INHERITANCE OF RESISTANCE TO BENOMYL IN VENTURIA INAEQUALIS

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ABSTRACT

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The ineffectiveness of benomyl (methyl-1-butylcarbamoyl -2-benzimidazole carbamate) as a systemic fungicide to control Venturia inaequalis in many apple orchards is attributed to a build-up of resistant strains in the pathogen population. To gain insight into the genetic mechanisms involved in benomyl resistance, isolates obtained from apple orchards with a history of benomyl resistance were crossed with artificially induced biochemical marker strains. Meiotic analysis of random ascospore progeny revealed the activity of at least two independent loci coding for resistance. The loci have been assigned to two linkage groups provided by D. Boone, University of Wisconsin. Ben5, conferring resistance at 500 ug/ml of benomyl was mapped 38.9 units from nic-1 on group I. Ben1, located on linkage group VIII was mapped 36.3 units from pur-6 and 38.8 units from bio-1. Forty percent of the progeny obtained from a cross between ben1 and ben5 conferred a higher resistance level than either parental, indicating additive effects in progeny containing both loci for resistance.

TO MY FATHER

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INTRODUCTION

The development of the systemic benzimidazole fungicides in the late 1960s provided an opportunity to control a wide range of phytopathogenic fungi. These compounds provide post-infection control through local systemic action and often reduce the number of applications needed per season. Although they are broad spectrum fungicides, they act as specific-site inhibitors, unlike the multi-site inhibitors available before their introduction.

Carbendazim (methyl benzimidazol-2-yl carbamate), the fungitoxic derivative of benomyl, was demonstrated by Hammerschlag and Sisler (15) to form complexes with tubulin, the primary protein necessary for microtubule formation. Inhibition of microtubule formation, resulting in mitotic disruptions, was subsequently demonstrated in a number of test organisms (7, 9, 15, 29). Davidse (10) proposed a correlation between the level of sensitivity expressed by the target organism and the differential binding activity between carbendazim and tubulin. By virtue of this specific mode of action, naturally occurring resistant strains of the target organisms were detected after only three years of application in the field (16).

Venturia inaequalis (Cke.) Wint., the causal organism of apple scab, has a high propensity for developing resistance to fungicides. Two extensively used fungicides, benomyl and dodine, can no longer be used in many areas of the United States because of the development of resistant strains of the pathogen. Resistance to dodine, a selective protective fungicide, was first reported in 1969 by Szkolnik and Gilpatrick (35) in New York orchards, only ten years after its introduction. Polach (27, 28) demonstrated the presence of at least two genes operating in naturally occurring dodine resistant strains of V. inaequalis. The genes conferred different levels of resistance and were additive. Modifier or secondary genes were also suggested to be operating.

Benomyl resistance, unlike isolated incidences of dodine resistance, has been well documented in most major apple producing regions of the world (16, 17, 24, 37). Wicks (39) has observed a common occurrence of cross resistance to other benzimidazole fungicides. The resistance of V. inaequalis to low concentrations of benomyl have been reported to result from the mutation at a single gene (16). However, the involvement of more than one gene at higher concentrations cannot be ruled out.

Since the use of dodine and benzimidazole fungicides have been discontinued in many apple orchards, it would be fortuitous to obtain a better understanding of the genetic mechanisms involved before alternative fungicides

are introduced. Although the new ergosterol biosynthesis inhibiting fungicides are themselves structurally unrelated, they have a similar chemical mode of action. If resistance results to one compound, then strains will be resistant to the other fungicides as well. It is apparent that organisms are capable of evolving to fit their environment; without understanding the mechanisms of this process, predictions of resistance build-up in a pathogen population cannot be made. Finally, the development of new techniques to study these mechanisms are necessary to quickly detect future problems. Therefore, economic considerations should be made not only to growers due to crop loss and companies developing new fungicides, but importantly the possibility of compounding already existing resistance problems.

