Genetics, Pathogenicity, and Stability of Carbendazim-Resistant Isolates of *Venturia pirina*

E. Shabi and T. Katan

Division of Plant Pathology, Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.

Contribution 153-E (1978 Series) from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.

This research was supported by the Ben-Aziz and Tobolsky Memorial Research Grant. The technical assistance of S. Elisha is gratefully acknowledged.

Accepted for publication 18 September 1978.

ABSTRACT

SHABI, E., and T. KATAN. 1979. Genetics, pathogenicity, and stability of carbendazim-resistant isolates of Venturia pirina. Phytopathology 69:267-269.

Nine single-spored cultures of carbendazim-resistant *Venturia pirina* were isolated from different pear orchards in which benomyl failed to control scab. These cultures retained resistance in the absence of the fungicide, produced typical sporulating lesions on Spadona pear plants, and transmitted the carbendazim resistance to the new conidiospores. All isolates had the same resistant phenotype. Crosses between resistant isolates and sensitive wild types, as well as among different resistant isolates, showed that carbendazim-resistance is conferred by a mutation in a single Mendelian gene and is not affected by modifying genes or cytoplasmic components.

Additional key words: Benzimidazole, MBC, systemic fungicides, thiabendazole, thiophanate-methyl, tolerance.

In the last decade benzimidazole fungicides were used extensively to control many phytopathogenic fungi. However, the appearance of increasing numbers of fungal strains resistant to those fungicides has caused much concern (1–3,6). Since 1970, benzimidazole fungicides have been used extensively in Israel for the control of scab (caused by *Venturia pirina* Aderh.) in pear (*Pyrus communis* L.) orchards. In 1975, resistance to these fungicides was found in *V. pirina* from two pear-growing regions in Israel (8). Within 2 yr, resistant phenotypes were detected in pear orchards throughout the country (Fig. 1).

This article reports the results of studies of the pathogenicity of carbendazim-resistant isolates, the stability of resistance, and its mode of inheritance.

MATERIALS AND METHODS

Media. Cultures were maintained on potato-dextrose agar (PDA) (Difco). Clear plates of malt agar, composed of 0.5% malt extract (Difco) and 2% bacto-agar (Difco) were used for the isolation of single germinating spores. Potato-dextrose-decoction agar (Pd), containing quarter-strength PDA, 2% bacto-agar and 25% Spadona pear leaf decoction, and malt-decoction agar (Md), containing 0.5% malt extract, 2% bacto-agar and 50% leaf decoction served as crossing media (5). All media were prepared with deionized water and autoclaved for 20 min. Carbendazim (technical grade, BASF AG, Ludwigshaven, Germany) was added to media used in resistance determination immediately after sterilization.

The organism. *Venturia pirina*, race II (9), was isolated from infected Spadona pear fruits collected from orchards in different regions of Israel. Spores were collected from the scab lesions and their germination morphology was examined on PDA amended with carbendazim (8). In this survey we identified carbendazim-sensitive, carbendazim-resistant, and mixed populations which became the source of material for genetic studies (Fig. 1).

Six sensitive and nine resistant, independent single-spore isolates (see below) were selected for phenotype determinations. Three sensitive and seven resistant isolates, which originated from five pear-growing regions, were used for genetic studies. The resistant isolates and their geographical sources were: R-2-1 (Dovev, Upper Galilee); R-3-1 and R-3-2 (Dalia, Mt. Carmel); R-5-1 (Kfar Szold, Upper Hula Valley); R-6-13 (Rosh Pina, Lower Hula Valley); R-8-2 (Tel Zoffit, Judean foothills); and R-9 (Zarit, Upper Galilee).

Preparation of single-spore cultures and determination of pathogenicity. Dilute spore suspensions were plated on malt agar medium 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 at 20 C. The ability of an isolate to infect Spadona pear leaves was tested by the filter paper disk technique (9).

Determination of carbendazim resistance. Wild-type, carbendazim-sensitive spores of *V. pirina* form short, distorted germ tubes on carbendazim-amended agar medium. In contrast, the germination and growth of resistant spores is not impaired (Fig. 2) (4,8). Preliminary studies showed the threshold of carbendazim sensitivity of different wild-type isolates to be at 0.1–0.2 μM. Thus, unless stated otherwise, sensitive and resistant spores were distinguished routinely by the shape of their germ tubes after incubation 1–2 days at 20 C on media supplemented with 0, 0.5, 5, and 50 μM of carbendazim.

Crosses. Matings were done in screw-capped vials, either on slants of Pd or Md (5) or on propylene-oxide sterilized Spadona pear leaf disks placed on watersoaked autoclaved vermiculite (7). Conidial suspensions of single-spore cultures were mixed as pairs and pipetted onto each of the three crossing media. The vials were incubated at 15 C for 2 wk, to allow mycelial growth, and then transferred to 8 C until perithecia with ascospores had developed (5).

