Ceratocystis ulmi Tolerance to Methyl-2-Benzimidazole Carbamate and Other Related Fungicides

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ABSTRACT

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An isolate of *Ceratocystis ulmi* which was tolerant to methyl-2-benzimidazole carbamate (MBC) concentrations as high as 2,000 μg/ml, but sensitive to lower concentrations of several other benzimidazole fungicides was discovered in a routine bioassay plate. The inheritance of this high level of MBC tolerance was controlled by a single gene. American elm (*Ulmus americana*) seedlings inoculated in the greenhouse with the MBC-tolerant isolate developed typical Dutch elm disease (DED) symptoms, but of a greater intensity than that produced with an MBC-sensitive wild-type isolate. Fungicide-treated greenhouse-grown elm seedlings also developed typical DED symptoms when inoculated with the MBC-tolerant isolate. Comparisons of 205 *C. ulmi* isolates from untreated elms and elms treated with various benzimidazole or thiourea-type fungicides failed to indicate increases in the frequency of MBC or TBZ tolerance as a result of the treatment. The results of inoculation and reisolation studies with greenhouse-grown American elm seedlings pretreated with benomyl, MBC-H_3PO_4, M&B 21914C, or TBZ-H_2PO_2 suggested that benomyl, but not the other compounds tested, induced a significant level of tolerance in a wild-type *C. ulmi* isolate.

Additional key words: chemotherapy.

Increasing numbers of reports of fungus tolerance to the substituted 2-amino benzimidazole and related antifungal compounds (5) have prompted researchers studying Dutch elm disease (DED) to investigate the occurrence and significance of *Ceratocystis ulmi* (Buism.) C. Moreau isolates tolerant to methyl-2-benzimidazole carbamate (MBC). Brasier and Gibbs (2) found *C. ulmi* strains tolerant to 0.5 μg/ml MBC-HCl with a frequency of about 1.3×10^8 conidia in both aggressive and nonagressive isolates from England. None of them, however, was tolerant to 5 μg/ml of MBC-HCl. On the basis of mating studies with their isolates, they suggested that inheritance of MBC tolerance was controlled by a single gene.

Schreiber and Townsend (11) reported that six of 29 *C. ulmi* isolates obtained from various locations throughout the United States which had no prior exposure to the benzimidazole fungicides grew on potato-dextrose agar (PDA) amended with MBC-HCl at concentrations ranging from 1.0 to 1,000 μg/ml. Growth rates of these tolerant strains, however, were greatly reduced at the higher concentrations.

Schafer and Campana (10) reported tolerance of *C. ulmi* to 0.7 μg/ml after successive mycelial disk transfers during a conditioning period of 26 wk on PDA amended with MBC-HCl concentrations varying from 0 to 4.5 μg/ml. However, 5.6% of ascospores exposed once to MBC-HCl were tolerant to 4.0 μg/ml MBC-HCl. Schafer (9) also reported that the cultures derived from ascospores exposed to MBC-HCl demonstrated a greater level of MBC tolerance than cultures from conidia similarly exposed.

In routine branch bioassays, utilizing the technique of Smalley et al (13), of American elms (*Ulmus americana* L.) trunk-injected with MBC-HCl, three colonies of *C. ulmi* suspected of being MBC tolerant were isolated from inhibition zones formed by the diffusion of fungicide from the twig into the agar medium. Since conidia of wild-type aggressive isolate 99, originally derived from a diseased elm from Brown County, Minnesota, had been used to seed these bioassay plates, the isolates were coded 99-T1, 99-T2, and 99-T3. Preliminary experiments demonstrated ED_{50} levels for inhibiting radial growth of about 0.3 μg/ml for isolate 99-T1 and 1.0 μg/ml for isolate 99-T3 as compared to about 0.1 μg/ml for isolate 99. Isolate 99-T2, however, was not inhibited by MBC even at levels as high as 2,000 μg/ml.

We report in this paper the results of laboratory, greenhouse, and field studies on: (i) the tolerance of isolate 99-T2 to several benzimidazole related fungicides, (ii) genetic inheritance of this tolerance, and (iii) the frequency of tolerant isolates obtained from diseased seedlings and trees treated with systemic fungicides.

