. These data are in full agreement with the idea of a single gene for benomyl resistance in V. inaequalis, and the absence of sensitive progeny from the cross of the two resistant isolates indicates that the same gene for resistance was present in the two parents. To account for the six different levels of benomyl resistance recognized in their earlier studies (12), the authors proposed the operation of modifying genes and/or cytoplasmic factors. Utilizing such traits as colony morphology, mycelial coloration, and sporulation, colonies of benomyl-resistant isolates could not be distinguished from those of sensitive isolates, and the examination of progeny from crosses between resistant and sensitive isolates differing in these respects showed no evidence of linkage. Likewise, as in the

aforementioned case of V. pirina (21), it was shown that benomyl resistance in V. inaequalis is not linked to mating-type.

Concurrent with the report from West Germany was a similar report from France in which Martin et al. (14) showed 1:1 segregation of resistance and sensitivity in the progeny of each of ten crosses between benomyl-resistant and sensitive French isolates of V. inaequalis. Resistance was attributed to the action of a single gene, and although high and low levels of resistance were noted, the study only treated those isolates having high resistance. Following UV-irradiation of a conidial suspension from a benomyl-resistant isolate, a benomyl-resistant white mutant was obtained. In subsequent crosses the traits of white mycelium and benomyl resistance segregated independently showing that they were not linked.

The genetic basis underlying different levels of benomyl resistance in V. inaequalis was studied by Shabi et al. (22) using benomyl-resistant Israeli isolates of the fungus and later Katan et al. (9) who considered those same isolates along with benomyl-resistant isolates from New York. Recognizing four levels of resistance according to growth-rate and sporulation on benomyl-amended media, the inheritance of different levels of resistance was investigated. This work showed that discrete levels of resistance are heritable according to the rules of Mendelian segregation. Analyzing the progeny of twenty crosses made between resistant and sensitive isolates and among different resistant isolates, it was shown that different levels of resistance could be accounted for by different alleles of a single gene. Neither modifying genes nor cytoplasmic inheritance as suggested by Kiebacher and Hoffmann (13) were necessary in the explanation.

The newest group of fungicides of importance are the sterol inhibitors. These chemicals offer promise in the control of numerous diseases. Most remarkable is their effectiveness at very low rates of application, and almost all

possess some degree of systemicity which results in curative activity. In field trials, results of the use of sterol inhibitors against V. inaequalis has been favorable (20). As yet, many sterol-inhibiting fungicides are still in the developmental stage, but it is expected that they will be used extensively on apple in the near future.

Resistance to sterol-inhibiting fungicides in the field has not yet been confirmed. Adaptation to triforine has been obtained in the laboratory for some triforine-sensitive non-obligate plant pathogens, but tolerance was lost upon further subculturing on triforine-free medium (4). Increased levels of tolerance both in the greenhouse and the field have been reported for Erysiphe graminis f. sp. hordei to tridomorph (31). While isolates varied both in level of tolerance and pathogenicity, tolerance was in most cases stable in laboratory culture (32). Under laboratory conditions, mutants showing permanent resistance to sterol inhibitors have been obtained for both pathogens and non-pathogens by either mutagenic treatment or by selection from wild-type populations. Triarimol-resistant mutants of Cladosporium cucumerinum and Aspergillus fumagatus were obtained from colonies growing in high concentration of the fungicide (25). Mutagenesis through UV irradiation has yielded triarimol-resistant mutants of C. cucumerinum, Verticilium albo-atrum (4), and Ustilago maydis (24). Similarly induced triforine-resistant mutants have been obtained for C. cucumerinum (4). Nitrosoguanidine-induced mutagenesis has yielded isolates of Penicillium expansum resistant to imazalil (30). In a comparison of rates of spontaneous, UV-, and nitrosoguanidine-induced mutation for Aspergillus nidulans, imazilil resistance was found to occur much more frequently in all three cases than resistance to either benomyl or carbonxin (30).

While resistance to sterol inhibitors is rather easily induced in the laboratory, the levels of resistance are generally lower than those obtained for

many other fungicides. Van Tuyl (29) isolated imazalil-resistant mutants of Aspergillus nidulans having ED_{50} values 43 and 133 times greater than wild-types, respectively. As a rule, cross-resistance occurs among sterol-inhibiting fungicides and is interpreted as presumptive evidence for a similarity in the mode of action of these compounds. A number of cases of cross-resistance to sterol inhibitors have been summarized by Fuchs et al. (3).

As yet, the only genetic study of resistance to sterol-inhibiting fungicides has been the work of van Tuyl (29,30) with imazalil-resistant mutants of A. nidulans. Among 21 mutants examined genetically, seven originated spontaneously, while eight were UV-induced and six were induced with nitrosoguanidine. All 21 mutants possessed single gene mutations of which 11 were allelic to one locus, four were allelic to a second locus, and six represented another six loci. Two mutants selected for cycloheximide resistance were found to be also imazilil-resistance and represent two additional loci. The total of ten genes for imazalil resistance was allocated to six of the eight linkage groups (chromosomes) of A. nidulans. As further shown in mapping, the genes for imazalil resistance were not clustered, but instead distributed throughout the genome. Different levels of resistance were found among the different genes as well as among their different allelic forms. Individually, single gene mutations produced relatively low levels of resistance with a maximum 10-fold increase in minimal inhibitory concentration over sensitive isolates. However, the combination of genes for resistance resulted in positive interaction. In one recombinant strain with two genes for resistance and a modifier gene, which while not conferring resistance itself enhanced the level of resistance, a 100-fold increase over sensitive isolates was obtained in the minimal inhibitory concentration.

In recent years, stringent governmental regulation and rising costs of development have diminished the availability of new fungicides. If the sterol inhibitors are suitable replacements for dodine and benomyl, as expected, they will be well received. To prevent the loss of yet other fungicides to resistance, it is clearly desirable to gain a greater understanding of the problem; its genetic basis, the concomitant physiological changes in the pathogen, and the epidemiology. Such understanding would aid in devising management strategies for delaying or preventing the development of resistance, and ultimately might contribute to the rational development of new fungicides for which resistance will be less likely. The research presented here contributes to an understanding of fungicide resistance in V. inaequalis. The objectives were to i) investigate the genetic basis for the different levels of benomyl resistance in V. inaequalis and ii) to determine if the reduced sensitivity to sterol-inhibiting fungicides in certain field isolates of V. inaequalis extended across a range of sterol-inhibiting fungicides and if this differential response was genetically controlled.

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PART I

GENETICS OF BENOMYL RESISTANCE IN *VENTURIA INAEQUALIS* FROM NORTH AND SOUTH AMERICA, EUROPE, AND NEW ZEALAND

ABSTRACT

Fifty-nine benomyl-resistant isolates of Venturia inaequalis from Australia, Chile, Germany, Italy, New Zealand, and five states in the United States were compared with sensitive isolates for growth on benomyl-amended media. Isolates which failed to grow on media amended with 0.1 µg/ml benomyl were considered sensitive. Resistant isolates were divided into three phentotypes: low resistance, isolates grew on media amended with 1 µg/ml but not with 10 µg/ml benomyl; medium resistance, growth at 10 µg/ml but not at 25 µg/ml benomyl; and high resistance, good growth on media amended with up to 500 µg/ml benomyl. Genetic analysis of 23 resistance isolates corroborates recent reports that different levels of resistance are determined by alleles of a single gene and supports the conclusion that although benomyl resistance in V. inaequalis developed independently in several areas of the world, resistance was determined by the same gene in each area. No linkage was detected between the gene for benomyl resistance and four genetic markers for auxotrophy or three colony color markers.

