Biology of Benomyl-Tolerant Strains of Cercospora beticola from Sugar Beet

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ABSTRACT

Benomyl-tolerant strains of *Cercospora beticola* as a group were no different from benomyl-sensitive strains in growth and sporulation in vitro, or in virulence and sporulation in vivo. Strains differed in their degree of tolerance, the tolerance level being stable for at least 1 year in vitro. Tolerance of one strain was unchanged after three passages through sugar beet. In mixed inoculations of sugar beet with a sensitive strain, a tolerant strain population declined, but never disappeared. Degree of cross-tolerance as measured by linear growth of three tolerant strains tested against benomyl, thiabendazole, and thiophanate depended on isolate, fungicide, and fungicide concentration. Conidial germination of a tolerant and a sensitive strain tested against three benzimidazole derivatives and six protectant fungicides showed that the sensitive strain generally was more sensitive to all the fungicides at concentrations of 10 μg or more per ml, and the protectants were somewhat more inhibitory to both strains than were the systemics. Benomyl was more toxic to conidia than to hyphae of a tolerant strain, whereas the reverse was true for thiabendazole. Ultraviolet irradiation of conidia from a sensitive strain failed to induce benomyl-tolerant mutants.

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Benzimidazole derivatives have shown phenomenal effectiveness as systemic chemotherapeutants for controlling many plant diseases. Recently, however, benzimidazole-tolerant strains have been reported for several plant pathogenic fungi that previously were sensitive to these fungicides (1, 2, 5, 7, 8, 13, 15, 16, 17).

Benomyl-tolerant strains of *Cercospora beticola* Sacc. were first isolated in 1972 from leaf spot-infected sugar beets (*Beta vulgaris* L.) in Greece (3). We isolated similar strains from sugar beets grown in northern Texas in 1973 (10). In both isolations, the tolerant strains appeared after an almost exclusive use of benzimidazole fungicides for leaf spot control for 3 years.

The efficacy of the benzimidazoles in controlling Cercospora leaf spot (11, 14), and their past superiority over protectant-type fungicides in sugar beet (11) has led to their widespread use in all sugar beet growing areas of the USA where leaf spot is endemic. The appearance of benomyl-tolerant strains of *C. beticola* raised several questions on the biology of these strains. The present study was conducted to compare growth, sporulation, and spore germination of tolerant and sensitive strains. Also, more information was desired on cross-tolerance of benomyl-tolerant strains to benzimidazole and protectant-type fungicides, and on the induction and stability of tolerant strains.

MATERIALS AND METHODS.—Monospore isolates of *C. beticola* were obtained from leaf spot-infected sugar beets grown in 1973 near Hereford, Texas, and their sensitivity to benomyl was determined (10). Benomyl-tolerant (T) and -sensitive (S) isolates used in this study included H1-12T (ATCC 28058), H4-5T, H5-12T, HB-6T (ATCC 28053), HB-28T (ATCC 28054), HB-30T (ATCC 28055), HC-10S (ATCC 28056), and HC-15S. Cultures were maintained on potato-dextrose agar (PDA) slants and transferred monthly to fresh medium. Sporulation was induced on sugar beet leaf extract agar (SBLEA), and sugar beets were inoculated as reported elsewhere (9). Isolate C-1 (ATCC 24078), a benomyl-sensitive isolate from Colorado, was included in sensitivity tests to determine the effectiveness of fungicide-amended media.

Fungicides used were as follows: (i) benomyl wettable powder (WP), 50% active ingredient (a.i.); (ii) chlorothalonil emulsifiable concentrate, 54% a.i.; (iii) copper oxychloride sulfate WP, 75% a.i.; (iv) cupric hydroxide WP, 86% a.i.; (v) maneb WP, 80% a.i.; (vi) thiabendazole (TBZ) WP, 60% a.i.; (vii) thiophanate-methyl WP, 70% a.i.; (viii) triphenyltin hydroxide WP, 47.5% a.i.; (ix) zinc polyethylenethiuràm disulfide WP, 80% a.i. All concentrations in the text refer to active ingredient.

