

## Genetics of Resistance to Benomyl in *Venturia inaequalis* Isolates from Israel and New York

Talma Katan, E. Shabi, and J. D. Gilpatrick

First and second authors, Division of Plant Pathology, Agricultural Research Organization, The Volcani Center, P.O. Box 6, Bet Dagan 50 250, Israel; previous address of third author (deceased), Department of Plant Pathology, Cornell University, NYS Agricultural Experiment Station, Geneva, NY.

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### ABSTRACT

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Fourteen single-spore cultures of *Venturia inaequalis* isolated from apple scab lesions at two sites in Israel and at three sites in New York state, United States, showed resistance to benomyl. Four levels of resistance were determined in vitro: five isolates with low resistance grew at 0.5 but not at 5  $\mu\text{g}$  of benomyl per milliliter; one moderately resistant isolate grew at 5 but not at 50  $\mu\text{g}/\text{ml}$ ; three highly resistant isolates grew and sporulated at 50  $\mu\text{g}/\text{ml}$  but more slowly than at 5  $\mu\text{g}/\text{ml}$ ; and five isolates with very high

resistance grew and sporulated abundantly at benomyl concentrations higher than 50  $\mu\text{g}/\text{ml}$ . In crosses between different resistant isolates and between sensitive wild-types and resistant isolates, the levels of benomyl resistance are controlled by a polymorphic series consisting of four allelic mutations, in a single Mendelian gene. No effect of modifying genes or cytoplasmic components on benomyl resistance was evident in the isolates from either country.

*Additional key words:* apple scab, fungicide resistance.

Resistance of the apple (*Malus sylvestris* Mill.) scab pathogen *Venturia inaequalis* (Cke.) Wint. to benzimidazole fungicides has been reported from several countries. In some populations of *V. inaequalis*, different levels of resistance were noted when isolates were tested in vitro on varying concentrations of benomyl and other benzimidazole fungicides (2,3,5,7,10). Nevertheless, resistant isolates with different resistance levels produced typical scab lesions when inoculated on unsprayed apple plants and on plants sprayed with benomyl (150 mg/L), although wild-type sensitive isolates infected only the unsprayed plants; the newly formed conidia retained the parental phenotypes (7).

Inheritance of benomyl resistance has been studied with *V. inaequalis* isolates from Michigan (2), Germany (4), and Israel (9), and in each of these studies a single Mendelian gene controlled resistance. Genetic analysis of 10 resistant strains from Israel revealed three alleles conferring three different levels of resistance to benomyl; there was no evidence for modifiers or cytoplasmic determinants (9).

Similarity of genes for benomyl resistance in *V. inaequalis* from different countries can be determined by crossing resistant isolates from geographically remote sources. The purpose of our research was to determine the number of genes governing the different resistance levels of isolates from New York, their mode of inheritance, and their relationship with the benomyl-resistance gene of *V. inaequalis* isolates from Israel.

### MATERIALS AND METHODS

**Media.** Cultures were maintained on potato-dextrose agar (PDA). Benomyl (Benlate 50 WP) was added for determination of resistance, before autoclaving the media for 20 min.

**The pathogen.** *V. inaequalis* strains WIS(+), WIS(-), and WIS-Gr(+) (green colony) provided by D. M. Boone of the University of Wisconsin-Madison served as reference cultures for mating type

determination. WIS-Gr(+) was used also as a sensitive parent in crosses. Other sensitive cultures were GH-5 and GH-8, which had been maintained for several years by successive transfers to greenhouse-grown apple seedlings without fungicides; S-9, isolated from Red Delicious; and GS-3, isolated from Granny Smith apples. Six resistant isolates were obtained from Red Delicious apple trees at two sites on the Golan Heights in Israel, which had been treated with benzimidazole fungicides. These were isolate IMS-11 from an orchard with about 10% benomyl resistance in the *V. inaequalis* population and isolates IOT-82, IOT-83, IOT-95, IOT-96, and IOT-97 from a site with about 2% resistance in the population. Eight resistant isolates were obtained from McIntosh apple orchards in western New York, which had been treated with benomyl but showed apple scab. These were NYP-6 and NYP-9 from one orchard; NYM-1, NYM-4, NYM-5, NYM-7, and NYM-13 from a second orchard; and NYML-9 from a third orchard. Each isolate originated from a separate scab lesion.