MATERIALS AND METHODS

The fungicides utilized in this study were Benlate® (benomyl) 50 W [methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate], Lignasan® -BLP 0.8S [methyl-2-benzimidazole carbamate phosphate] and MBC (carbendazim) technical [methyl-2-benzimidazole carbamate] from E.I. duPont de Nemours & Co., Wilmington, DE 19898; M&B 21914C 95 technical [1-methoxycarbonyl-3-(2 dimethylaminoacetamidophenyl) thiourea hydrochloride] from Rhodia, Inc., Chipman Div., New Brunswick, NJ

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08903; TBZ (thiabendazole) technical [2-(4-thiazolyl) benzimidazole] and Arbotect® (TBZ-H_2PO_220S) from Merck & Co., Inc., Rahway, NJ 07065; and Topsin M® (thiophanate methyl) 70 W [1, 2-bis (3-methoxycarbonyl-2-thioureido) benzene] from Pennwalt Corp., Tacoma, WA 89401.

In vitro tolerance.—Test fungicides were prepared in a graded concentration series with distilled water (except MBC and TBZ where 0.1 N HCl was the solvent) so that 0.25 ml added to each petri dish containing melted, freshly made PDA from a single batch resulted in concentrations of 0.1, 0.5, 1.0, 5.0, 10.0, 50.0, and 100 μg/ml. Except with MBC, the fungicides at 100, 500, 1,000, and 2,000 μg/ml were weighed individually and added to each petri dish without previously being suspended in water or HCl and mixed by swirling after the melted agar was added to each dish. These concentrations of MBC were prepared by adding 1% (w:v) MBC-HCl (in 0.1 HCl) stock solution at the rate of 50, 100, and 200 ml/liter PDA, respectively. To determine the effect of the solvent alone at these high concentrations similar volumes of 0.1 N HCl only were added to a control series of plates.

Single, germinated conidia, incubated on 2% Difco Bacto water agar in the dark for 14 hr at 24 C, of isolates 99 and 99-T2 were placed on opposite sides, about 0.5 cm from the side, of 9.0 cm diameter petri dishes which contained 25 ml of fungicide-amended PDA. Two radial measurements were made of each colony after allowing them to grow for 12 days at 24 C. Five replications were made of all treatments.

Inheritance of MBC tolerance.—Matings for studies on the inheritance of MBC tolerance were made on debarked elm twigs. Disks, 1 cm thick, cut from twigs 1.5 to 2.0 cm in diameter were placed in petri dishes over two moist filter paper disks and autoclaved. Pairings were made by inoculating the elm disks with agar cubes (~ 1 mm each edge) from cultures of opposite mating types. Cultures were incubated at room temperature in the dark for about 14 days.

Ascospores were isolated from mature perithecia by first spreading perithecial ooze on sterile 22 × 40 mm coverslips coated with a thin layer of 2% Agarose® (Nutritional Biochemicals Corp., Cleveland, OH 44128). Then single ascospores were picked off with a pneumatic micromanipulator equipped with a hand-drawn ultrafine glass tip and transferred to a second coverslip coated with a thin layer of Agarose. The coverslip with the ascospore was placed on 1.5 cm wide PDA strips in petri dishes so the ascospore was in direct contact with the agar strip. Four-millimeter diameter mycelial/agar plugs from the colony arising from the ascospore was transferred after 5 to 7 days to PDA plates amended with 0, 10, and 1,000 μg/ml MBC-HCl to determine their tolerance to MBC.

Virulence on fungicide-treated seedlings.—Plastic cups (100-ml capacity) were attached to the stems of 6-mo-old greenhouse-grown American elm seedlings for treatment with fungicides. The cups were supported and rendered water-tight with a combination of masking tape, modeling clay, and melted paraffin. Each cup was filled with 60 ml of either benomyl, M&B 21914C, or TBZ-H_2PO_2 (at 1 or 10 g/liter) or MBC-H_3PO_4 (at 1 or 8 g/liter) after a diagonal scalpel cut in the stem had been made near the bottom of the cup.

The seedlings were inoculated with isolate 99 (wild-type) or isolate 99-T2 (MBC-tolerant) 1 wk after treatment at six points at 25-cm intervals along the stems above the point of treatment. Inoculations were done by introducing a heavy conidial suspension with a hypodermic syringe into a wound made with a 1.3 mm diam × 5.0 mm long drill. Xylem isolations were made after 3 wk at 5-cm intervals above each point of inoculation by plating two slivers of wood from each sample point onto PDA acidified with lactic acid (five drops of 20% lactic acid to 25 ml PDA). A total of 48 wood slivers were used for isolations from each seedling. The presence of fungicide in each 25-cm section of stem was determined by the bioassay technique described by Smalley et al (13).