The objective of this study was to determine and locate the loci involved in the inheritance of benomyl resistance in naturally occurring resistant strains of *V. inaequalis*.

LITERATURE REVIEW

Systemic benzimidazole fungicides benomyl, thiabendazole, carbendazim, and thiophanate-methyl have been used extensively to control many genera of fungal pathogens inciting disease on a wide range of agronomic crops. The virtue of these compounds, compared to protective fungicides, included the ability to enter plant cells to provide post-infection control and a reduction in the number of applications per season, necessary to obtain control.

Fungitoxic activity of benomyl was demonstrated by Hammerschlag and Sisler to cause mitotic interference following its breakdown into the toxic derivative methyl benzimidazol-2-yl carbamate (MBC) (15). Carbendazim (MBC) operates as a single-site inhibitor by binding to fungal tubulin, the primary protein involved in microtubule formation (10). Richmond and Phillips (29) have demonstrated antimitotic activity of benomyl in hyphal cells of Botrytis cinerea and in root tips of onions (Allium cepa). Inhibition of microtubule formation resulting in mitotic and other subcellular disruptions have been described in Ustilago maydis, Saccharomyces cerevisiae (15) and Aspergillus nidulans (9). Davidse (10) has suggested that

the differential affinity of fungal tubulin for carbendazim may have a direct correlation to the levels of sensitivity expressed by the target organisms in the presence of the toxin. Resistant strains of A. nidulans were found by Davidse (10) to have lower binding activity than sensitive strains. Van Tuyl (38) demonstrated the mutation in benomyl resistant strains of A. nidulans was controlled by one gene, B-tubulin, and that carbendazim resistance was attributed to altered B-tubulin, resulting in lowered binding activity.

Due to the high specificity of the benzimidazole fungicides, in disrupting a single or few metabolic processes in the target organisms, the development of resistant strains would result from a mutation involving a single or few genes. This mechanism exists in a number of organisms subjected to benzimidazole fungicides in vitro (38).

Development of naturally resistant strains of many phytopathogenic fungi to benomyl have been well documented since its introduction in the late 1960s (12, 17, 26, 30, 31). The economic impact of the breakdown in benomyl control in major agricultural areas stimulated research into the genetic mechanisms involved. Organisms such as A. nidulans, Neurospora crassa, Cladosporium cucumerinum, U. maydis, Hypomyces solani and V. inaequalis, whose genetic mechanisms have been well documented, afforded ideal cases for studying the inheritance of benomyl resistance. However,

only certain of these are of economic importance. Artificial induction of resistant mutants has been performed in vitro using ultraviolet radiation and yielded from 1.2 to 100 per 10^7 survivors (38).

Genetic analysis of UV induced mutants of A. nidulans by Hastie and Georgopoulos (14) revealed the activity of two independent genes, ben A, resulting in a high level of resistance to benomyl and ben B, imparting a low level of resistance. Resistance in strains containing both genes appeared to be additive. In 1975, Van Tuyl (38) confirmed these findings and identified an additional gene, ben C, which conferred a low level of resistance. Cross resistance to thiabendazole was also observed in certain benomyl resistant strains by a single resistant gene. Georgopoulos (13) postulated that the level of resistance to benzimidazole compounds is determined by which of the three types of tubulin is being coded for at the ben A locus.

Results of crosses with five resistant strains of U. maydis, in which no recombinants were observed, indicated benomyl resistance to be determined by one gene. Thiabendazole resistant strains exhibiting increased benomyl sensitivity also appeared to be controlled by a single gene (38). Similar results have been obtained by Broch and Braymer (6) and Ben-Yephet et al. (2) with N. crassa and Ustilago hordei, where the mutation of a single gene is responsible for benomyl resistance.

