Naturally Occurring Tolerance in Isolates of Ceratocystis ulmi to Methyl 2-Benzimidazolecarbamate Hydrochloride

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ABSTRACT

Twenty-nine isolates of *Ceratocystis ulmi* from various locations throughout the United States were tested for tolerance to methyl 2-benzimidazolecarbamate hydrochloride (MBC-HCl). Tolerance was measured by the growth of the isolates in a series of petri plates containing potato-dextrose agar amended with several concentrations of MBC-HCl. Six isolates (from Colorado, Massachusetts, New York, North Carolina, Tennessee, and Virginia) grew on agar amended with MBC-HCl concentrations ranging from 1 to 1,000 μg/g, whereas growth of the other 23 isolates was inhibited by 1 μg/g. The chemical tolerance of *C. ulmi* to MBC-HCl occurred naturally, and did not arise following exposure to MBC-HCl or other benzimidazole compounds.

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Numerous reports have appeared recently in the literature on the failure of benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate] and other benzimidazole compounds to control diseases in locations where these fungicides had been used successfully for several years. These failures have been traced to the development of fungus strains with high levels of tolerance to the fungicides (2, 5, 7, 9). Also, Benyephet et al. (1) developed benomyl-tolerant mutants of *Ustilago hordei* by ultraviolet irradiation. There are, however, few reports of naturally occurring tolerant fungus strains such as that of *Verticillium fungicola* (*V. malthousei*) reported by Wuest et al. (10).

Methyl 2-benzimidazolecarbamate hydrochloride (MBC-HCl) has demonstrated greater prophylactic and therapeutic activity than the commercially available wettable powder. Because of the emphasis given to benomyl and, more recently, the MBC derivative, as control agents for Dutch elm disease, we initiated this study to determine the existence and degree of tolerance to MBC-HCl of isolates of *Ceratocystis ulmi* (Buism.) C. Moreau.

MATERIALS AND METHODS.—Isolates of *C. ulmi* were obtained from Alabama (AL), Colorado (CO-D49, CO-D53, CO-D58, and CO-FM). Illinois (IL-C, and IL-D), Iowa (IA55), Maine (ME, and ME-O), Massachusetts (MA-A, MA-D, MA-B, MA-N, and MA-NH), Missouri (MO), New Hampshire (NH1), New Jersey (NJ4), New York (NY1, NY2, and NY4), North Carolina (NC56), North Dakota (ND), Ohio (OH66), Pennsylvania (PA12), Tennessee (TE), Virginia (VA2, and VA-B), and Wisconsin (WI). Some were isolated from diseased wood in 1971; others had been in culture for 6-10 years. Conidia of these isolates were screened for ability to grow on potato-dextrose agar (PDA) amended with 1 μg/g MBC-HCl.

The MBC-HCl was prepared according to the method of McWain and Gregory (6), sterilized through a Gelman GA-6 Metricel filter, incorporated (1 μg/g) into melted 2% PDA, and poured into petri plates. The pH of amended and nonamended agar in this, and subsequent, experiments varied from 5.6 to 5.9. Schleicher and Schuell (S and S) filter paper disks (12.7 mm in diameter) were placed on the PDA surface. A drop of a standardized conidial suspension of the test isolate was placed on each pad, and the diameter of the growing colony was measured periodically. To prepare conidial suspensions for tests, we placed a small amount of mycelium in 60 ml of potato-dextrose broth in 125-ml Erlenmeyer flasks, and agitated the flasks on a rotary shaker for 3 days. These suspensions were diluted to produce standard suspensions. We used three replications for the control, and six replications for each treatment. *Ceratocystis ulmi* isolates tolerant to MBC-HCl at 1 μg/g were tested first at 10 and 100 μg/g, and later at 10, 100, 500, and 1,000 μg/g in two subsequent experiments.

To determine if the fungitoxic effects of the treatments were fungicidal or fungistatic, the TE and OH66 isolates that failed to grow on PDA amended with MBC-HCl at 10 or 100 μg/g after 7 days were transferred to nonamended PDA, and their subsequent growth was recorded.

RESULTS.—Six of the 29 *C. ulmi* isolates including CO-D49, MA-A, NY4, NC56, TE, and VA-B grew on PDA amended with MBC-HCl at 1 μg/g. These isolates were obtained from areas where benzimidazoles had not been used (J. Faut, R. J. Stipes, and W. Sinclair, *personal communication*). Their growth rates (expressed as a percent of the controls grown on nonamended agar) were 39, 88, 95, 89, 0, and 41%, respectively, after 6 days, and 63, 100, 103, 90, 11, and 83%, respectively, after 13 days.