Conidia from a random selection of 10 isolates from each treatment were used to determine whether tolerance to MBC might have been induced or selected for by any of the treatments. A total of 10^9 conidia were screened for each treatment by the method described by Brasier and Gibbs (2). However, the conidia were seeded on PDA plates rather than Oxoid malt agar plates.

Natural occurrence of MBC and TBZ tolerance.—Two-hundred and five *C. ulmi* isolates previously maintained on either elm sawdust or branch sections and stored at −25 C were tested for MBC and TBZ tolerance at 1, 10, 100, and 1,000 μg/ml of MBC- and TBZ-amended PDA. One-hundred and nine of the isolates were obtained from elms previously treated with benzimidazole or thiourea-based antifungal systemics, and the remainder were obtained from untreated diseased trees. Most of the cultures were from isolations made 8 mo after artificial inoculation and subsequent fungicide treatment. The tolerance of the isolates was determined by transferring two 4-mm diameter mycelial/agar plugs from 1-wk-old cultures to each of a series of PDA plates amended with 0, 1, 10, 100, and 1,000 μg/ml of MBC-HCl and TBZ-HCl in the manner previously described. Any radial growth of the test isolate after 10 days indicated tolerance to the particular treatment.

RESULTS

In-vitro tolerance.—Isolate 99 grew more rapidly (3.5 mm/day) than isolate 99-T2 (2.9 mm/day) on PDA (Fig. 1). Concentrations of 1% (v:v) 0.1 N HCl used for MBC up to 100 μg/ml did not inhibit either isolate. The increased quantities of HCl used for 1,000 and 2,000 μg/ml MBC, however, had increasing inhibitory effects on radial growth of 99-T2.

All compounds had comparable inhibiting activity against isolate 99 (no growth at 0.5 μg/ml) although there was slight growth at 0.5 and 1.0 μg/ml of thiophanate methyl and M&B 21914C. Against isolate 99-T2, however, there was a significant difference in activity between MBC and the other fungicides. There was no growth of 99-T2 on PDA amended with benomyl, thiophanate methyl, or TBZ at 50 μg/ml and M&B 21914C at 500 μg/ml. Growth of 99-T2 on PDA amended with 500 and 1,000 μg/ml MBC was reduced by about 20% when compared with its growth on the HCl controls but at 2,000 μg/ml its growth after 12 days on the MBC-amended PDA (18.1 mm) exceeded that of the HCl control (10.4 mm).

Inheritance of MBC tolerance.—The cross between isolates 99-T2 and 116 (wild-type) produced 49 sensitive and 55 tolerant F_1 progeny (Table 1). Backcrossing a tolerant F_1 isolate to the tolerant parent produced all tolerant progeny. Backcrossing a sensitive isolate to the tolerant parent produced 63 sensitive and 59 tolerant progeny, and backcrossing a tolerant F_1 isolate to the sensitive parent produced 57 sensitive and 66 tolerant progeny.

Virulence on fungicide-treated seedlings.—Untreated elm seedlings and those previously treated with fungicides were inoculated with isolates 99 or 99-T2. The untreated control seedlings developed typical DED symptoms, including chlorosis, wilting, premature defoliation and vascular discoloration both above and below the point of

inoculation. In all cases the seedlings inoculated with 99-T2 developed more intense and wider spreading vascular discoloration than did seedlings inoculated with isolate 99. The pathogen was readily isolated from points immediately adjacent to the point of inoculation, but the success of other isolations from distances up to 25 cm from the point of inoculation varied depending on the isolate, the fungicide used, and its concentration. Except for the benomyl treatment, the stems from all treated seedlings formed large and distinct inhibition zones in the bioassay plates indicating high fungicide levels. The benomyl-treated seedlings formed only slight inhibition zones and had reduced numbers of coremia on the cut stem ends compared to those of the controls.