Venturia inaequalis, the causal organism of apple scab, is an example of the development of resistance to two extensively used fungicides, dodine and benomyl. In 1969, Szkolnik and Gilpatrick (34, 35) reported the development of resistance in New York orchards after only ten years of dodine usage. Dodine (n-dodecylguanidine acetate), acts by altering the permeability of the protoplast membrane (8, 36). Genetic inheritance or resistance to dodine was observed by Kappas and Georgopoulos (19) in Nectria haematococca f. cucurbitae to involve four independent genes, giving additive effects when recombined. They also proposed the presence of modifying genes. In 1973, Polach (27, 28) demonstrated the presence of at least two genes, each conferring different levels of resistance, in naturally occurring dodine resistant strains of V. inaequalis collected in New York state. The possibility modifier genes were also operating was suggested.

Unlike dodine resistance, which is restricted to the northeast United States and southeastern Canada, benomyl resistant strains of V. inaequalis have developed in many areas of the world (16, 17, 24, 32, 37, 39). Benomyl was used extensively to control V. inaequalis in major fruit growing areas in the United States until the development of resistant strains in 1975. Cross resistance in V. inaequalis to the benzimidazole fungicides, thiophanate-methyl and carbendazim is common (35, 39). Benzimidazole resistant

strains were found to tolerate 1000 times the minimum concentration needed to control sensitive strains (16). The resistance of V. inaequalis to benomyl was reported to involve a single gene at 25 ug/ml (16). However, because the progeny were only tested at one concentration of benomyl, other loci may be functioning at higher or lower concentrations. Crosses between naturally resistant isolates and artificially induced biochemical markers would enable the number of loci involved in the inheritance of benomyl resistance to be determined and located.

The genetics of V. inaequalis have been well documented and this fungus provides a useful tool for the study of benomyl resistance (1, 11, 18, 21). The sexual stage can be obtained in vitro and the eight haploid ascospores isolated in order of their position in the ascus. By virtue of the haploid state throughout its life cycle, inherited traits are expressed immediately without the problem of dominance found in other diploid or dicaryotic organisms. Biochemical, morphological, and color mutants are readily induced by treatment with ultraviolet radiation or nitrogen mustard (3, 4, 5, 20, 23, 25, 33). Biochemical mutants were induced by treating conidia with either nitrogen mustard methyl-bis-(B-chloroethyl) amine or ultraviolet radiation and required amino acids, nitrogen bases or vitamins. The deficiency of each mutant was controlled by a single gene (4, 5, 22, 23). By crossing naturally resistant isolates

of V. inaequalis with artificially induced biochemical markers, linkage groups may be determined.

MATERIALS AND METHODS

Isolates of V. inaequalis were obtained from Michigan apple orchards with a history of extensive benomyl spray programs except for isolate WL-2. Isolates WL-2 and Saur-79 were sensitive wild-type strains, while isolates NU-4C, FS-24, CR-2C and KV-3C were resistant to benomyl. Monoconidial isolates were obtained by streaking leaf lesions across petri plates containing 4% Difco potato-dextrose agar (PDA).

Biochemical mutants, provided by D. M. Boone, University of Wisconsin, were obtained by treating conidia of wild-type line 365-4 with ultraviolet radiation, except 934 Pyrimidine-1 was induced by nitrogen mustard (4, 5, 22). Mutant strains required nicotinic acid (U-2599), biotin (U-2570), purine (U-2892), pyrimidine (U-1907, N-934). The deficiency in each mutant was controlled by a single gene (23).

Testing of Isolates

Both biochemical and resistant parental (P_1) isolates were tested for benomyl resistance and auxotrophy before and after crossing to detect reverse mutations. The first felial (F_1) generation of random ascospores were tested for inherited traits using the same methods. Small

squares of agar with sporulating mycelium were cut with a sterilized spatula from the margin of colonies grown on Difco potato-dextrose agar. The mycelial squares were transferred to 13 x 100 mm pyrex test tubes containing 2 ml sterile deionized water. The agar was crushed with a small sterile spatula and the test tubes were vortexed at 6 rpm for 30 sec with a deluxe mixer to loosen the conidia.