INTRODUCTION

Excellent control of Venturia inaequalis (Cooke) Wint., the pathogen causing apple scab, was possible when benzimidazole fungicides became available for use on apple (Malus sylvestris Mill.). However, in Michigan and in many other apple growing regions of the world, benzimidazoles became ineffective for scab control about 3 years after their introduction (3,5,11,13-15,17-20). An explanation for the failure of benzimidazoles came with the demonstration of heritable resistance in isolates of V. inaequalis from orchards where these fungicides were ineffective. Investigations in Michigan (4), Germany (10), France (12), and Israel (6,17) have all attributed benomyl resistance in V. inaequalis to mutations in a single gene. To explain widely differing levels of resistance among different resistant isolates (4,8,13,18), the influence of modifying genes and cytoplasmic factors was suggested (10), but recent work provides evidence for a multi-allelic system in which different alleles of a single gene confer different levels of benomyl resistance (6,17).

In this study, 59 benomyl-resistant isolates of V. inaequalis from Australia, Chile, West Germany, Italy, New Zealand, and five states in the United States were tested for their level of resistance. Twenty-three of the resistant isolates were analyzed genetically to determine if resistance is always determined by the same gene regardless of the source of the isolate. Linkage between the gene conferring benomyl resistance and other genetic markers was also investigated, using auxotrophic and colony color mutants.

MATERIALS AND METHODS

Cultures. The original culture designation and geographical origin of each isolate used in this study is given in Table 1. Several sources provided benomyl-reistant isolates, but the majority were provided by E. I. duPont de Nemours & Co., Wilmington, DE 19898. The laboratory-induced mutants used in the linkage study were supplied by D. M. Boone, University of Wisconsin, Madison. To ensure genetic purity, a single conidium or hyphal tip was taken from each original culture and used to start a stock culture. Stock cultures, used in all testing and crosses, were maintained at 18°C and subcultured by transfer of single conidia every 5-7 weeks.

Media. Cultures were grown on modified potato-dextrose agar (modified PDA: 40 g potatoes steamed 45 min in 200 ml distilled water and then homogenized, 17 g agar, 5 g dextrose, and sufficient distilled water to make a final volume of 1 L) (1). To make a substrate suitable for inducing the sexual phase of V. inaequalis, a decoction of overwintered apple leaves was included in the modified PDA as described by Keitt and Langford (7). All benomyl-amended media were prepared from Difco PDA (Difco Laboratories Inc., Detroit, MI 48201). Benomyl (Benlate 50% WP, E. I. dePont de Nemours & Co., Wilmington, DE 19898) was added to the media prior to sterilization in the autoclave at 121°C for 20 min. Minimal medium for testing the auxotrophic mutants was prepared with distilled water according to Boone et al. (2) as follows (g/L): KNO_3, 3.12; K_2HPO_4, 0.75; KH_2PO_4, 0.75; $MgSO_4 \cdot 7H_2O$, 0.5; NaCl, 0.1; $CaCl_2 \cdot H_2O$, 0.1; dextrose, 5; trace element solution, 1 ml, agar, 17. The trace

Table 1. Variation in levels of benomyl resistance among isolates of Venturia inaequalis subjected to a series of benomyl concentrations in potato-dextrose agar

Isolate and Origin	Level of Resistance[b]	Source
U.S.A., Michigan		
WB(+)[a]	S	A. L. Jones
FS6C-12 (+)	S	A. L. Jones
WRR (-)	S	A. L. Jones
WM (-)	S	A. L. Jones
WL (+)	S	A. L. Jones
SPINKS 79 (-)	LR	E. I. duPont de Nemours & Co.
RH-4 (-)	HR	E. I. duPont de Nemours & Co.
RH1-11B (-)	HR	E. I. duPont de Nemours & Co.
SH1-9B (-)	HR	E. I. duPont de Nemours & Co.
SH2-10B	HR	E. I. duPont de Nemours & Co.
SH2-9B	HR	E. I. duPont de Nemours & Co.
KV3C (+)	HR	A. L. Jones
CR2C (-)	HR	A. L. Jones
GAVIN 43	HR	E. I. duPont de Nemours & Co.
VAN DRESSEN 1	HR	E. I. duPont de Nemours & Co.
PAJ-13C-P3	HR	E. I. duPont de Nemours & Co.
FS-17C-P3	HR	E. I. duPont de Nemours & Co.
VAN DRESSEN 2	HR	E. I. duPont de Nemours & Co.
NU-26 DPX-5	HR	E. I. duPont de Nemours & Co.
BG-7C	HR	E. I. duPont de Nemours & Co.

Table 1. (cont.)

BIGLER	HR	E. I. duPont de Nemours & Co.
AB-2C-B1	HR	E. I. duPont de Nemours & Co.
FP-TC 139-4	HR	E. I. duPont de Nemours & Co.
SH1-2B	HR	E. I. duPont de Nemours & Co.
U.S.A., Minnesota		
MINN FRUIT ACRES 7 (-)	HR	E. I. duPont de Nemours & Co.
MINN 6-1B	HR	E. I. duPont de Nemours & Co.
MINN LAUTZ 4 (-)	HR	E. I. duPont de Nemours & Co.
OLD HICKORY 9-2	HR	E. I. duPont de Nemours & Co.
MINN 5C	HR	E. I. duPont de Nemours & Co.
MINN 5-3B	HR	E. I. duPont de Nemours & Co.
LAUTZ 10-5	HR	E. I. duPont de Nemours & Co.
MINN 8-2B	HR	E. I. duPont de Nemours & Co.
OLD HICKORY 6-3	HR	E. I. duPont de Nemours & Co.
MINN 5-4B	HR	E. I. duPont de Nemours & Co.
MINN 3	HR	E. I. duPont de Nemours & Co.
U.S.A., New York		
#42 MINNS 118 (-)	LR	J. D. Gilpatrick
#74 PRESTON 7 (+)	LR	J. D. Gilpatrick
#6 MITCHELL 94-1 (-)	LR	J. D. Gilpatrick
#32 MINNS 86-8	HR	J. D. Gilpatrick
#60 MINNS 86-8	HR	J. D. Gilpatrick
VAN DUSER 3	HR	E. I. duPont de Nemours & Co.
VERDINE 2	HR	E. I. duPont de Nemours & Co.
U.S.A., Maine		
V-558 (+)	MR	M. Zuck

Table 1. (cont.)