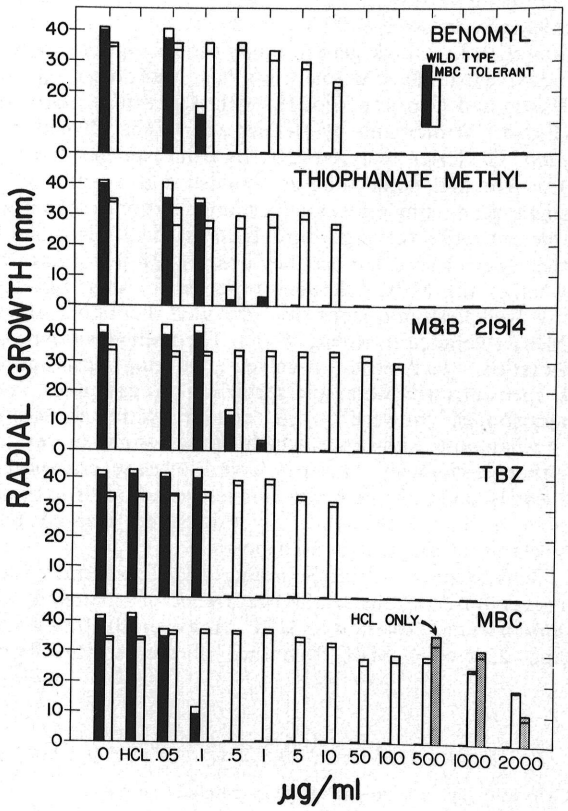

Fig. 1. Radial growth of *Ceratocystis ulmi* wild-type isolate 99 and MBC tolerant isolate 99-T2 after 12 days at 24 C on fungicide-amended potato-dextrose agar (PDA). Values represent the mean radii of five replicates; caps on bars indicate the lower 95% confidence interval. The shaded bars labeled "HCl ONLY" refers to growth of 99-T2 on PDA without MBC but containing HCl in amounts equal to those amended with MBC-HCl.

Isolate 99-T2 was more readily isolated from all fungicide treatments (except benomyl at the lower dose) and the controls (Table 2). The only treatments that significantly reduced the success of isolation of 99-T2 were 1% M&B 21914C and 1% TBZ-H_2PO_2. In comparison, the success of isolation of isolate 99 was significantly reduced by 0.1 and 0.8% MBC-H_3PO_4, 1% M&B 21914C, and 0.1% TBZ-H_2PO_2.

The frequency of MBC-tolerant isolates obtained from inoculated seedlings pretreated with systemic fungicides is summarized in Table 3. The order of the treatments based on an increasing frequency of tolerant spores induced or selected for was: water < TBZ-H_2PO_2 < M&B 21914C < MBC-H_3PO_4 < benomyl. The frequencies ranged from one tolerant conidium in 2×10^8 conidia for the water treatment to one in 1.9×10^5 conidia for benomyl.

Natural occurrence of MBC and TBZ tolerance.—Of 96 isolates originating from untreated elms, three were tolerant to 1 µg/ml, one to 10 µg/ml, and none to 100 and 1,000 µg/ml of MBC (Table 4). Only one of the MBC-tolerant isolates also was tolerant to TBZ. Seven other isolates tolerant to 1 µg/ml TBZ were not tolerant to the same concentration of MBC.

There were fewer tolerant isolates originating from fungicide-treated elms than there were from untreated elms. Only one isolate from a fungicide-treated elm proved to be tolerant to 1 µg/ml MBC although it was sensitive to higher MBC concentrations and to all levels of TBZ. Two other isolates were tolerant to 1 µg/ml TBZ, but were sensitive to higher TBZ concentrations and to all levels of MBC. All tolerant isolates which grew at the concentrations described did so at lower rates than on nonamended PDA.

DISCUSSION

The maximum level of MBC tolerance of isolate 99-T2 could not be determined because at 2,000 µg/ml, the inhibition attributable to the HCl solvent was greater than the inhibition due to MBC. In fact, at this level MBC seemed to have acted as a buffer in suppressing the inhibitory effect of the HCl.

The mechanism of tolerance of isolate 99-T2 was not investigated in this study, but most probably involved a change in the site of binding on tubulin protein (4) that specifically excluded the MBC molecule. If this was so,

TABLE 1. Segregation ratios for progeny of the methyl-2-benzimidazole carbamate (MBC)-tolerant × wild-type cross and backcrosses of F_1 to parents in *Ceratocystis ulmi*

Cross	Observed Progeny[a] S (no.)	Observed Progeny[a] T (no.)	Observed Ratio S:T	Expected Progeny S (no.)	Expected Progeny T (no.)	Expected Ratio S:T	Chi-square ($\chi^2_{P=0.05}$)	P^b
Cross:								
99-T2(T) × 116(S)	49	55	1:1.1	52	52	1:1	.17	0.60–0.70
Backcross: Parent / F_1								
99-T2(T) × M92(T)	0	194	0:1.0	0	194	0:1	...	...
116(S) × M80(S)	142	0	1.0:0	142	0	1:0	...	...
99-T2(T) × M76(S)	63	59	1:0.9	61	61	1:1	.07	0.80–0.90
116(S) × M47(T)	57	66	1:1.1	61.5	61.5	1:1	.33	0.50–0.60