Test for Fungicide Resistance

Resistance levels were determined by pipetting 0.20 ml of the mycelial suspension into each section of 100 x 15 cm plastic, quadrant petri dishes containing PDA amended with 0, 25, 100, 500 and 800 ug/ml benomyl. The benomyl (Benlate WP 50% ai., E. I. Dupont de Nemours and Co., Wilmington, DE.) was added to the media before autoclaving. Suspensions were streaked across the surface of the media with a bent glass rod. Isolates that failed to grow after 2 wk at 21 C were considered susceptible, while isolates that grew on media with benomyl were considered resistant.

Test for Auxotrophy

Isolates were tested for auxotrophy by pipetting 0.20 ml of the mycelial suspension onto 10 ml minimal media in 60 x 15 cm petri dishes. The minimal media as suggested by Boone (5) consisted of the following (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, 1.0 ml. The trace element solution contained (mg/400 ml):

$FeC_6H_5O_7 \cdot 3H_2O$, 214.2; $ZnSO_4 \cdot 7H_2O$, 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; and distilled water to make 400 ml (25). The media was adjusted to pH 5.6 with 1N NaOH. Isolates that exhibited little or no growth after 14 day at 21 C on minimal media were tested for the required vitamin or nitrogen base. Two 4 mm diameter plugs of the prestressed mycelium were transferred to tri-section petri dishes containing 5 ml of minimal, supplemented and complete (PDA) media. Supplemented media (5) was minimal media with one of the following supplements (mg/L): adenine sulfate, 40; uracil, 10; nicotinic acid, 0.5; and biotin, 0.005. Solutions containing each supplement were filter sterilized and added to cooled media after autoclaving. Plates were held at 21 C for 14 day before growth was recorded.

Conidial Production

Conidia were produced by growing isolates in 8 oz prescription bottles containing 30 ml of a 4% Difco malt extract solution and cheesecloth wicks as described by Keitt (21). After 14 to 20 days, conidia were harvested by pouring off the malt solution and adding 30 ml sterile deionized water. The bottles were shaken vigorously to loosen conidia and the suspension was filtered through two layers of cheesecloth into a 50 ml sterilized Erlenmeyer flask. Concentrations were determined using a hemacytometer, and were approximately 3 to 5 x 10^5 conidia/ml.

Crossing of Isolates and Isolation of Ascospores

Isolates were mated using the methods of Keitt and Langford (21). Conidial suspensions containing 1 ml each of two single-spored isolates were mixed in 100 x 15 cm petri dishes. Cooled 2.5% PDA amended with 0.5% malt extract and apple leaf decoction (40) was poured over the spore suspension and swirled to distribute the spores throughout the agar. Plates were held at 21 C for 14 day on the laboratory bench, then transferred to dark incubators at 8 C until mature perithecia and asci were observed, usually 6 to 8 mo after crossing.

Mature perithecia were transferred to sterilized pyrex culture tubes containing 5 ml sterile deionized water and crushed with a sterile rod to release the ascospores. One ml samples of spore suspension were transferred to petri dishes containing PDA. Single germinated ascospores were removed with a sterile glass needle to individual petri dishes (60 x 15 mm) containing complete media and placed at 21 C until colonies were large enough for testing.

RESULTS

Two isolates WL-2 and Saur-79 were inhibited at 25 ug/ml and were considered sensitive or wild type strains. Isolates NU-4C, FS-24, CR-2C and KV-3C grew at levels up to 500 ug/ml and were considered resistant (Table 1). None of the isolates grew at 800 ug/ml. Biochemical mutants 934 Pyrimidine-1, 2599 Nicotinic acid, 1907 Pyrimidine-2, 2570 Biotin and 2892 Purine-6 were all sensitive at 25 ug/ml of benomyl.