SIS-16 (+)	MR	M. Zuck
V-560 (+)	MR	M. Zuck
V-565 (+)	MR	M. Zuck
MAINE 8 (+)	MR	E. I. duPont de Nemours & Co.
MAINE 6 (+)	MR	E. I. duPont de Nemours & Co.
MAINE 2B (+)	MR	E. I. duPont de Nemours & Co.
U.S.A., North Carolina		
N. C. STATE 1 298	HR	T. B. Sutton
Italy		
15-1 (-)	HR	E. I. duPont de Nemours & Co.
17-1 (+)	HR	E. I. duPont de Nemours & Co.
14-1	HR	E. I. duPont de Nemours & Co.
16-1	HR	E. I. duPont de Nemours & Co.
11-1	HR	E. I. duPont de Nemours & Co.
New Zealand		
75-78	LR	G. J. Samuels
75-150 (+)	HR	G. J. Samuels
75-71	HR	G. J. Samuels
75-26	HR	G. J. Samuels
Australia		
RI	HR	E. I. duPont de Nemours & Co.
RIII	HR	E. I. duPont de Nemours & Co.
West Germany		
GERMANY G3-2 (-)	HR	E. I. duPont de Nemours & Co.
Chile		
CHILE 24B (+)	MR	E. I. duPont de Nemours & Co.

Table 1. (cont.)

Unknown origin		
S	HR	E. I. duPont de Nemours & Co.
Laboratory-induced mutants[c]		
U-2340 PYRIMIDINE-3 (-)	S	D. M. Boone
U-3010 PURINE-8 (-)	S	D. M. Boone
U-2599 NICOTINIC ACID-1	S	D. M. Boone
U-3069 ARGININE-3 (-)	S	D. M. Boone
U-2570 BIOTIN-1	S	D. M. Boone
1906 PYRIMIDINE-6	S	D. M. Boone
U-1907 PYRIMIDINE-2	S	D. M. Boone
1261 HISTIDINE-1	S	D. M. Boone
U-1905 CHOLINE (-)	S	D. M. Boone
U-2129 PURINE-3	S	D. M. Boone
N-156 WHITE-2 (-)	S	D. M. Boone
N-1 BROWN-1	S	D. M. Boone
N-282 BROWN	S	D. M. Boone
N-151 PALE	S	D. M. Boone
N-10 GREEN (+)	S	D. M. Boone
N-222 YELLOW (+)	S	D. M. Boone

[a]Mating-type of isolates used in crosses is included in parentheses.

[b]S=sensitive to benomyl at 0.1 µg/ml; LR = low resistance, growth at 1 µg/ml but not at 10 µg/ml; MR = medium resistance, growth at 10 µg/ml but not at 25 µg/ml; HR = high resistance, growth at 500 µg/ml.

[c]First 10 mutants listed are auxotrophs and remaining 6 mutants are color mutants.

element solution was prepared as follows (mg/400 ml): $ZnSO_4 \cdot 7H_2O$, 58.4; $CuSO_4 \cdot 5H_2O$, 31.6; $MnSO_4 \cdot 4H_2O$, 16.2; H_3BO_3, 11.4; MoO_3, 7.0; $Fe(C_6H_5O_7) \cdot 3H_2O$, 214.2.

Benomyl resistance and auxotrophy. To determine levels of benomyl resistance, all isolates were subjected to a series of benomyl concentrations in PDA ranging from 0.1-1200 µg/ml benomyl. Two methods were used. In the first method, a 2- to 3-week-old colony was excised from its agar substrate, macerated in 4 ml of sterile distilled water with a 7 ml tissue grinder, and 0.5 ml aliquots of the homogenate were spread over the surface of benomyl-amended and unamended PDA in 60 X 15 mm petri plates. Growth was evaluated after 3 weeks and rated relative to the unamended control. In the second method, bits of mycelium (<1 mm in diameter) were taken from the mid-radium area of 2- to 3-week-old colonies and inoculated onto benomyl-amended and unamended PDA plates. Twenty-four isolates per petri plate were tested simultaneously at each concentration. Growth was evaluated after 3 weeks. Resistant isolates were retested 3-5 times to confirm that the level of resistance was stable from test to test. Using bits of mycelium as inoculum, all isolates were tested for growth on minimal medium to confirm the presence of nutritional deficiencies in the auxotrophic mutants and the prototrophy of field isolates.

Crosses. Matings were made according to the procedure of Keitt and Langford (7) except colony homogenates rather than spore suspensions were used as inoculum. Individual 2- to 3-week-old colonies were removed from their agar substrates and macerated in 2 ml sterile distilled water. To make the crosses, a 0.5 ml aliquot of homogenate of each member of the cross was added to a standard 100 X 15 mm petri plate, 30 ml of warm (42°C) modified PDA with apple leaf decoction was added, and the homogenate and agar were mixed by swirling the plate. Inoculated petri plates were incubated 7-10 days at 20°C in

an incubator until mycelial growth was evident on the surface of the agar, then sealed with Parafilm (American Can Co., Greenwich, CT 06830), inverted, and held at 8°C for 5-6 months as required for pseudothecia formation and ascospore maturation. All isolates used in crosses were also selfed to confirm self-incompatibility.

Testing of progeny. From each fertile pairing, 12-20 pseudothecia were removed from the agar substrate with the aid of a needle and a dissecting microscope and crushed in 1 ml of sterile distilled water using a 7 ml tissue grinder. Portions of the resultant ascospore suspension were plated on 2% water agar and incubated at about 21°C for 24-48 hours. Randomly chosen germinated ascospores were transferred to individual culture tubes containing modified PDA. After 2-3 weeks growth, the single-ascospore colonies were tested on PDA with 0, 1, 10, and 500 µg/ml benomyl, using bits of mycelium for inoculum as described earlier. Progenies from crosses with auxotrophic mutants were also tested on minimal medium to determine if they were prototrophic or auxotrophic.

RESULTS

Levels of benomyl resistance. Testing of isolates on benomyl-amended PDA resulted in the identification of four readily distinguishable groups based on the concentration of benomyl required to inhibit the growth of each isolate (Table 1). Isolates were considered sensitive (S) if they failed to grow on media amended with 0.1 µg/ml benomyl. This group included five isolates from Michigan and the 16 laboratory-induced mutants. Among the 59 resistant isolates, five grew on media amended with 1 µg/ml but not with 10 µg/ml benomyl, eight grew at 10 µg/ml but not at 25 µg/ml benomyl, and 46 showed good growth on media amended with up to 500 µg/ml benomyl. The three phenotypes were designated as having low resistance (LR), medium resistance (MS) and high resistance (HR), respectively.

Analysis of crosses. None of the isolates produced pseudothecia when selfed, confirming their self-incompatibility. All 212 ascospores from three crosses among five sensitive isolates were sensitive to 1 µg/ml benomyl (Table 2, Fig. 1A). To determine whether resistance was controlled by more than one gene, 4922 ascospores were examined from 25 crosses of 21 different resistant isolates with sensitive isolates. In all but one cross, resistant and sensitive progeny were found in a 1:1 ratio, and in each cross the level of resistance of the resistant progeny and the resistant parent always corresponded regardless of the level of resistance involved (Table 2, Fig. 1B). This indicates that in each of the LR, MR, and HR isolates tested, resistance was determined by a single Mendelian gene. In the cross KV3C X WRR (HR X S) segregation deviated from

Table 2. Segregation for benomyl resistance among ascospore progenies of Venturia inaequalis

Crosses[a]	Progeny tested (no.)					X^2 Values (1:1)[b]
		Phenotype				
	Total	HR	MR	LR	S	
S X S						
FS6C-12 X WM	110				110	
WB X WRR	20				20	
WM X WL	82				82	
HR X S						
RH-4 X WB	238	119			129	0.40
RH1-11B X WB	252	112			140	3.11
RH1-11B X WL	150	62			88	4.51
SH1-9B X WRR	248	110			138	3.16
I5-1 X WB	249	116			133	1.16
MINN FRUIT ACRES 7 X WB	250	118			132	0.78
I7-1 X WM	236	110			126	1.08
I7-1 X WRR	241	118			123	0.10
GERMANY G302 X WL	240	122			118	0.07