A preliminary test indicated that autoclaving benomyl-amended PDA did not alter its fungitoxic properties. Therefore, in tests with benomyl alone, the fungicide was added to the medium before autoclaving. When several fungicides were used, all were added after autoclaved media were cooled to approximately 45 C.

In growth studies on solid media, 4-mm diameter agar-mycelium disks were transferred to the test media (6-cm diameter petri dishes with 10 ml medium per dish) from the margins of 7-day-old cultures growing on PDA. Colony diameter was measured after incubation at 25 C for 7 days.

A randomized complete block design with six replications was used to compare growth and sporulation of benomyl-tolerant and benomyl-sensitive strains in vitro. Three 4-mm diameter mycelium disks from 5-day-old sporulating cultures were vigorously agitated for 15 seconds in 5 ml sterile distilled water. Average spore concentration of each isolate was calculated from six subsamples per replication with the aid of a hemacytometer. The experiment was repeated once.

To determine in vivo sporulation of benomyl-tolerant and -sensitive strains, five 3-mm diameter sporulating lesions from infected sugar beet (highly susceptible cultivar R & G Pioneer) were agitated in 5 ml of sterile distilled water. Average spore concentration of each strain was determined as described above. A randomized complete block design with four replications was used, and the experiment was repeated once.

Stability of benomyl tolerance was tested in three ways. First, tolerant strains were grown in PDA slant cultures for 12 months at 25 C, with monthly transfers to fresh medium and medium containing benomyl. Two in vivo tests included (i) three passages through sugar beet of a tolerant strain alone and (ii) in mixed inoculations with a sensitive strain. In the first in vivo test, strain HB-6T was inoculated to sugar beet. When lesions developed in 10-14 days, sporulation was induced by placing leaf pieces in moisture dishes for 48 hours at 25 C, and 30 monospore isolations were made as previously described (9). These isolates were grown on PDA for 7 days, and then transferred to PDA containing 5 µg benomyl/ml. Mycelial suspensions from all the isolates were prepared, bulked, and used to initiate new spore cultures on SBLEA (9). A spore suspension from the SBLEA cultures was used to inoculate sugar beet again. Sugar beets were inoculated and the spores were reisolated three times. A similar procedure was followed in the second in vivo test to inoculate sugar beet with an equal concentration of spores from strains HB-6T and HC-10S. In this test, 60 monospore cultures were obtained from sporulating lesions developing after each inoculation. After reisolated cultures were tested on benomyl-amended PDA, tolerant and sensitive isolates were used to initiate spore cultures. Equal concentrations of spores from each strain were mixed and used as inoculum (aqueous suspension).

To test cross-tolerance of benomyl-tolerant strains to benomyl, TBZ, and thiophanate, the strains were grown on PDA amended with those fungicides at concentrations of 1-, 10-, 50-, and 100-µg/ml. Three replications were used in a randomized complete block design. The experiment was repeated once. In another test with three replications, conidia of a benomyl-tolerant and a benomyl-sensitive strain were mixed with solutions of two benzimidazoles, thiophanate-methyl, and six protectant type of fungicides at concentrations of 0.1-, 1-, 10-, 100-, and 1,000 µg/ml. Percentage of germination of 100 conidia per treatment was determined after 16 hours of incubation at 25 C. A conidium was considered germinated if it had one germ tube twice as long as the spore diameter.