Auxotrophy was determined by transferring mycelial plugs to minimal, supplemented and complete media. Isolates with an auxotrophic mutation showed no growth or a marked reduction in growth on minimal media after a 14-day period (Table 1). The mutant requiring nicotinic acid did not grow on minimal media and was complete for its deficiency. Those mutants requiring pyrimidine, biotin and purine were incomplete (leaky) and showed no growth or a reduction in growth on minimal media when compared to supplemented or complete media.

Meiotic mapping of resistant loci was performed by crossing naturally occurring resistant strains with biochemical marker strains. All isolates were selfed and resulted in either no perithecia development or perithecia

Table 1.--Response of parentals to different concentrations of benomyl and to minimal media.

Isolates	PDA with benomyl (ug/ml)					Minimal Media	Strain
	0.0	25	100	500	800		
KV-3C	+	+	+	+	-	+	Ben1
NU-4C	+	+	+	+	-	+	Ben2
FS-24	+	+	+	+	-	+	Ben3
WL-2	+	-	-	-	-	+	$Ben^{+}4$
CR-2C	+	+	+	+	-	+	Ben5
Saur-79	+	-	-	-	-	+	$Ben^{+}4$
934 Pyrimidine-1	+	-	-	-	-	- (uracil)[a]	Pyr-1
2599 Nicotinic Acid	+	-	-	-	-	- (nic acid)[a]	Nic-1
1907 Pyrimidine-2	+	-	-	-	-	- (uracil)[a]	Pyr-2
2570 Biotin	+	-	-	-	-	- (biotin)[a]	Bio-1
2892 Purine-6	+	-	-	-	-	- (adenine)[a]	Pur-6

+ = ability; - = inability or reduced growth on media containing the benomyl concentration and required supplement for growth on minimal media.

[a]Supplement required for growth.

but no ascospore formation. Random ascospore analysis of the F_1 progeny revealed linkage in three of eight crosses. Ben5, conferring resistance at 500 ug/ml, was mapped 38.9 units from the nic-1 locus (Table 2). A deviation from the expected 1:1 allele ratio of the nic-1 locus was observed. The segregation ratio differed significantly ($\beta = 0.005$) from an expected 1:1 ratio. The cross between nic-1 and ben$^+$4, a wild type strain, yielded no recombinants and was highly significant at the 1:1 ratio (Table 2). Only parental ditypes were recovered, indicating the segregation of the nic-1 locus.

Ben1 was located 36.3 units from the pur-6 locus and 38.8 units from the bio-1 locus (Table 3). Segregation ratios of these two crosses deviated significantly from a 1:1 ratio and fewer progeny were observed of the parental type requiring purine or biotin than of the parental type containing the benomyl resistant locus. However, only a significant deviation from the expected 1:1 allele ratio for pur-6 was observed. In cross 4, the recombinant classes deviated from the expected 1:1 ratio. None of the benomyl resistant progeny obtained in crosses 1, 3 and 4 differed in the level of benomyl resistance from the two parental types. Only one parental type was obtained from a cross between ben3 and pyr-1 (Table 4). All 333 spores were resistant to benomyl at 500 ug/ml.

The ratio obtained from a cross between pyr-2 and ben$^+$6 did not differ significantly from a 1:1 (Table 4).

Table 2.--Meiotic mapping of loci determining resistance in *Venturia inaequalis*.

Cross 1: *nic*-1 x *ben*5			Cross 2: *nic*-1 x *ben*$^{+}$4		
Progeny genotype		number	Progeny genotype		number
+	nic	106	+	nic	252
ben	nic	92	+	+	127
+	+	27			379
ben	+	81			
		306			
Allele Ratio					
198 nic : 108 +					
173 ben : 133 +					
Recombination		(%)	Recombination		(%)
nic-1--*ben*5		38.9	*nic*-1--*ben*$^{+}$4		0
X^2 (1:1) =		46.8*	X^2 (1:1) =		42.0*

*$p < .005$.