KV3C X WRR	243	154			89	17.39
KV3C X WM	248	138			110	3.16
CR2C X WL	292	160			132	2.68
CR2C X WB	214	114			100	0.92
MINN LAUTZ 4 X WB	245	127			118	0.33
75-150 X WRR	211	98			113	1.07
MR X S						
CHILE 24B X WRR	242		116		126	0.41
V-558 24B X WRR	242		116		115	1.53
SIS-16 X WRR	229		97		126	0.41
V-560 X WRR	94		45		49	0.17
V-565 X WRR	98		52		46	0.37
MAINE 8 X WRR	94		45		49	0.17
MAINE 2B X WRR	88		43		45	0.05
LR X S						
SPINKS 79 X W	96			52	44	0.67
#74 PRESTON 7 X WRR	103			59	44	2.18
#6 MITCHELL 94-1 X WB	99			44	55	1.22
HR X HR						
CR2C X Sh1-9B	250	250				

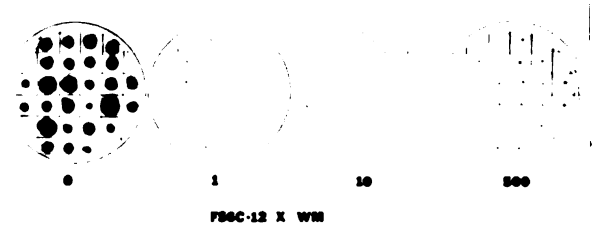
CR2C X I7-1	230	230			
CR2C X KV3C	253	253			
GERMANY G3-2 X KV3C	100	100			
CR2C X 75-150	88	88			
RH-4 X SH1-9B	214	214			
RH1-11B X SH1-9B	210	210			
I5-1 X SH1-9B	227	227			
SH1-9B X MINN FRUIT ACRES 7	237	237			
GERMANY G3-2 X SH1-9B	83	83			
SH1-9B X MINN LAUTZ 4	142	142			
LR X LR					
SPINKS 79 X #74 PRESTON 7	120			120	
#74 PRESTON 7 X #6 MITCHELL 94-1	77			77	
HR X MR					
CR2C X CHILE 24B	253	131	122		0.32
CR2C X CHILE 24B	253	131	122		0.32
CR2C X V-558	252	113	139		2.68
CR2C X SIS-16	20	10	10		0
CR2C X MAINE 6	225	110	115		0.11

HR X LR					
KV3C X #6 MITCHELL 94-1	103	58		45	1.64
CR2C X #74 PRESTON 7	96	49		47	0.04
MR X LR					
V-565 X #6 MITCHELL 94-1	161		88	73	1.40
V-560 X #42 MINNS 118	127		69	58	0.95
V-560 X #6 MITCHELL 94-1	99		56	43	1.71
CHILE 24B X #6 MITCHELL 94-1	135		59	76	2.14

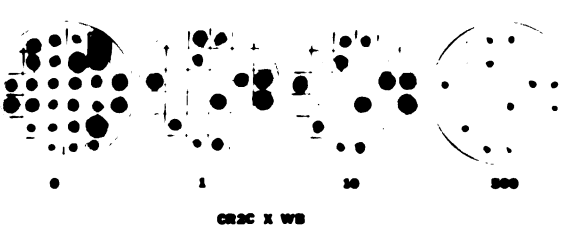
[a]Crosses are grouped according to parental phenotypes, i.e., S X S = sensitive X sensitive, HR X S = high resistance X sensitive, ...MR X LR = medium resistance X low resistance.

[b]Expected value at P=0.01 is 6.63.

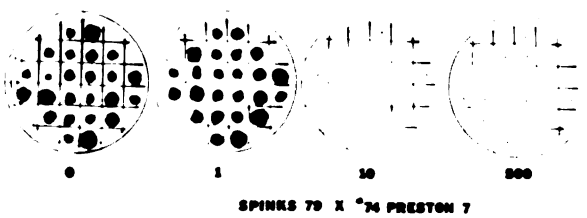
Figure 1. Progeny of Venturia inaequalis tested on potato dextrose agar amended with 0, 1, 10 and 500 µg/ml benomyl. Twenty-four progeny per plate were evaluated simultaneously at each concentration using bits of mycelium from single-ascospore colonies. Examples show growth 3 weeks after inoculation with progenies from four crosses; A) sensitive X sensitive, B) high resistance X sensitive, C) low resistance X low resistance, D) high resistance X medium resistance.


0
1
10
500
FSOC-12 X WH

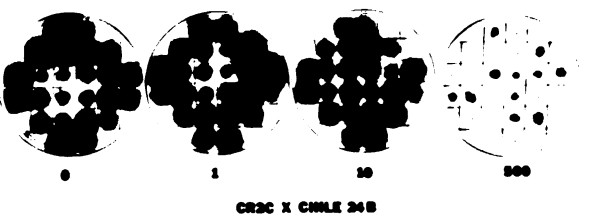
A


0
1
10
500
CR2C X WB

B


0
1
10
500
SPINKS 79 X °76 PRESTON 7

C


0
1
10
500
CR2C X CHILE 24B

D

a resistant:sensitive ratio of 1:1. However, no deviation from expected ratios was observed in other crosses involving KV3C.

From 23 crosses between 21 different resistant isolates, 3702 ascospores were examined to determine whether the mutations for benomyl resistance occurred at the same locus in all isolates. No sensitive progeny were detected in any cross between resistant parents (Table 2). When the parents had the same level of resistance, all of the progeny had a corresponding level of resistance (Fig. 1C), and when the parents had different levels of resistance, the same two levels were found in the progeny in a 1:1 ratio (Table 2, Fig. 1D). The absence of any sensitive recombinant or any recombinant showing higher levels of resistance than the parents indicates allelism at a single locus.

To investigate possible linkage of the gene for benomyl reistance with other genes, each of the 10 auxotrophic and six color mutants were crossed with each of the HR isolates of opposite mating type, KV3C and CR2C. Fertile pairings were obtained with seven mutants, but no indication of linkage between any genetic markers and the gene conferring benomyl resistance was found.

Each of the crosses of resistant isolates with the auxotrophic mutants U-2340 PYRIMIDINE-3, U-3010 PURINE-8, and U-1905 CHOLINE yielded the four phenotypes in approximately a ratio of 1:1:1:1, indicating an independent assortment of the markers (Table 3). Segregation of progeny of the cross with U-3069 ARGININE-3 deviated from a 1:1:1:1 ratio, but the recovery of similar numbers of progeny in the parental and recombinant classes indicates that the ben and arg-3 markers recombined freely.