Unsuccessful attempts to "train" sensitive *C. beticola* strains to grow on benzimidazole-amended medium were reported (10). To attempt ultraviolet (UV) induction of benomyl-tolerant mutants, conidial suspensions of a sensitive isolate (C-15) were irradiated for 1.5-, 2-, and 3-

minutes by the methods of Ben-Yephet, Henis, and Dinoor (1). A sample of irradiated conidia was plated on PDA to determine % kill, whereas the remainder was plated on PDA containing 5 μg benomyl/ml. A total of approximately 3.7×10^7 conidia (approximately 1.2×10^7/test) was tested against benomyl after irradiation. With an average of approximately 15 cells per conidium, a total of 5.6×10^7 nuclei were irradiated.

To compare the virulence of a benomyl-tolerant and -sensitive strain, 3-month-old sugar beets [highly susceptible cultivar R & G Pioneer, and an experimental hybrid (52-334 × 51-319)] were inoculated with conidial suspensions (40,000 spores/cc). One half of the plants were irrigated with 300 ml/15-cm diameter pot of a benomyl solution (100 μg benomyl/ml) 24 hours before inoculation. Inoculated plants were held in a humidity chamber for 96 hours at 100% relative humidity and 27 C and then placed on the greenhouse bench. Disease severity was recorded 21 days after inoculation on a disease index (D.I.) scale of 0 to 5 (0 = no leaf spot, and 5 = complete defoliation). A randomized complete block design with three replications was used, and the experiment was repeated once.

Data were subjected to analysis of variance, and mean separations were calculated by Duncan's multiple range test at $P = 0.05$. Where similar results were obtained in repeated experiments, data from one representative test are presented.

RESULTS.—*Growth and sporulation without benomyl.*—Significant differences in linear growth were found among benomyl-tolerant and -sensitive strains in vitro. However, as a group, tolerant strains were no different from sensitive strains. Mean colony diameters from two trials on PDA were 22 mm for strain HB-28T, 23 mm for HC-10S, 26 mm for HC-15S, and 22 mm for HB-6T. Spore production in two trials on SBLEA ranged from 1.6 to 3.6×10^5 spores/cm². In one trial, strains did not differ significantly. In the second trial, strain HC-10S produced significantly more spores and HB-28T significantly fewer spores than the other strains. Strains HC-15S and HB-6T did not differ significantly from each other. Spore production on leaf lesions, from 3.2 to 6.5×10^4 spores per cm², did not differ significantly.

Growth of tolerant strains on benomyl-amended PDA.—A factorial design with three replications was used in each of two tests to compare growth of six benomyl-tolerant strains on PDA containing 0-, 1-, 10-, 50-, and 100-μg benomyl per ml. Results of both tests were similar. Significant differences in growth among strains were found at each concentration of benomyl, Table 1. The strain × concentration interaction was highly significant, indicating that strain behavior partly depended on benomyl concentration.

In another test with six replications, the strain most tolerant to benomyl (H1-12T) was plated on PDA containing 100-, 500-, 1,000-, and 5,000 μg/ml of benomyl or TBZ. No growth occurred on TBZ at these concentrations whereas the ED_{50} (concentration causing 50% growth inhibition) on benomyl-amended PDA was between 1,000- and 5,000 μg/ml.

Stability of benomyl tolerance.—Six benomyl-tolerant strains retained their relative tolerance through 12 monthly transfers to PDA in the absence of benomyl. When a single tolerant strain was cultured in vivo, all

TABLE 1. Colony diameter of benomyl-tolerant strains of *Cercospora beticola* grown on different concentrations of benomyl in potato-dextrose agar for 7 days at 25 C

Fungus strain	Response of strains of *Cercospora* to benomyl				
	Mean diameter of colonies (mm)[a]				
	None	1 (μg/ml)	10 (μg/ml)	50 (μg/ml)	100 (μg/ml)
H1-12T	23.0 A	22.4 A	21.4 A	17.4 A	15.1 A
HB-28T	22.0 B	21.9 A	21.0 A	16.2 B	14.0 B
HB-30T	21.0 C	20.7 B	12.2 D	0 D	0 E
HB-12T	19.7 D	20.4 B	19.7 B	16.6 B	11.6 C
H5-12T	17.4 E	16.0 D	6.7 E	0 D	0 E
HB-6T	17.2 E	17.2 C	13.3 C	4.7 C	1.0 D

[a]Means followed by the same letter in any column did not differ significantly by Duncan's multiple range test at $P = 0.05$.