Table 3.--Meiotic mapping of ben1.

Cross 3: pur-6 x ben1			Cross 4: bio-1 x ben1		
Progeny			Progeny		
genotype		number	genotype		number
+	pur	77	+	bio	90
ben	pur	50	ben	bio	87
+	+	52	+	+	48
ben	+	102	ben	+	123
		281			348
Allele Ratio			Allele Ratio		
127 pur : 154 +			177 bio : 171 +		
152 ben : 129 +			210 ben : 138 +		
Recombination (%)			Recombination (%)		
pur-6--ben1		36.3	bio-1--ben1		38.8
X^2 (1:1) =		25.1*	X^2 (1:1) =		33.4*

*p < .005.

Both strains were sensitive to benomyl at 25 ug/ml and segregation for the pyr-2 locus was observed. A cross between a strain conferring resistance at 500 ug/ml and a wild type, sensitive at 25 ug/ml was made to determine the presence of secondary genes. The two types of progeny obtained were both parental types and did not differ significantly from a 1:1 ratio. None of the F_1 generation was more resistant than the parentals. Results indicated the segregation of a single gene, ben2.

To test possible additive effects greater than 500 ug/ml, a cross was made between two loci, ben5 and ben1,

Table 4.--Meiotic mapping of ben^{+}6 and ben3.

Cross 5: pyr-2 x ben^{+}6		Cross 6: pyr-1 x ben3	
Progeny		Progeny	
genotype	number	genotype	number
+ pyr	107	+ pyr	0
+ +	118	ben +	333
	225		333
Recombination	(%)	Recombination	(%)
pyr-2--ben^{+}6	0	pyr-1--ben3	0
X^2 (1:1) =	0.54ns		

ns = not statistically significant.

both conferring benomyl resistance up to 500 ug/ml (Table 5). From the 278 random ascospores of the F_1 progeny tested, 111 or 40% were resistant at 800 ug/ml of benomyl, while 167 or 60% were resistant at 500 ug/ml. None of the progeny scored were less resistant than either of the two parentals.

Linkage Groups

Boone (D. M. Boone, personal communication) has mapped four of the five biochemical mutants used in this study to linkage groups. The nic-1 locus has been located 8.3 map units from the centromere on linkage group I. Ben5 was mapped 38.9 units from nic-1 and located on group I. Both pur-6 and bio-1 have been located on linkage group VIII, with pur-6 close to the centromere. Meiotic analysis indicated the ben1 locus to be linked to pur-6 by 36.3 units

Table 5.--Detection of allelic or additive effects between benomyl resistant strains.

Cross 7: ben2 x ben^{+}4		Cross 8: ben1 x ben5	
Progeny		Progeny	
phenotype	number	phenotype	number
+	127	ben^{+a}	111
ben	133	ben^{b}	167
	260		278
Recombination	(%)		
ben2--ben^{+}4	?		
X^2 (1:1)	0.14ns		

ns - not statistically significant.

^{a}ben^{+} = resistance at 800 ug/ml benomyl.

^{b}ben = resistance at 500 ug/ml benomyl.

and to bio-1 by 38.8 map units. The assignment of ben3 to a linkage group was not possible and further crosses are necessary.

DISCUSSION

Genetic analysis of the F_1 progeny from three of the eight crosses revealed linkage of loci in naturally resistant strains of V. inaequalis to biochemical markers. Location of the two resistant genes have been mapped to two of the linkage groups proposed by Boone (D. M. Boone, personal communication). Ben5 was located 38.9 units from the nic-1 locus and has been assigned to linkage group I. All F_1 progeny obtained from a cross between nic-1 and ben$^+$4 were of parental ditype. This would be expected due to the segregation of only the nic-1 locus. Similarly, the cross between pyr-2 and ben$^+$6 resulted in a 1:1 segregation of the pyr-2 locus. Boone (personal communication) has mapped pyr-2 close to the centromere on linkage group VII. Ben1 was linked to both pur-6, mapping close to the centromere, and bio-1 on linkage group VIII. However, further crossing is necessary to determine if these genes are on the same side of the centromere. Segregation ratios of a cross between ben2 and ben$^+$4 indicated the activity of a single gene conferring resistance to benomyl.