[a]Abbreviations: S = MBC-sensitive, and T = MBC-tolerant. Tested on MBC-HCl (1,000 µg/ml)-amended potato-dextrose agar.
[b]P = probability of agreement between expected and observed ratios.

then the sensitivity of 99-T2 to benomyl, thiophanate methyl, and M&B 21914C, whose activities often are attributed to their hydrolysis to MBC, might be due to the antifungal activity of the unhydrolyzed molecule or to its hydrolytic products. The former may be possible if the change in the tubulin protein was small enough to specifically exclude only the MBC molecule but not such molecules as the unhydrolyzed parent molecule or TBZ.

In fact, a secondary hydrolytic product from benomyl was reported by Hammerschlag and Sisler (6) who identified butyl isocyanate as a volatile breakdown product in the air over moistened benomyl and attributed this compound to the differential action of benomyl and MBC on *Ustilago maydis* (DC.) Cda. and *Saccharomyces cerevisiae* (Meyen) Hansen. Similar breakdown products of thiophanate methyl and M&B 21914C also may be responsible for the differential action between these compounds and MBC.

It seems very likely that the single-gene control of MBC tolerance in isolate 99-T2 is the result of a specific alteration in a single gene at a single locus. Similar single-locus benzimidazole tolerance has been reported by Hastie and Georgopoulos (8) with *Aspergillus nidulans* (Eidam) Winter and by Brasier and Gibbs (2) with *C. ulmi*. The latter analyzed progeny from pairings of eight sensitive and eight tolerant isolates and postulated a single-gene, single-locus inheritance despite sensitive: tolerant ratios varying from 1.1:1.0 to 0.5:1.0. Because of the lower order tolerance they observed, it is not known whether the MBC tolerance reported for their isolates involved the same locus that provided the higher order MBC tolerance in isolate 99-T2. Their single-ascospore isolation technique involved diluting ascospore suspensions with water and spreading on agar plates. The ascospores, however, are coated with a sticky mucilaginous substance which causes some spores to adhere tenaciously. This may have allowed several spores to be isolated as one by the above procedure. Isolations with a micromanipulator in this study insured the selection of only single ascospores.

This study confirms Schreiber and Townsend's (11) earlier observations of the occurrence of isolates of *C. ulmi* naturally tolerant to MBC. However, the frequency and degree of MBC tolerance of the isolates from

TABLE 2. Survival of wild-type and methyl-2-benzimidazole carbamate (MBC)-tolerant isolates of *Ceratocystis ulmi* inoculated separately to 6-mo-old *Ulmus americana* seedlings treated with systemic fungicides

Formulation	Concentration (%)	Successful isolations[w] from seedlings inoculated with: Wild-type (%)	MBC-tolerant (%)
Benomyl	0.1	91.4 a[x]	81.6 ab
	1.0	82.7 ab	98.3 a
MBC-H_3PO_4	0.1	5.8 c	97.5 a
	0.8	10.3 c	71.9 ab
M&B 21914C	0.1	59.0 b	89.8 ab
	1.0	12.5 c	58.1 b
TBZ-H_2PO_2	0.1	8.3 c	72.7 ab
	1.0	...[y]	14.6 c[z]
Water control	...	77.5 ab	97.9 a

[w]Based on 144 isolations onto acidified potato-dextrose agar from three seedlings per treatment except for the water control which is based on 280 isolations from six seedlings.

[x]Means in each column followed by the same letter do not differ significantly ($P = 0.05$) by Duncan's multiple range test.

[y]Severe phytotoxicity, no seedlings were used.

[z]Severe phytotoxicity, only one seedling (48 isolations) was used.