**Preparation of single-spore cultures and determination of benomyl resistance.** Dilute suspensions of conidia were plated on PDA and incubated at 20 C for 1-2 days to allow germination. Germlings were picked up with a stainless steel needle under microscopic observation, transferred individually to PDA plates, and allowed to grow and form sporulating colonies.

Wild-type sensitive strains of *Venturia* do not grow, and their spores form short, distorted germ tubes on media amended with benomyl. In contrast, the germination and growth of resistant strains are not impaired (2,8). Preliminary studies showed the threshold of benomyl sensitivity of different wild-type strains to be at 0.15-0.3  $\mu\text{g}/\text{ml}$ . Thus, unless stated otherwise, sensitive and resistant strains were distinguished routinely by their germination and growth at 20 C on media supplemented with benomyl at 0, 0.5, 5, or 50  $\mu\text{g}/\text{ml}$ .

**Crosses.** Matings were done on sterilized Red Delicious apple leaf disks placed on 2% water agar in 60  $\times$  15 mm petri plates. Conidiating mycelia of single-spore cultures were inoculated as pairs on the leaf disks. The plates were incubated at 15 C for 2 wk, to allow mycelial growth, and then transferred to 8 C until perithecia with ascospores developed (6).

**Determination of ascospore phenotypes and progeny**

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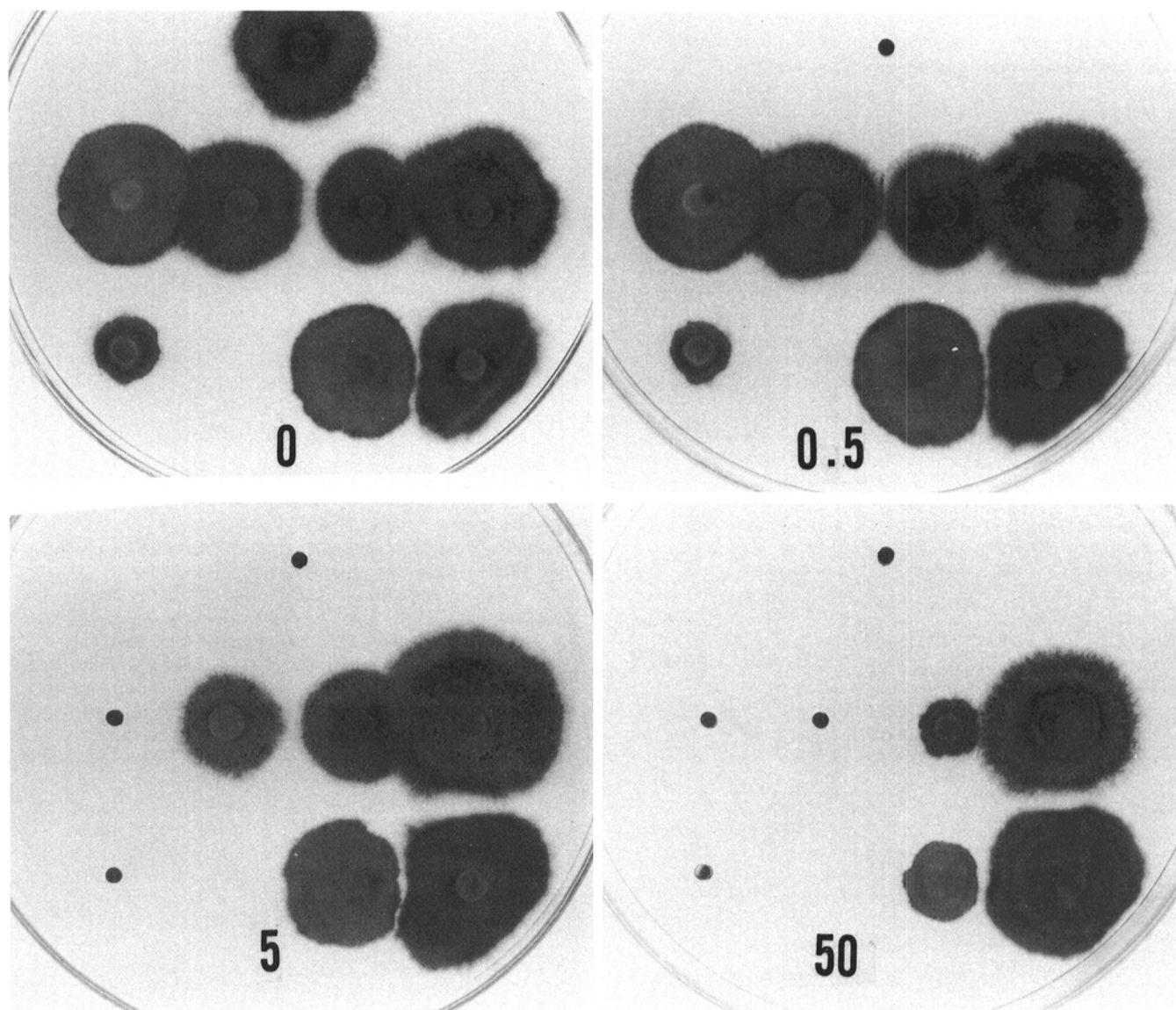
**segregation.** Mature perithecia were picked up with a needle from the leaf disks and crushed in a drop of sterile water on a glass slide. The resulting ascospore suspension was diluted and plated on PDA or 2% water agar, and incubated 24–48 hr at 20 C. Germinating ascospores were transferred individually to new plates and allowed to form sporulating colonies. Spores and hyphae of these colonies were tested for germination, growth, and sporulation on media supplemented with 0, 0.5, 5, or 50  $\mu\text{g}$  of benomyl per milliliter.