Determination of ascospore phenotypes and progeny segregation. Ascospore phenotypes were determined simultaneously by two methods. In the *direct method* leaf disks bearing perithecia were attached to the underside of a lid of a petri dish containing malt agar with 5 μM carbendazim. Ascospores discharged from the perithecia onto the medium and germinated. Forty eight to 72 hr after discharge the germlings were examined microscopically and the resistant ones were distinguished from the sensitive ones by the response to the carbendazim in the medium (Fig. 2-[C, D]). In the *indirect method* the ascospores similarly

00031-949X/79/000048$03.00/0
©1979 The American Phytopathological Society

discharged from leaf disks onto fungicide-free malt medium. The germinating ascospores were transferred individually to new plates and allowed to form sporulating colonies. Spores and hyphae of these colonies were tested for germination and growth on carbendazim-amended medium. Perithecia produced in Md or Pd slants were picked up with a needle and crushed in a drop of sterile water on a glass slide. The resulting ascospore suspensions were diluted and plated on malt agar and the ascospore germlings were isolated and tested as above.

RESULTS

Phenotypic expression of carbendazim resistance. Carbendazim had no effect on spore germination, hyphal growth, or sporulation of nine independent resistant isolates. Occasionally growth rate was slowed slightly, but this was quickly overcome. All the resistant isolates were normal in respect to growth rate, sporulation, and morphology and all were also resistant to 50 μM thiabendazole in PDA.

Quantitative aspects of carbendazim resistance. Spore germination, hyphal growth, and sporulation of the nine resistant isolates were examined on PDA amended with increasing concentrations of carbendazim. In comparison with nonamended PDA, carbendazim at 0.5, 5, 50, 500, 1,000, 2,500, and 5,000 μM did not affect germination (the significance of the three highest concentrations is doubtful, since carbendazim crystallizes at these levels). Hyphal growth of all resistant isolates was somewhat retarded at 500 μM, although hyphal morphology was not altered. Accordingly, sporulation was delayed at 500 μM and above, but not at lower concentrations. The responses of nine resistant isolates to carbendazim at any concentration were indistinguishable.

Stability and pathogenicity of carbendazim-resistant isolates. The resistant isolates were subcultured on fresh, fungicide-free media about once each month for over 1 yr, without loss of resistance. All the resistant isolates were pathogenic, as indicated by the development of typical sporulating lesions similar to those of the sensitive isolates on leaves of Spadona pear. In order to test if the resistance is retained or lost upon infection of the host plant, conidia from lesions produced by the resistant isolates were examined on carbendazim-amended medium. Hyphae produced by the new spores were as resistant as those from the original inoculum, the resistance was transmitted throughout consecutive infections of host plants.

Perithecial development and heterothallism. Perithecia were evident in part of the matings after 2-3 mo at 8 C. Once formed, perithecia appeared on all three crossing media bearing a given pair of isolates. Perithecia were not formed in single-spored cultures and in some of the matings under the same conditions. According to presence or absence of perithecia, all mated isolates, both carbendazim-sensitive and carbendazim-resistant, were grouped into two mating-types (5). The groups have been arbitrarily designated *mt A* and *mt a* and a designation letter was added to each isolate number (Table 1).

Progeny segregation and progeny phenotypes. Progeny segregations of crosses between five different resistant isolates and three sensitive ones (the sensitive isolates are not specified, because all reacted similarly—see below) were determined by testing at least 100 progenies from each cross on medium amended with 5 μM carbendazim. The results (Table 1) clearly fit a 1:1 ratio of resistant:sensitive, indicating that in each mutant, resistance is controlled by a single Mendelian gene.

All the resistant progenies grew and sporulated on 5 μM carbendazim-amended medium like their resistant parents. Two hundred and five randomly-selected resistant progenies, representing the five crosses listed in Table 1, also were examined on 500 μM carbendazim. All were as resistant as their resistant parents in