TABLE 2. Colony diameter of benomyl-tolerant strains of *Cercospora beticola* grown on varied concentrations of benomyl, TBZ, or thiophanate in potato-dextrose agar for 7 days at 25 C

Fungus strain	Fungicide	Response of strains of *Cercospora* to fungicides			
		Mean diameter of colonies (mm)[a]			
		1 (μg/ml)	10 (μg/ml)	50 (μg/ml)	100 (μg/ml)
H1-12T	Benomyl	20.0	17.7	15.3	12.0
	TBZ	19.3	16.0	6.7	0
	Thiophanate	19.7	17.7	16.7	15.7
HB-30T	Benomyl	18.0	3.0	0	0
	TBZ	18.0	15.3	7.0	0.3
	Thiophanate	18.3	5.7	0	0
H4-5T	Benomyl	15.0	2.0	0	0
	TBZ	15.3	15.0	3.7	0
	Thiophanate	15.0	2.7	0	0

[a]Means of three replications.

monospore cultures obtained after each reisolation showed the cultural characteristics of HB-6T, and were benomyl-tolerant. In the test with mixed inocula of a tolerant and sensitive strain, monospore cultures obtained after each inoculation of sugar beet and reisolation on PDA could be distinguished by pigmentation in the medium or mycelium. Pigmentation was either "green" (similar to HB-6T) or "red" (similar to HC-10S). Green cultures tended to be tolerant to benomyl, whereas red cultures tended to be sensitive to benomyl. The ratios of green:red cultures after three reisolations were 13:47, 6:48 (six contaminated cultures were discarded), and 21:39, whereas the ratios of tolerant:sensitive cultures were 9:51, 5:49, and 19:41, with all tolerant isolates being green in color.

Cross-tolerance to benzimidazoles and thiophanate at varied concentrations.—Strains H1-12T, HB-30T, and H4-5T showed cross-tolerance to benomyl, TBZ, and thiophanate in varied degrees, depending on fungicide concentration, Table 2. Analysis of variance showed highly significant differences ($P = 0.01$) among fungicides, isolates, and concentrations. All the interactions also were highly significant. Strain H1-12T generally was more tolerant to the fungicides than were HB-30T and H4-5T.

Effect of nine fungicides on conidial germination.—Conidia of benomyl-sensitive strain HC-15S were more sensitive to all fungicides tested than were conidia of benomyl-tolerant strain H1-12T, except for maneb and chlorothalonil at 0.1- and 1 μg/ml and copper oxychloride sulfate at 0.1 μg/ml. Protectant fungicides chlorothalonil, cupric hydroxide, and triphenyltin hydroxide were the most fungitoxic compounds tested, regardless of strain. Further, all protectants were more fungitoxic to both strains than were the benzimidazoles (benomyl and thiabendazole) and thiophanate at concentrations above 10 μg/ml. The ED_{50} for chlorothalonil, copper oxychloride sulfate, cuprice hydroxide, and triphenyltin hydroxide was $<$ 0.1 μg/ml for both strains. The ED_{50} for maneb was $<$ 0.1 for the benomyl-tolerant strain and between 0.1 and 1 for the benomyl-sensitive strain. With zinc polyethylenethiuram disulfide, the ED_{50} was between 1 and 10 for the tolerant strain and approximately 1 for the sensitive strain. The ED_{50} for benomyl was between 100 and 1,000 for the tolerant strain and $<$ 0.1 for the sensitive strain. With both TBZ and thiophanate-methyl, the ED_{50} was $>$ 1,000 for the tolerant strain, whereas for the sensitive strain the ED_{50} was $<$ 0.1 with TBZ and between 0.1 and 1.0 with thiophanate-methyl.