## RESULTS

**Phenotypic expression of benomyl resistance.** Spore germination, hyphal growth, and sporulation of the 14 resistant isolates were examined on PDA amended with increasing concentrations of benomyl (Fig. 1). At 0.5  $\mu\text{g}$ /ml, benomyl had no effect on germination, growth, or sporulation of the 14 isolates. At 5  $\mu\text{g}$ /ml of benomyl, isolates NYP-6, NYP-9, NYM-5, NYM-13, and NYML-9 (from New York) behaved the same as sensitive strains did on 0.5  $\mu\text{g}$ /ml; the phenotype of these isolates was termed low resistance (LR), to indicate that they were resistant to 0.5

$\mu\text{g}$ /ml but sensitive to 5  $\mu\text{g}$ /ml benomyl. Isolate IOT-82 from Israel was resistant to benomyl at 5  $\mu\text{g}$ /ml but sensitive to 50  $\mu\text{g}$ /ml and thus had a moderately resistant (MR) phenotype. The other eight resistant isolates germinated, grew, and sporulated at 50  $\mu\text{g}$ /ml. However, the germ tubes and hyphae of isolates NYM-1, IMS-11, and IOT-83 grew slower at 50 than at 5  $\mu\text{g}$ /ml; this growth response was designated R' and the phenotype was termed highly resistant (HR). Isolates NYM-4, NYM-7, IOT-95, IOT-96, and IOT-97 grew and sporulated at 50  $\mu\text{g}$ /ml as fast as at 5  $\mu\text{g}$ /ml; this growth response was designated R and the phenotype was termed very highly resistant (VHR). With benomyl at 500  $\mu\text{g}$ /ml, VHR isolates grew more slowly than at 50  $\mu\text{g}$ /ml, but both HR and VHR isolates grew at this concentration, maintaining the relative difference between them that was observed at 50  $\mu\text{g}$ /ml.