With crosses involving the color mutants N-10 GREEN and N-222 YELLOW, progeny of parental and recombinant phenotypes were recovered in a ratio of 1:1:1:1 (Table 3). In a cross with N-156 WHITE-2, whitish pseudothecia were produced in abundance, but they were often void of asci or contained mostly

Table 3. Segregation for benomyl resistance and genetic markers among Venturia inaequalis progenies

Crosses	Progeny tested: Total number	Segregation by phenotype: Phenotypes		Number	X^2 Values (1:1:1:1:)*
KV3C X U-2340 PYRIMIDINE-3	159	+	ben	46	5.35
		pyr-3	+	48	
		+	+	32	
		pyr-3	ben	33	
KV3C X U-3010 PURINE-8	67	+	ben	20	10.43
		pur-8	+	10	
		+	+	26	
		pur-8	ben	11	
KV3C X U-1905 CHOLINE	237	+	ben	52	10.71
		cho	+	47	
		+	+	58	
		cho	ben	80	
KV3C X U-3069 ARGININE-3	198	+	ben	92	112.67
		arg-3	+	17	

		+	+	81	
		arg-3	ben	8	
CR2C X N-10 GREEN	254	+	ben	64	2.19
		gr	+	70	
		+	+	66	
		gr	ben	54	
CR2C X N-222 YELLOW	240	+	ben	73	5.40
		ye	+	55	
		+	+	49	
		ye	ben	63	
KV3C X N-156 WHITE-2	31	+	ben	17	31.58
		wh-2	+	0	
		+	+	14	
		wh-2	ben	0	

*Expected value at P = 0.01 is 11.3

four-spored asci. All ascospores were pale and thin-walled, and the majority failed to germinate. The two white colony phenotypic classes were absent, indicating that the wh-2 mutation was lethal to those progeny which inherited it. Progeny of the other two phenotypic classes were recovered in similar numbers indicating free recombination.

DISCUSSION

When 59 benomyl-resistant isolates of V. inaequalis from Australia, Chile, Germany, Italy, New Zealand, and five states in the United States were screened for variation in the level of benomyl resistance, three phenotypes were distinguished. The three phenotypes correspond well to those described by Katan et al. (6) in a study of V. inaequalis from Israel and New York, except HR isolates were not subdivided into an additional very highly resistant phenotype based on growth rate.

The predominance of HR isolates among the isolates screened for resistance may be a consequence of the technique used in collecting the primary isolates and therefore not accurately reflect the distribution or prevalence of the LR and MR strains in natural populations. For example, media containing 5 or 25 µg/ml benomyl were commonly used to monitor for resistant strains (4,21). At 25 µg/ml benomyl the LR and MR strains would not be detected and at 5 µg/ml benomyl the LR strains would not be detected.

Fifty-one crosses involving 23 different resistant isolates were analyzed. The results show that discrete levels of resistance are inherited according to Mendelian rules. In crosses between resistant and sensitive isolates or between isolates having different levels of resistance, only parental phenotypes in a ratio of 1:1 were recovered among the progeny. In several crosses using the same resistant isolate with a different sensitive partner, the expression of the gene for resistance was not altered by the genetic background of the sensitive parent. Crosses between resistant isolates having the same level of resistance produced

only resistant progeny of the same phenotype. It is concluded that the three levels of benomyl resistance are determined by three alleles of a single Mendelian gene.

Benzimidazole-resistant and sensitive isolates of V. inaequalis are indistinguishable from each other in culture except on media amended with benzimidazole fungicides. Kiebacher and Hoffmann (9,10) attempted to correlate traits such as colony morphology, mycelial coloration, sporulation, and mating-type with resistance, but the examination of progeny from crosses between resistant and sensitive isolates differing in these traits provided no evidence for their linkage with benomyl resistance. Martin et al. (12) isolated a benomyl-resistant white mutant following UV-irradiation of a benomyl-resistant isolate. Genetic analysis of this mutant showed that the traits of white mycelium and benomyl resistance segregated independently. Similarly, Shabi et al. (17) found no evidence of linkage of the gene for benomyl resistance and a gene for green colony color.

Sixteen laboratory-induced mutants were utilized in an attempt to localize the gene for benomyl resistance relative to other genetic markers. Failure to obtain fertile pairings with nine of the mutants is attributed to physiological disorders of these mutants, since the HR isolates with which they were crossed were suitable partners in many other matings. Analysis of progenies of crosses between resistant isolates with four auxotrophic and three colony color mutants, did not reveal linkage between any of the genetic markers and the gene for benomyl resistance. Progenies of crosses with U-3069 ARGININE-3 and N-156 WHITE-2 failed to show the 1:1:1:1 phenotypic ratios, but parental and recombinant phenotypes were found in similar numbers indicating free recombination between markers. The two aberrant phenotypic ratios can be related to the abortion of ascospores carrying the mutant alleles.

Ascospore abortion with mutant N-156 WHITE-2 has been reported previously (22).

Genetic analysis of a large number of isolates from a wide geographical range fully corroborate the recent finding (6,17) that different levels of benomyl resistance in V. inaequalis are controlled by different alleles of the same gene and are not the result of interactions between different genes for resistance.

In a comparison of two V. inaequalis isolates, one showing low benomyl resistance and the second with an isolate showing high benomyl resistance, Shabi and Gilpatrick (16) reported that the two isolates did not differ in pathogenicity and that both caused typical scab lesions on benomyl-sprayed apple seedlings. Consequently, in contributing to the failure of benzimidazole fungicides to control apple scab, the level of resistance may be less important than the fitness of the particular resistant strain involved.

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PART II

REDUCED SENSITIVITY TO STEROL-INHIBITING FUNGICIDES IN FIELD ISOLATES OF *VENTURIA INAEQUALIS*

ABSTRACT

For eight sterol-inhibiting fungicides, minimal inhibitory concentrations for preventing colony formation by individual conidia were 4 to 8 times higher for Venturia inaequalis isolates from one West German orchard than for isolates from a second West German orchard and from orchards in the United States. Reduced sensitivity was exhibited to BAS 454 06 F (1-(2,4-dichlorophenyl(-2-(1H-1,2,4-triazol-1-yl)), bitertanol, CGA 71818 (1-[2-(2,4-dichlorophenyl)-pentyl] -1H-1,2,4-triazole), DPX H6573 (bis (4-fluorophenyl)methyl-(1H-1,2,4-triazol-1-yl-methyl)silane), etaconazole, fenarimol, Ro 15-1297, and triflumizole. Isolates with reduced sensitivity to sterol inhibitors did not show increased sensitivity to dodine. Dodine, sodium lauryl sulfate, and the respiratory inhibitor carbonyl cyanide 3-chlorophenylhydrazone did not potentiate fenarimol toxicity toward either sensitive isolates or isolates with reduced sensitivity. Genetic analysis of nine isolates with reduced sensitivity, involving 3261 ascospores from 15 crosses, indicates that reduced sensitivity is determined by a single Mendelian gene.

INTRODUCTION

Several sterol-inhibiting fungicides are now available for agricultural use, and a number of others are being developed. These fungicides are a chemically diverse group of compounds which specifically inhibit ergosterol biosynthesis (12,16). They are effective at very low concentrations against a broad spectrum of higher fungi, and almost all exhibit some degree of systemicity. Sterol-inhibiting fungicides have proven useful in the control of several important apple diseases (13). With excellent postinfection activity, these compounds offer much needed alternatives to dodine and to the benzimidazole fungicides whose use has declined following the development of resistance in Venturia inaequalis (Cooke) Wint., the cause of apple scab. Due to their favorable characteristics, sterol-inhibiting fungicides will probably be used extensively on apple in the near future.