UV irradiation.—UV exposures of 1.5, 2, and 3 minutes killed 0, 5, and 95% of the conidia, respectively. All conidia appeared morphologically normal after irradiation, but no benomyl-tolerant mutants were detected on benomyl-amended PDA.

Virulence of benomyl-tolerant and benomyl-sensitive strains.—Mean disease severity did not differ significantly between cultivars (D.I. = 1.5), nor did virulence between the tolerant (H1-12T) and sensitive (HC-15S) strains (D.I. = 1.4 vs. 1.5, respectively). Disease severity in plants treated with benomyl was significantly reduced (D.I. = 0.7), compared to nontreated plants (D.I. = 2.3), regardless of fungus strain. However, the degree of control was always greater when plants were inoculated with a benomyl-sensitive strain than with the tolerant strain.

DISCUSSION.—Bollen and Scholten (2) reported that a benomyl-tolerant strain of *Botrytis cinerea* was less vigorous in culture than was the sensitive parent isolate. Similarly, a benomyl-tolerant isolate of *Verticillium malthousei* showed less reproductive and pathogenic capacity than sensitive isolates (17). Except for normal cultural variation among strains, benomyl-tolerant strains of *C. beticola* did not differ from sensitive strains in growth and sporulation in vitro or in virulence and sporulation in vivo. In mixed culture in vivo, like the benomyl-tolerant mutants of *Ustilago hordei* in vitro (1), the population of a tolerant strain of *C. beticola* tended to decline, but never disappeared completely. More work is needed on the competitive ability of benomyl-tolerant strains of *C. beticola*. However, it seems unlikely that tolerant strains will disappear whether or not benzimidazoles are used, as suggested by Wuest et al. (17) for *V. malthousei*.

The in vitro and in vivo stability of benomyl tolerance in strains of *C. beticola* suggests a mutational evolution, rather than an "adaptive-type" mechanism, as shown by some isolates of *Fusarium oxysporum* f. sp. *melonis* (12). The varied degrees of tolerance shown by the strains and the significant interactions among isolates, benzimidazoles, and fungicide concentrations indicate that genetic control of tolerance may be quite complex. Like benzimidazole-tolerant strains of *Aspergillus nidulans* (6), different mutated genes may confer varied tolerance levels in strains of *C. beticola*.

Tests wtih benomyl and TBZ on the most tolerant isolate (H1-12T) showed an inverse relationship between spore germination and linear growth. Benomyl was more toxic to conidia (ED_{50} = 10 to 100 μg/ml) than hyphae (ED_{50} = 1,000 to 5,000 μg/ml), whereas TBZ was more toxic to hyphae (ED_{50} = 10 to 50 μg/ml) than conidia (ED_{50} = $>$ 1,000 μg/ml). Differential permeability of spores and hyphae to the compounds or solubility differences of the chemicals could account for this reversal. A similar relationship was reported with TBZ and a *Penicillium* sp. and an *Aspergillus* sp. (4). Such reversals, and the significant interactions mentioned above, show the need for both growth and germination tests of several isolates at varied chemical concentrations in determining the toxicological properties of a potential fungicide.

The general superiority of protectant over the systemic fungicides in spore germination tests contradicts results of field tests where systemics were more effective in the control of sugar beet leaf spot.

The origin of benomyl-tolerant strains of *C. beticola* is still unresolved. We were unable to "train" isolates to tolerate benomyl by growing them on increasing amounts of benzimidazoles in the medium (10). Also, tolerant strains have not been reported from many areas where these selective compounds have been widely used. Thus, the chemicals do not appear to be mutagenic to *C. beticola*. However, benomyl-induced, stable, tolerant mutants of *Fusarium oxysporum* and *F. solani* have been reported (8, 15). The role of benzimidazoles in the development of tolerant strains in the field needs to be determined.

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