**Crosses.** Perithecia were evident in some of the matings after 4–6 mo at 8 C. According to perithecia formation in crosses with the reference cultures and in other combinations, a (+) or (–) mating type could be assigned to each of the crossed isolates (Table 1). Twenty fertile crosses, consisting of 10 combinations of parental phenotypes, were analyzed at three benomyl concentrations (Table



**Fig. 1.** Growth response of eight *Venturia inaequalis* isolates at varying benomyl concentrations. Each plate was inoculated with 4-mm mycelial disks of sensitive (upper row) and resistant cultures from Israel (middle row) and New York (lower row) and incubated at 20 C for 47 days. The numbers in the figure indicate concentration of benomyl ( $\mu\text{g}/\text{ml}$ ) in potato-dextrose agar plates. Phenotypes (left to right) indicate low, moderate, high, and very high resistance to benomyl.

1). Progeny segregation of crosses 1–5, between five sensitive parents and four parents with low resistance, clearly fit a 1:1 resistant-sensitive ratio, indicating that in each LR mutant, resistance is controlled by a single Mendelian gene, which determines also the phenotype of the haploid ascospore progenies. In these crosses and in crosses 6 and 7, the phenotypes of the resistant progenies were identical to those of the resistant parents.

All the progenies of crosses 9–15 and 17, in which resistant parents with different phenotypes were crossed with each other, had only parental phenotypes. No recombinants were found among 905 progenies of eight such crosses, and progeny segregations for parental phenotypes on the discriminating benomyl concentrations clearly fit the 1:1 ratio.

Crosses 8, 16, and 18–20, in which resistant parents with the same phenotypes were crossed, yielded only parental type progenies; ie, no recombination or segregation was evident among 583 progenies tested.

## DISCUSSION

Previous genetic studies with 10 benomyl-resistant isolates of *V. inaequalis* from Israel showed that MR, HR, and VHR phenotypes were governed by an allelic series in a single Mendelian gene (9). The availability of two collections of resistant isolates from New York and Israel enabled us to study and compare the inheritance of resistance within each group and also to cross and analyze additional combinations of parental phenotypes. Whereas HR and VHR phenotypes were present in both collections, the MR

phenotype was present only in the collection from Israel, and the LR phenotype only in the collection from New York (an LR strain was isolated in Israel, after completion of this study; see Fig. 1).

Crosses between resistant isolates from western New York and sensitive wild-types proved that benomyl resistance had originated through mutations. A segregation ratio of 1:1 in crosses 1–5 showed that in each LR isolate resistance was controlled by a single Mendelian gene, and the absence of recombination or segregation in cross 8 (between two LR isolates) indicated allelism. No recombinant phenotypes were found among the F<sub>1</sub> progenies of crosses 1–7, between resistant and sensitive isolates, or in crosses 10 and 13 between resistant isolates from New York with different resistant phenotypes. The presence of only parental phenotypes among the progenies of any one cross, their 1:1 segregation ratio at the discriminating benomyl concentrations, and the lack of segregation in crosses between isolates with the same resistant phenotype, showed clearly that the various levels of resistance were governed by different alleles in a single gene. The deviation in crosses 6 and 7 of progeny segregation from a 1:1 ratio is considered experimental error because when the same HR and VHR isolates participated in other crosses (10, 16, and 17; and 13, 19, and 20, respectively), the results clearly fit the one-gene hypothesis. Crosses 9, 11, 12, and 14–20, in which resistant isolates from New York were crossed with resistant isolates from Israel, showed that in spite of the geographic remoteness, the same gene had mutated in both countries. By analogy with other fungi, this could be the gene coding for tubulin, in which various amino acid substitutions might result in variant polypeptides with reduced affinity to

TABLE 1. Segregation for resistance to benomyl among ascospore progenies of *Venturia inaequalis*