The specific mode of action common to the sterol inhibitors suggests the potential for problems of resistance under conditions of intense field use (3-5). Under laboratory conditions, mutants resistant to sterol-inhibiting fungicides are readily induced and cross-resistance often occurs (1,6,8,14,15). Fenarimol resistance in the non-pathogen Aspergillus nidulans (Eidam) Wint. was reported to be negatively correlated with dodine resistance (7). If this same relationship were to occur with pathogens in the field, there would be the possibility of using mixtures of alternative applications of dodine to combat the problem of resistance.

Reduced sensitivity to tridemorph has been reported in strains of Erysiphe graminis f. sp. hordei collected from the greenhouse and field (18), but as yet, there is no report of reduced sensitivity to sterol-inhibiting fungicides in natural strains of V. inaequalis. Recently, isolates of V. inaequalis were obtained from the Federal Republic of Germany which formed sporulating colonies on media amended with sterol-inhibiting fungicides, while isolates from other sources failed to grow at similar fungicide concentrations. This study was conducted to determine if the differential response of these isolates was stable in culture and extended across a range of sterol-inhibiting fungicides. Also, it was determined whether this reduced sensitivity to the sterol-inhibiting fungicides was inherited by Mendelian rules.

MATERIALS AND METHODS

Cultures. Twelve monoconidial isolates of V. inaequalis, taken from separate leaf lesions, were obtained from the Federal Republic of Germany. Nine of the isolates, designated B1...B9, were collected from one apple orchard, and three isolates, designated W10...W12, were from a second orchard. Sterol-inhibiting fungicides had been used experimentally in both orchards. Among four monoconidial isolates from Michigan, WB and WRR were opposite mating types and GAVIN 29 and GAVIN 32 were dodine-resistant. The Michigan isolates were collected from orchards where no sterol-inhibiting fungicides had been used.

Isolates were grown at 18°C on modified potato-dextrose agar (modified PDA: 40 g potatoes steamed 45 min in 200 ml distilled water and then homogenized, 17 g agar, 5 g dextrose, and distilled water to make a final volume of 1 L) (2). At 4-6 wk intervals single conidia were subcultured.

Fungicides and chemicals. The fungicides used in this study were: 1-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-yl) (BAS 454 06 F 25% EC) from BASF Wyandotte Corp., Parsippany, NJ 07054; bitertanol (Baycor 25% WP) from Bayer AG, Leverkusen, Federal Republic of Germany; 1-[2-(2,4-dichlorophenyl)-pentyl]-1H-1,2,4-triazole (CGA 71818 10% WP) and etaconzaole (Vangard 10% WP) from Ciba-Geigy Corp., Greensboro, NC 27409; dodine (100% technical grade) from American Cyanamid Co., Princeton, NJ 08540; bis(4-fluorophenyl)-methyl(1H-1,2-4-triazol-1-ylmethyl)silane (DPX H6573 40% EC) from E. I. duPont de Nemours & Co., Inc., Wilmington, DE 19898; fenarimol (Rubigan 12.5%

EC) from Eli Lilly and Co., Greenfield, IN 46140; Ro 15-1297 (48% EC) from Maag Agrochemicals, Vero Beach, FL 32960; triflumizole (A-815 30% WP) from Uniroyal, Naugatuck, CT 06770; and triforine (Funginex 18.2% EC) from EM Laboratories, Hawthorne, NY 10532. Sodium lauryl sulfate (SLS) and carabonyl cyanide 3-chlorophenylhydrazone (CCCP) were purchased from Sigma Chemical Co., St. Louis, MO 63178.

Minimal inhibitory concentrations. Minimal fungicide concentrations needed to inhibit colony formation were determined by plating conidia on 2% water agar and after 12 to 24 hours transferring individual germinated conidia to 100 X 15 mm petri plates containing PDA (Difco Laboratories Inc., Detroit, MI 48201) amended with a series of fungicide concentrations. Concentrations ranged from 0 to 5 µg/ml for all fungicides except triforine which was in the range of 20 to 44 µg/ml. Using 95% ethanol to assist in dissolution, fungicides were added to molten PDA (42°C) after sterilization. The final ethanol concentration never exceeded 1% in either treatment or control. Up to 12 isolates were tested simultaneously in each petri plate (Fig. 1). Colony growth was evaluated after 3 weeks incubation at 20-22°C.

Dodine sensitivity. Response to dodine was tested using two methods. In the first method, conidia were germinated on 2% water agar and transferred individually to dodine amended PDA. Dodine concentrations ranging from 0 to 1.0 µg/ml in 0.1 µg/ml increments were prepared by adding dodine in ethanolic solution to molten PDA (42°C) after sterilization. The concentration of ethanol never exceeded 1% in either treatment or control. Dodine resistant isolates GAVIN 29 and GAVIN 32 were included for comparison. Colony growth was evaluated after 3 weeks. In the second method, isolates were compared for inhibition of growth around assay discs treated with 0, 50, and 300 µg/ml dodine. Sterile 13 mm assay discs (Schleicher and Schuell, Inc., Keene, NH 03431) were

saturated with 100 µl of distilled water or dodine solution and allowed to dry. For each isolate tested, a 2- to 3-week-old colony was excised from its agar substrate and homogenized in 4 ml of sterile distilled water using a 7 ml tissue grinder. Aliquots (0.5 ml) of homogenate were used to seed the surface of PDA in each of three 100 X 15 mm petri plates, and a treated disc was placed in the center of each plate with light pressure to ensure contact with the agar. Diameters of zones of inhibition were measured after 25 days incubation at 20-22°C.

Interaction of fenarimol and other chemicals. To investigate the effect of certain chemicals on fenarimol toxicity, the crossed paper strip method was used (7). Filter paper strips (1 cm wide) were dipped into a methanolic solution of 1487 µg/ml fenarimol and then allowed to dry. Similarly, strips were prepared with 14,300 µg/ml dodine, 57,600 µg/ml SLS, and 307 µg/ml CCCP. Strips were transferred to the surface of PDA plates previously seeded with 0.5 ml aliquots of colony homogenate and incubated 3 days at 20-22 C. In each plate, a fenarimol treated strip was crossed with one of the other chemically treated strips. After an additional 14 days incubation, zones of inhibition about the strips were measured near the edge of the petri plate and near the center where the strips crossed.

Crosses. Isolates were crossed using the procedure of Keitt and Langford (9), except colony homogenates rather than spore suspensions were used as inoculum. Individual 2- to 3-week-old colonies were removed from their agar substrates and homogenized in 2 ml of sterile distilled water using a 7 ml tissue grinder. A 0.5 ml aliquot of homogenate of each member of the cross was added to a 100 x 15 mm petri plate, 30 ml of warm (42°C) modified PDA with apple leaf decoction (9) was added, and the homogenate and agar were mixed by swirling the plate. The plates were incubated 7-10 days at 18°C until mycelial

growth was evident on the surface of the agar, then sealed with Parafilm (American Can Co., Greenwich, CT 06830), inverted, and held at 8°C for 5-6 months as required for pseudothecia formation and ascospore maturation. All isolates used in crosses were also selfed to confirm self-incompatibility.