TABLE 1. Diameter of colonies of tolerant *Ceratocystis ulmi* isolates exposed to methyl 2-benzimidazolecarbamate hydrochloride (MBC-HCl) in potato-dextrose agar (PDA)

Isolates	Colony diameter (mm) after growth in PDA containing MBC-HCl for: 4 days — Concentration of MBC-HCl (μg/g) 0	10	100	500	1,000	11 days — Concentration of MBC-HCl (μg/g) 0	10	100	500	1,000
Experiment I										
CO-D49	27	0	0	...	...	70[a]	0	0	...	...
MA-A	27	2	0	...	...	70	25	8	...	...
NC56	24	18	8	...	...	70	38	21	...	...
NY4	43	22	6	...	...	70	48	22	...	...
VA-B	32	6	3	...	...	62	34	22	...	...
Experiment II										
NC56	17	...	5	6	2	48	...	16	17	6
NY4	32	...	8	9	6	61	...	23	21	14
VA-B	29	...	4	2	1	67	...	26	15	11

[a]The maximum net growth measured was 70 mm.

TABLE 2. Diameter of colonies of isolates of *Ceratocystis ulmi* transferred to unamended potato-dextrose agar (PDA) after 7 days of exposure on media amended with (MBC-HCl)

Isolate	Colony diameter (mm) attained on nonamended PDA after: 6 days — following 7 days on PDA with MBC-HCl at: 0 (μg/g)	10 (μg/g)	100 (μg/g)	9 days — following 7 days on PDA with MBC-HCl at: 0 (μg/g)	10 (μg/g)	100 (μg/g)	13 days — following 7 days on PDA with MBC-HCl at: 0 (μg/g)	10 (μg/g)	100 (μg/g)	17 days — following 7 days on PDA with MBC-HCl at: 0 (μg/g)	10 (μg/g)	100 (μg/g)
TE	59	0	0	70[a]	34	0	70	64	0	70	70	0
OH66	56	0	0	70	0	0	70	4	0	70	19	0

[a]The maximum net growth measured was 70 mm.

Tolerance of the isolates to MBC-HCl was not correlated with their growth rates on nonamended agar. Of the least rapid-growing isolates (MA-N, CO-FM, VA2, NJ4, and NH1), none was tolerant of MBC-HCl at 1 μg/g. Of the most rapid-growing isolates (VA-B, ND, and CO-D58), only VA-B was tolerant at 1 μg/g.

Ceratocystis ulmi isolates tolerant to 1 μg/g MBC-HCl were tested on PDA amended with 10, 100, 500, or 1,000 μg/g (Table 1). In Experiment I, CO-D49 failed to grow at 10 μg/g after 11 days. Isolates NC56, NY4, and VA-B grew after 4 days, and MA-A after 11 days at 100 μg/g. In Experiment II, isolates NC56, NY4, and VA-B all started to grow within 4 days on 100,500, and 1,000 μg/g MBC-HCl.

Growth rates of the isolates varied following transfer to nonamended PDA after exposure for 7 days to either 10 or 100 μg/g MBC-HCl (Table 2). Isolate TE was more tolerant than isolate OH66, because it grew to 49% of the control within 9 days, but OH66 grew only to 6% of the control after 13 days. Neither isolate grew within 17 days after exposure to 100 μg/g MBC-HCl.

DISCUSSION.—This is the first report of a widely distributed plant pathogen tolerant to a benzimidazole compound in the absence of prior exposure to benzimidazoles or mutagenic agents. *Ceratocystis ulmi* isolates vary widely in tolerance to MBC-HCl. Six of 29 isolates grew on media containing 1 to 1,000 μg/g MBC-HCl. None of the tolerant isolates were from areas where benzimidazoles had been used so tolerance did not arise after chemical treatment. Therefore, genes giving tolerance to MBC-HCl are present in natural populations of *C. ulmi*. The wide variability in tolerance of the isolates suggests multiple-gene control for this character.

Tolerant isolates were found in widely dispersed collections from Colorado, Virginia, Massachusetts, New York, North Carolina, and Tennessee. Tolerance also varied among isolates within the same state (Colorado, Massachusetts, New York, and Virginia), or even the same city (Denver isolates CO-D49, CO-D53, and CO-D58).

The significance of fungicide-tolerant isolates in disease control has been questioned. Wuest et al. (10) found a benomyl-tolerant isolate of *V. malthousei* to have less reproductive and pathogenic capability than sensitive strains. Warren et al. (9) and Littrell (5) questioned the ability of tolerant strains that arise in chemical control areas to survive in competition with the sensitive strains. The naturally occurring tolerant strains of *C. ulmi* used in this study were pathogenic (i.e., having been isolated from diseased elms) and appeared well adapted for survival among populations containing sensitive strains.

From our data and studies showing the development of tolerance to benzimidazoles in disease-control areas, it may be assumed that the effectiveness of benomyl and

MBC-HCl in controlling DED may decline as use of these fungicides increases. Benomyl and MBC-HCl are fungitoxic to *C. ulmi* in vitro at concentrations as low as 1 μg/g, whereas they are not phytotoxic to elm trees in concentrations in excess of 2,000 μg/g (3, 4, 8). Although several of the isolates we tested grew at about 30% of the controls, on media amended with 1,000 μg/g MBC-HCl, there is no way to compare concentrations in these in vitro studies with those in the branches of treated elms. Therefore, in vivo studies are needed to obtain a more accurate picture of the impact of MBC-HCl-tolerant *C. ulmi* isolates on chemical control of Dutch elm disease.

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