Cross	Parental phenotypes <sup>a</sup>	Parental isolates <sup>b</sup>	Total	Benomyl in medium (µg/ml)							X <sup>2</sup> <sup>f</sup>
				0.5		5		50		R	
				S <sup>c</sup>	R <sup>d</sup>	S	R	S	R <sup>e</sup>		
S × LR											
1		S-9(+) × NYM-13(-)	120	64	56	120	0	120	0	0	0.53
2		GH-5(-) × NYP-6(+)	117	58	59	117	0	117	0	0	0.01
3		GH-8(-) × NYP-9(+)	70	31	39	70	0	70	0	0	0.91
4		GS-3(+) × NYML-9(-)	91	39	52	91	0	91	0	0	1.86
5		WIS-Gr(+) × NYML-9(-)	126	55	71	126	0	126	0	0	2.03
S × HR											
6		WIS-Gr(+) × NYM-1(-)	126	47	79	47	79	47	79	0	8.13
S × VHR											
7		WIS-Gr(+) × NYM-7(-)	124	46	78	46	78	46	0	78	8.25
LR × LR											
8		NYP-6(+) × NYM-13(-)	149	0	149	149	0	149	0	0	NS <sup>g</sup>
LR × MR											
9		NYP-9(+) × IOT-82(-)	91	0	91	40	51	91	0	0	1.33
LR × HR											
10		NYP-9(+) × NYM-1(-)	120	0	120	56	64	56	64	0	0.53
11		NYP-9(+) × IOT-83(-)	101	0	101	55	46	55	46	0	0.80
12		NYM-5(-) × IMS-11(+)	107	0	107	51	56	51	56	0	0.23
LR × VHR											
13		NYP-9(+) × NYM-7(-)	133	0	133	56	77	56	0	77	3.32
14		NYM-13(-) × IOT-95(+)	108	0	108	46	62	46	0	62	2.37
15		NYML-9(-) × IOT-96(+)	127	0	127	72	55	72	0	55	2.28
HR × HR											
16		NYM-1(-) × IMS-11(+)	107	0	107	0	107	0	107	0	NS
HR × VHR											
17		NYM-1(-) × IOT-96(+)	118	0	118	0	118	0	55	63	0.54
VHR × VHR											
18		NYM-4(-) × IOT-95(+)	133	0	133	0	133	0	0	133	NS
19		NYM-7(-) × IOT-95(+)	123	0	123	0	123	0	0	123	NS
20		NYM-7(-) × IOT-97(+)	71	0	71	0	71	0	0	71	NS

<sup>a</sup>S = sensitive to benomyl at 0.5 µg/ml; LR, MR, HR, and VHR = low, moderate, high, and very high resistance to benomyl at 0.5, 5, 50, and more than 50 µg/ml, respectively.

<sup>b</sup>NY = resistant isolates from New York, and I = resistant isolates from Israel. Mating types are indicated (+) and (-).

<sup>c</sup>S = sensitive reaction.

<sup>d</sup>R = resistant reaction.

<sup>e</sup>R' = resistant reaction with limited growth at 50 µg/ml.

<sup>f</sup>Expected value for a 1:1 ratio at  $P = 0.05$  is 3.84.

<sup>g</sup>NS = no segregation.

carbendazim—the mechanism implicated for benomyl resistance (1).

In studies of the inheritance of benomyl resistance in *V. pirina*, only a high level of resistance (due to a single gene mutation) was identified (8), whereas we observed great variation in resistance among resistant isolates of *B. inaequalis*. At a benomyl concentration of 25  $\mu\text{g}/\text{ml}$  progeny segregation of crosses between a sensitive strain and resistant isolates from Michigan revealed a single Mendelian gene for resistance in each resistant isolate; the parental resistance level was not specified, nor was it apparent whether the mutations were allelic (2). Similarly, one gene for resistance was identified at a benomyl concentration of 10  $\mu\text{g}/\text{ml}$  in resistant isolates from Germany (4). At present, there is no indication for more than one benomyl-resistance gene in *V. inaequalis*; likewise, there is no evidence that modifiers or cytoplasmic components are involved, since no new phenotypes appeared in  $F_1$ .

We concluded that various levels of resistance to benomyl in *V. inaequalis* are the manifestation of polymorphism in a single Mendelian gene. So far, four alleles have been recognized, in addition to the sensitive wild-type, which can fully account for the phenotypes found in New York and Israel. Because of allelism, the high level of resistance could not develop from interactions between low-resistance genes. Presumably, the different alleles originated with random and independent mutational events.

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