None of the F_1 progeny obtained from crosses between highly resistant strains with sensitive biochemical strains conferred higher benomyl resistance than either of the two

parentals. This would be expected when only one locus controlling benomyl resistance is present. However, when two strains both resistant at 500 ug/ml of benomyl were crossed, 40% of the progeny conferred a higher level of resistance (800 ug/ml) than either of the two parentals. Hastie and Georgopoulos observed increased resistance levels in progeny obtained from crosses between mutants of A. nidulans (14). When benA, conferring a high level of benomyl resistance was crossed with benB, imparting a lower level, the resistance level of resulting F_1 progeny containing both genes appeared to be additive. Similar additive effects were obtained by Polach (27, 28) from crossing strains of V. inaequalis, resistant to dodine at 0.25 ug/ml with strains resistant at 0.50 ug/ml of dodine. One would expect, however, a certain percentage of the tested F_1 progeny in cross 8 to be sensitive or wild type, but none were found.

Allele ratios for the nic-1 locus in cross 1 and the ben1 locus in cross 4 deviated significantly from the expected 1:1 ratio of mutant to wild type alleles. A selection for one allele over the other or problems encountered in determining genotypic ratios were considered to be the contributing factors. Similar results were noted by Van Tuyl with the pimA, palC and pA loci in A. nidulans (38).

All F_1 progeny scored from a cross between pyr-1 and ben3 were of the ben3 genotype. A reduction in the

viability of the auxotrophic mutant would explain the detection of only one parental type. Crosses of different benomyl and thiabendazole resistant strains of U. maydis resulted in progeny with only the benomyl resistant phenotype and Van Tuyl (38) attributed this deviation from a 1:1 ratio to a selective advantage of one parental type over the other.

Resistance to benomyl in V. inaequalis has been demonstrated to be controlled by several loci. At least two independent loci appear to be involved and confer additive effects, increasing resistance levels when combined. Hastie and Georgopoulos (14) revealed the activity of at least two unlinked genes benA and benB conferring benomyl resistance in A. nidulans. An additional gene, benC was detected by Van Tuyl (38) in the same organism. Genetic inheritance of dodine resistance in N. haematococca f. cucurbitae was observed by Kappas and Georgopoulos (19) to be controlled by four independent loci. Polach (28) demonstrated in V. inaequalis the activity of at least two genes conferring resistance at high concentrations of dodine.

The assignment of genes conferring resistance to benomyl on known linkage groups is still incomplete. Further crossing between biochemical and color markers that map close to the loci for benomyl resistance should be performed to give exact locations in terms of map units from these genes and also the centromere. To obtain this information, both random ascospore and tetrad analysis are suggested. Crossing F_1 progeny that contain both the benomyl and

biochemical mutations with another known marker strain would increase accuracy of mapping. Test crosses of the F_1 progeny with the wild type parental may reveal the activity of secondary or modifier genes that have previously been suppressed. Since additive effects were observed in the F_1 progeny in cross 8, further crossing of isolates conferring different levels of benomyl resistance would afford insight into these effects. Finally, analysis revealed the presence of two independent loci responsible for benomyl resistance located on two linkage groups. It would therefore provide additional linkage information, to continue crossing naturally occurring strains to marker strains that have previously been assigned to known linkage groups.

Results from this study demonstrates that although resistance to benomyl appears to be controlled by a single gene in each of the highly resistant strains tested, it is possible for mutations to occur at at least two independent loci. The ability of naturally occurring strains of V. inaequalis to mutate at different loci in response to increased selection by extensive benomyl spray programs would help explain the rapid build-up of resistant strains in apple orchards.

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