Testing of progenies. For each fertile pairing, 12-20 pseudothecia were removed from the agar substrate with the aid of a needle and a dissecting microscope and crushed in 1 ml of sterile distilled water. Portions of the resultant ascospore suspension were plated on 2% water agar and incubated at 20-22°C for 12-24 hours. Randomly chosen germinated ascospores were transferred to individual culture tubes containing modified PDA. After 2-3 weeks growth, bits of mycelium (< 1 mm in diameter) were taken from the mid-radius area of the colonies and inoculated onto PDA and PDA amended with 1.5 µg/ml fenarimol. Growth was evaluated after 3 weeks incubation at 20-22°C. Selected single-ascospore colonies were also tested for sensitivity to other sterol-inhibiting fungicides, for sensitivity to dodine, and for their response to fenarimol in the presence of dodine, SLS, and CCCP.

RESULTS

Minimal inhibitory concentrations. Although colony size of all isolates tested diminished with increasing fungicide concentration, isolates B1...B9 grew at higher concentrations of each sterol inhibitor except triforine than did isolates W10, W11, W12, WB, WRR, GAVIN 29, and GAVIN 32. Single-ascospore isolates derived from crosses between sensitive and less sensitive isolates behaved either like their sensitive or less sensitive parent. Within the less sensitive and sensitive groups, the response of difference members to different concentrations of each sterol inhibitor except triforine was uniform (Fig. 1, Table 2). In an amendment series with 32, 36, 40, and 44 µg/ml triforine, fewer and fewer colonies were produced, but differences in growth between less sensitive and sensitive isolates were inconsistent in four repetitions of the test.

Concentrations for differentiating those isolates with reduced sensitivity were selected for each sterol inhibitor except triforine (Fig. 1, Table 1). At these concentrations, sensitive isolates fail to grow, while less sensitive isolates form colonies. Except on Ro 15-1297 amended media, sporulation of colonies was observed in 3-4 weeks.

Response to dodine. When germinated conidia were transferred to dodine amended PDA, growth of isolates with reduced sensitivity to sterol inhibitors did not differ from the growth of sensitive isolates. Both groups showed the same diminution in colony size with increasing dodine concentration and cessation of growth at 0.2-0.3 µg/ml dodine. The dodine-resistant isolates, GAVIN 29 and GAVIN 32, grew at 0.5 and 0.7 µg/ml dodine but not 1 µg/ml.

Table 1. Minimal fungicide concentrations for preventing colony formation by germinated conidia of Venturia inaequalis with two levels of sensitivity to sterol-inhibiting fungicides. Suggested discriminatory concentrations inhibit colony formation by conidia from sensitive isolates but not by conidia from isolates with reduced sensitivity to sterol-inhibiting fungicides

Fungicides	Minimal inhibitory concentration (µg/ml)[a]		Suggested discriminatory concentration (µg/ml)
	Sensitive isolates	Isolates with reduced sensitivity	
BAS 454 06 F	0.4	2.0	0.9-1.0
bitertanol	1.0	5.0	0.5-1.0
CGA 71818	0.2	0.8	0.3-0.4
etaconazole	0.1	0.8	0.2
DPX H6573	0.1	0.6	0.2
fenarimol	0.4	3.0	0.5-1.0
Ro 15-1297	0.1	0.4	0.2
triflumizole	0.2	1.0	0.3-0.4
triforine	> 32.0	> 32.0	----

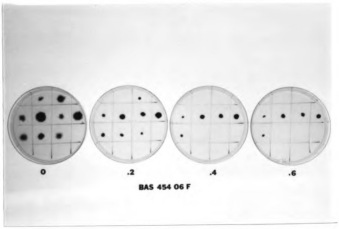
[a]Data are the results of numerous tests with field isolates that exhibited reduced sensitivity to these fungicides and with sensitive field isolates, as well as with single-ascospores isolates derived from crosses between sensitive and less sensitive strains.

Table 2. Inhibition of mycelial growth surrounding assay discs treated with two concentrations of dodine and placed on agar seeded with sensitive isolates of Venturia inaequalis or with isolates that exhibited reduced sensitivity to sterol-inhibiting fungicides

	Inhibition zone (mm)[a]			
	50 µg/ml dodine		300 µg/ml dodine	
Cross	Sensitive isolates	Less sensitive isolates	Sensitive isolates	Less sensitive isolates
B4 X WRR	21.0 (±4.6)	17.4 (±3.1)	31.8 (±4.4)	28.6 (±3.8)
B5 X WB	18.2 (±1.8)	15.0 (±1.0)	32.0 (±1.0)	23.6 (±3.3)
B6 X WRR	18.6 (±2.5)	15.0 (±1.9)	29.2 (±3.8)	24.2 (±4.8)
B7 X WRR	14.4 (±1.7)	14.4 (±1.5)	23.2 (±5.8)	23.0 (±2.0)
B8 X WRR	17.4 (±4.0)	15.2 (±3.0)	27.2 (±4.5)	25.4 (±2.9)
B9 X WRR	22.0 (±2.9)	18.0 (±3.3)	36.8 (±7.3)	27.8 (±3.8)

[a]Mean diameters and standard deviations are given for zones of inhibition of five sensitive isolates and five less sensitive isolates from each cross. Values include 13 mm diameter of assay disc.

Figure 1. Response of Venturia inaequalis tested on potato dextrose agar amended with 0, 0.2, 0.4, and 0.6 @g/ml BAS 454 06 F. Five isolates with reduced sensitivity to BAS 454 06 F and four sensitive isolates were tested simultaneously at each concentration. Three weeks after inoculation with single germinated conidia, isolates with reduced sensitivity but not sensitive isolates showed growth at the two higher concentrations.


0
.2
.4
.6
BAS 454 06 F

In tests with assay discs, no zones of inhibition developed around discs treated with water. Around discs treated with solutions containing dodine, growth was inhibited. At both concentrations of dodine, zones of inhibition were slightly less for the isolates with reduced sensitivity to the sterol inhibitors than for sensitive isolates (Table 2). However, according to the t test, differences in mean diameters of zones of inhibition for the two groups were not significantly different at $P = 0.05$.

Effect of chemicals on fenarimol toxicity. Tests were conducted with sensitive isolates, less sensitive isolates, and single-ascospore isolates derived from crosses between sensitive and less sensitive isolates. Zones of inhibition were observed around all strips except the water treated controls (Fig. 2). Around fenarimol treated strips, zones of inhibition were less distinct but wider than zones around dodine, SLS, and CCCP treated strips. Less sensitive isolates were inhibited less than sensitive isolates around fenarimol treated strips. Around dodine, SLS, and CCCP treated strips, no significant differences in the relative widths of zones of inhibition for sensitive and less sensitive isolates were observed. The three test chemicals did not alter the toxicity of fenarimol toward either sensitive or less sensitive isolates.

Analysis of crosses. None of the isolates produced pseudothecia when selfed, thereby confirming their self-incompatibility. Single-ascospore isolates derived from fertile pairings were readily separated into two groups based on their growth on PDA containing 1.5 µg/ml fenarimol. At this concentration, sensitive isolates failed to grow, while less sensitive isolates formed colonies. When crosses were made between sensitive isolates, isolates W10, W11, W12 were fertile with WRR, indicating that WB, W10, W11 and W12 were of the same mating-type (Table 3). All 361 single-ascospore isolates from the three crosses among four sensitive isolates were sensitive.