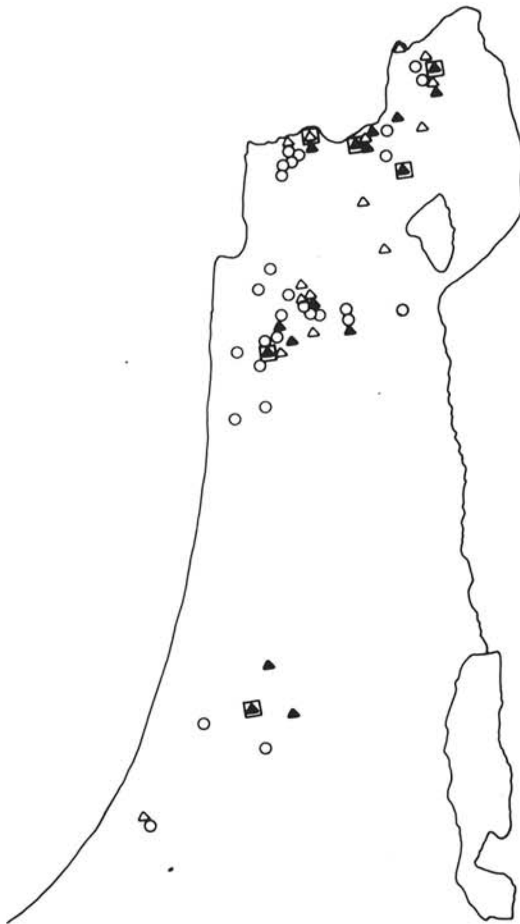

Fig. 1. Distribution of carbendazim-resistant *Venturia pirina* in pear orchards in Israel (1977). O = sensitive population; ▲ = resistant population; Δ = mixed population (mostly sensitive, few resistant). Six orchards, from which resistant isolates were used in genetic studies, are designated by triangles enclosed by a square.

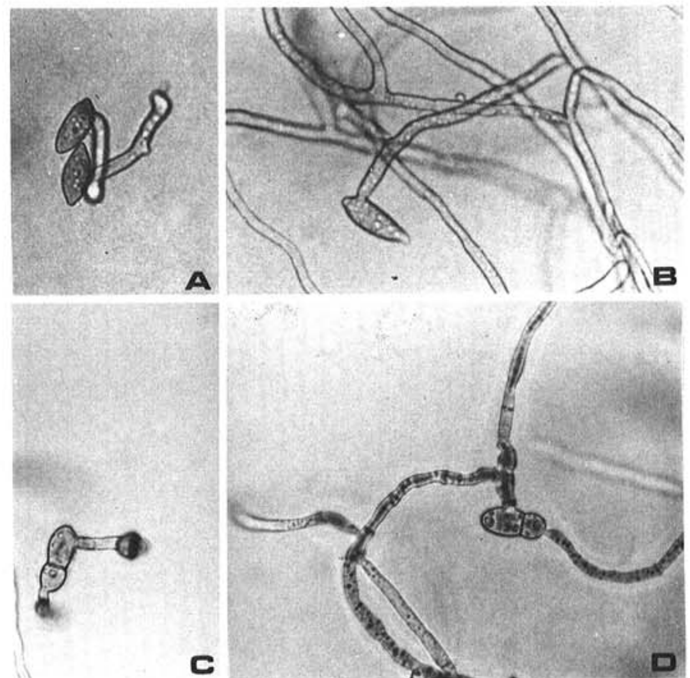

Fig. 2. Germinated conidia (A and B) and ascospores (C and D) of carbendazim-sensitive (A and C) and -resistant (B and D) *Venturia pirina* on 5 μM carbendazim-amended medium after 72 hr at 15 C (× 560).

TABLE 1. Segregation of *Venturia pirina* progenies on carbendazim-amended medium[a]

Resistant isolate[b]	Progenies tested[c] (no.)	Sensitive (%)	Resistant (%)	Chi-square values for 1:1 segregation[d]
R-3-2 *a*	112	54.4	45.6	0.89
R-5-1 *a*	221	46.6	53.4	1.02
R-6-13 *a*	305	51.8	48.2	0.4
R-8-2 *a*	296	49.7	50.3	0.01
R-9 *A*	115	50.4	49.6	0.01

[a] Malt agar medium with 5 μM carbendazim.
[b] *A* and *a* indicate mating-types.
[c] Represented are progenies produced on three crossing media.
[d] Expected value at $P = 0.05$ is 3.84.

TABLE 2. Segregation of mating type (*mt*) alleles among carbendazim-sensitive and resistant *Venturia pirina* progenies

Resistant parent	F_1				Parental:Nonparental ratio
	Resistant		Sensitive		
	mt A	*mt a*	*mt A*	*mt a*	(%:%)
R-3-2 *a*	11	14	13	13	53:47
R-5-1 *a*	9	4	8	4	48:52
R-6-13 *a*	13	14	12	17	46:54
R-8-2 *a*	18	14	11	19	40:60
R-9 *A*	12	6	19	7	43:57

spite of the interaction at the sexual stage with genomes and cytoplasms of sensitive mates; nor did the sensitive progeny gain any degree of resistance, as indicated by growth inhibition at 0.5 μM carbendazim of 72 randomly selected sensitive progenies of the same crosses.

Crosses among four resistant mutants (R-2-1 *A*, R-3-1 *A*, R-6-13*a*, R-8-2*a*), of which over 2,000 progenies were examined, produced no sensitive recombinants.