TABLE 3. Tolerance to methyl-2-benzimidazole carbamate (MBC) in wild-type *Ceratocystis ulmi* inoculated to 6-mo-old *Ulmus americana* seedlings treated with systemic fungicides

Pretreatment: Fungicide	Concentration (%)		One MBC tolerant[a] conidium in:	P^c
Benomyl	0.1		1.8×10^{5}[b]	
	1.0		2.0×10^{5}[b]	
		Mean	1.9×10^{5}	$P < .001$
MBC-H_3PO_4	0.1		8.3×10^{7}	
	0.8		7.1×10^{7}	
		Mean	7.4×10^{7}	$P < .300$
M&B 21914C	0.1		8.3×10^{7}	
	1.0		1.4×10^{8}	
		Mean	1.1×10^{8}	$P < .400$
TBZ-H_2PO_2	0.1		1.4×10^{8}	
	1.0		1.6×10^{8}	
		Mean	1.5×10^{8}	$P < .400$
Water	...		2.0×10^{8}	...

[a]Based on 10 isolates per treatment (water = 20 isolates), and testing each isolate (5×10^9 conidia per isolate) on potato-dextrose agar amended with 1 μg/ml MBC-HCl and an equal number of conidia on 10 μg/ml HMB-HCl; seedlings treated 1 wk prior to inoculation.

[b]Estimates, colonies were too numerous for exact count.

[c]Data analysis by Student's *t*-test. The value of *P* indicates the probability that the frequency of tolerance in isolates from fungicide treated seedlings is the same as that from water-treated seedlings.

TABLE 4. Frequency of methyl-2-benzimidazole carbamate (MBC)- and thiabendazole-tolerant *Ceratocystis ulmi* isolated from fungicide-treated and untreated *Ulmus americana* trees[a]

Tree source	Total number	Number of isolates tolerant to: MBC (μg/ml) 1 (μg/ml)	10 (μg/ml)	100 (μg/ml)	1,000 (μg/ml)	TBZ (μg/ml) 1 (μg/ml)	10 (μg/ml)	100 (μg/ml)	1,000 (μg/ml)
Untreated	96	3	1	0	0	8	0	0	0
Treated	109	1	0	0	0	2	0	0	0

[a]Determined by radial growth on fungicide-amended potato-dextrose agar.

untreated elms in our study were considerably lower than they reported. Only about 3% (three of 96) of our isolates were tolerant to 1 μg/ml MBC-HCl as compared to 21% (six of 29) of their isolates. The highest level of naturally occurring MBC tolerance found in this study was 10 μg/ml (one of 96 isolates). Schreiber and Townsend, however, found three of their 29 isolates tolerant to 1,000 μg/ml MBC-HCl. Although the frequency of naturally occurring MBC tolerance was not their emphasis, we feel that their results may not be representative of *C. ulmi* populations either with or without prior exposure to benzimidazole-type fungicides.

From our studies there was no evidence that induction or selection for MBC or TBZ tolerance took place in large elms as a result of treatment with fungicides. However, when the wild-type isolate was inoculated into 6-mo-old American elm seedlings pretreated with the fungicides, there was evidence that benomyl (but not TBZ-H_2PO_2, MBC-H_3PO_4, or M&B 21914C) actually induced MBC tolerance. If, in fact, benomyl does induce MBC tolerance in *C. ulmi* its failure to do so in larger elms may be a reflection of the limited numbers of samples used in this study.

Hastie (7) showed that benomyl induced genetic instability in diploid strains of *A. nidulans*. Dassenoy and Meyer (3) concluded that 24- and 48-hour exposure to benomyl caused mutations which resulted in nutritional deficiencies in *Fusarium oxysporum* f. sp. *melonis* (Leach & Currence) Snyder & Hansen. Seiler (12), using the technique of Ames (1), found that MBC and other benzimidazole derivatives possessed significant levels of mutagenicity. Unfortunately, the relative mutagenicity of benomyl and MBC were not compared in the same biological test system.

The significance of the presence in nature of isolates of *C. ulmi* tolerant to benzimidazole fungicides in DED control is not certain. Brasier and Gibbs (2) concluded that it is unlikely that this would be a problem, but Schreiber and Townsend (11) felt that the effectiveness of benomyl and MBC for DED control may decline with the increased use of these chemicals. Caution is appropriate in the widespread use of closely related fungicides for a single purpose. It is, however, imperative that a fungicide arsenal, including a variety of fungicides with different modes of action, be available in the event that tolerant *C. ulmi* isolates develop to the benzimidazole and related fungicides.

Proper sanitation measures will become more important with increased use of systemic fungicides for DED control. The prompt removal of unsuccessfully treated elms will be of utmost importance if the failure of the treatment was due to *C. ulmi* strains tolerant to the fungicides. The spread of such strains would then be minimized.

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