Table 3. Segregation of single-ascospore isolates of Venturia inaequalis into less sensitive and sensitive phenotypes according to growth on potato-dextrose agar amended with 1.5 µg/ml fenarimol

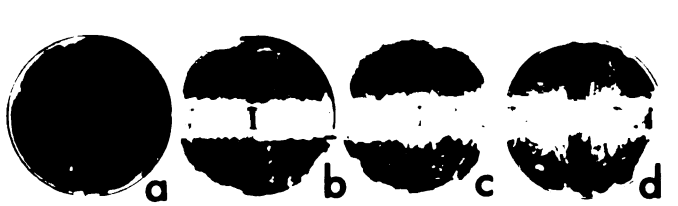
Cross	Progeny tested (no.)			X^2 Values (1:1)[a]
		Phenotype		
	Total	Less sensitive	Sensitive	
Sensitive isolates X Sensitive isolates				
W10 X WRR	90	0	90	
W11 X WRR	99	0	99	
W12 X WRR	172	0	172	
Totals	361	0	361	
Less sensitive isolates X Sensitive isolates				
B1 X WB	252	142	110	4.06
B2 X WB	228	97	131	5.07
B3 X WRR	96	43	53	1.04
B4 X WRR	250	116	134	1.30
B5 X WB	96	68	28	16.70
B6 X WRR	230	97	133	5.63
B7 X WRR	224	124	100	2.57
B8 X WRR	244	103	141	5.92
B9 X WRR	247	136	111	2.53
Totals	1867	918	949	0.12

Table 3. (cont.)

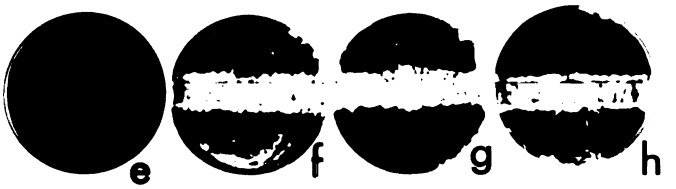
Less sensitive isolates X Less sensitive isolates			
B1 X B3	229	229	0
B1 X B4	246	246	0
B2 X B3	240	240	0
B2 X B4	246	246	0
B3 X B5	248	248	0
B4 X B5	185	185	0
Totals	1394	1394	0

[a]The expected Chi-square value at the 1% level of significance is 6.63.

Figure 2. Effects of dodine, sodium lauryl sulfate (SLS), and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) on the toxicity of fenarimol to Venturia inaequalis. Plates a-d were seeded with an isolate sensitive to sterol-inhibiting fungicides, while plates e-h were seeded with an isolate exhibiting reduced sensitivity to sterol inhibitors. Vertical strips were treated with fenarimol and horizontal strips a,e with distilled water; b,f with dodine; c,g with SLS; and d,h with CCCP. Inhibition zones around fenarimol treated strips were characterized by a lack of sporulation rather than a complete inhibition of growth. The lack of sporulation is not visible in the photographs although it was clearly visible in the original plates.



WRR



BS

To determine whether reduced sensitivity was controlled by more than one gene, 1867 single-ascospore isolates were tested from nine crosses between sensitive and less sensitive isolates (Table 3). Isolates B1, B2, and B5 were compatible with WB, and isolates B3, B4, B6, B7, B8, and B9 were compatible with WRR. In all but one cross, sensitive progeny were found in a 1:1 ratio with less sensitive progeny. The only ratio deviating from the 1:1 ratio was that of cross B5 x WB.

To determine whether the mutations for reduced sensitive to sterol-inhibiting fungicides occurred at the same locus, crosses between less sensitive isolates were examined (Table 3). A total of 1394 single-ascospore isolates were tested from six compatible pairings resulting from all possible pairings of isolates B1...B5. No sensitive progeny were detected. From the analysis of crosses between sensitive and less sensitive isolates and between the less sensitive isolates it was concluded that in the isolates studied, reduced sensitivity to the sterol-inhibition fungicides was determined by a single Mendelian gene. The gene for reduced sensitivity was not linked to mating type.

DISCUSSION

The concentration of sterol-inhibiting fungicides necessary to inhibit the growth of the West German V. inaequalis isolates B1...B9 ranged from 4 to 8 times that necessary to inhibit the growth of V. inaequalis isolates from a second West German orchard and from orchards in the United States. This differential response in sensitivity was not lost or changed in culture. The level of variation between less sensitive and sensitive isolates was comparatively small, and in this respect much like the difference between dodine-resistant and sensitive V. inaequalis (11) and unlike some benomyl-resistant V. inaequalis which grow at several hundred times the benomyl concentration which inhibits sensitive isolates (10). The variation in the sensitivity of V. inaequalis to the sterol inhibiting fungicides indicates a potential for the selection of less sensitive strains under orchard conditions. Using the suggested discriminatory concentrations given in Table 1, it should be possible to monitor conidial populations for shifts in sensitivity for establishing the incidence of strains with reduced sensitivity.

The inconsistent behavior of V. inaequalis on triforine amended media was manifested in two ways. Firstly, while the other eight sterol inhibitors prevented colony formation at concentrations of 5 µg/ml or less, concentrations in excess of 32 µg/ml were needed to prevent colony formation with triforine. Secondly, sensitive isolates could not be differentiated from less sensitive isolates in an amendment series. Despite evidence that triforine produces the accumulation of the same sterol intermediates as a number of other sterol inhibitors including fenarimol (17) and reports of cross-resistance between

triforine and other sterol inhibitors (8, 15, 16), isolates B1...B9 for some unexplained reason did not exhibit a reduced sensitivity to triforine.

De Waard and van Nistelrooy (7) reported that two laboratory-induced fenarimol-resistant mutants of Aspergillus nidulans were slightly more sensitive to dodine than a wild-type fenarimol-sensitive strain of the fungus. In our studies, strains of V. inaequalis with reduced sensitivity to fenarimol and sensitive strains were equally sensitive to dodine, and no correlation between reduced sensitivity to sterol inhibitors and increased dodine sensitivity was observed. This suggests that with V. inaequalis dodine may not provide any particular benefit in combating population shifts toward reduced sensitivity to sterol inhibitors. Also, dodine, SLS, and CCCP potentiated the toxicity of fenarimol toward both sensitive wild-type and fenarimol-resistant mutants of A. nidulans (7). However, in our studies no similar potentiation of fenarimol toxicity was observed toward sensitive isolates or toward isolates with reduced sensitivity to fenarimol. The differences between the results with V. inaequalis and A. nidulans may be explained in part by the fact that reduced sensitivity in V. inaequalis was controlled by a single gene, while a multigenic system existed for A. nidulans (17).

It is still too early to make unequivocal statements concerning the significance of reduced sensitivity to sterol inhibitors in V. inaequalis. As has been pointed out (4), an outbreak of disease depends on the overall fitness of a pathogen. However, the isolates with reduced sensitivity were obtained from infected apple leaves and thus were pathogenic. Since reduced sensitivity was controlled by a single gene, the potential for a buildup of these strains in the presence of prolonged exposure to sterol-inhibiting fungicides is greater than if several genes were required (3-5). In culture, these isolates grew and sporulated well, which suggests they may have sufficient fitness for survival among

populations containing sensitive strains. Based on current data, it seems likely that if sterol-inhibiting fungicides are used exclusively in apple scab control programs, there will be a shift in the sensitivity of the pathogen population. A possible method for proventing this shift in sensitivity is to use field rates for these fungicides that are sufficiently high to control both sensitive and less sensitive strains.

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