The mating-types of the progenies were determined by mating with two *mt A* and two *mt a* isolates and looking for the formation of perithecia. Considering the *carbendazim resistance* marker and the *mt* marker, all four possible combinations were present in our original collection of isolates and nonparental recombinants comprised about half of the progenies in each cross (Table 2). This indicated that these markers segregated freely from each other.

DISCUSSION

Crosses between carbendazim-resistant isolates of *V. pirina* and sensitive wild-types proved that carbendazim-resistance had originated with mutation. In all cases a segregation ratio of 1:1 showed that resistance was a distinct and inheritable feature controlled by a single Mendelian gene.

All the carbendazim-resistant isolates tested so far were pathogenic. This might be the consequence of the isolation procedure, namely, from naturally infected fruit. Resistance was stable in vivo as well as in vitro in the absence of the fungicide. This suggested a permanent change in the genetic material rather than conditional adaptation. The resistant phenotype was not greatly affected by the carbendazim concentration; ie, the resistant isolates germinated, grew and sporulated on fungicide concentrations at least 1,000 times higher than that inhibitory to the wild-type. Resistance was independent of the genetic background of the different isolates: no individuals with partial or intermediate resistance were found. This could not be attributed to extensive selection pressure by the fungicide in the orchard, because some of the resistant isolates were obtained from orchards in which the resistant type comprised only a small proportion of the pathogen population. The uniform response to carbendazim of all resistant *V. pirina* isolates suggested that only one gene was involved in the mutation and that the phenotypes were not affected by modifying genes.

No partially resistant recombinant genotypes were found among the F_1 progenies of crosses between resistant and sensitive isolates. This supported the conclusion that only one gene had mutated. Furthermore, intervention by modifying genes or cytoplasmic components was ruled out.

Although the resistant mutants shared a common phenotype, it was possible that the mutations had appeared at different loci. If that was the case, crossing those mutants would have given rise to some recombinant progeny sensitive to carbendazim. The absence of any sensitive progeny in crosses involving four resistant isolates, indicated that their mutations were allelic or very closely linked. The *mating-type* locus could not be used as a mapping marker, because it always segregated independent of the *carbendazim-resistance* gene (Table 2). All the alleles confer uniform resistance rather than polymorphism, and may be identical. It is possible that all the resistant isolates were derived from a single mutational event by gene migration, but the geographic pattern of the first appearances of carbendazim resistance in *V. pirina* (8) and its distribution thereafter do not favor this hypothesis. Alternatively, the mutation could have appeared independently in separate *V. pirina* populations. The pathogen may have only one gene mutation which will result in carbendazim resistance and still retain the degree of viability and of pathogenicity that will allow it to survive under field conditions. In vivo competition experiments, in which three resistant isolates were tested against sensitive counterparts, showed that in the absence of selection pressure by the fungicide, the mutants were not weaker and sometimes even tended to take over (Shabi, *unpublished*). The exclusive use of benzimidazole fungicides for the control of scab in pear orchards enhanced the establishment of the resistant phenotype through selection pressure.

LITERATURE CITED

1. DEKKER, J. 1976. Acquired resistance to fungicides. Annu. Rev. Phytopathol. 14:405-428.
2. GILPATRICK, J. D. 1976 (ed.) Symposium: Resistance of plant pathogens to chemicals. Proc. Am. Phytopathol. Soc. 3:47-98.
3. HARTILL, W. F. T., R. E. BEEVER, and P. J. BROOK. 1975. Fungicide resistance: an approach to the problem. Pages 130-132 in Proc. 28th N. Z. Weed and Pest Control Conference.
4. JONES, A. L., and G. R. EHRET. 1976. Tolerance to fungicides in Venturia and Monilinia of tree fruits. Proc. Am. Phytopathol. Soc. 3:84-89.
5. LANGFORD, M. H., and G. W. KEITT. 1942. Heterothallism and variability in Venturia pirina. Phytopathology 32:357-369.
6. OGAWA, J. M., and J. D. GILPATRICK. 1977. Review of plant pathogens resistant to fungicides and bactericides. FAO Plant Prot. Bull. 25:97-111.
7. ROSS, R. G., and S. A. HAMLIN. 1962. Production of perithecia of Venturia inaequalis (Cke.) Wint. on sterile apple leaf discs. Can. J. Bot. 40:629-635.
8. SHABI, E., and Y. BEN-YEPHET. 1976. Tolerance of Venturia pirina to benzimidazole fungicides. Plant Dis. Rep. 60:451-454.
9. SHABI, E., J. ROTEM, and G. LOEBENSTEIN. 1973. Physiological races of Venturia pirina on pear. Phytopathology 63:41-43.