Growth of *Ceratocystis ulmi* on Low Concentrations of Hydrochloride and Phosphate Salts of Methyl 2-Benzimidazolecarbamate and on Thiabendazole Hypophosphite

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The authors express appreciation to W. K. Kwolek and A. M. Townsend for assistance in the statistical analysis of the data.

Accepted for publication 22 April 1981.

ABSTRACT

Schreiber, L. R., and Gregory, G. F. 1981. Growth of *Ceratocystis ulmi* on low concentrations of hydrochloride and phosphate salts of methyl 2-benzimidazolecarbamate and on thiabendazole hypophosphite. Phytopathology 71:1261-1263.

We compared the growth of 27 isolates of the Dutch elm disease (DED) fungus, *Ceratocystis ulmi,* on potato-dextrose agar amended with MBC-HCl (methyl 2-benzimidazolecarbamate hydrochloride), Lignasan BLP (methyl 2-benzimidazolecarbamate phosphate) (MBC-H_3PO_4), and Arbotect 20-S (2-(4-thiazolyl)benzimidazole hypophosphite) (thiabendazole hypophosphite) at 0.1, 0.3, 0.9, and 2.7 μg/ml of the acid salts and at 0.3 and 0.9 μg/ml of the organic moieties. Increase in colony diameter of the isolates varied significantly within each treatment as well as between fungitoxicants. MBC-HCl was the most fungitoxic chemical followed by MBC-H_3PO_4 and thiabendazole hypophosphite based upon concentrations of acid salts. In comparisons of the organic moieties, MBC-HCl and MBC-H_3PO_4 were equally fungitoxic and both were more fungitoxic than thiabendazole hypophosphite. All isolates (except one) that were tolerant to one fungicide also were tolerant to the others; the exceptional one was more sensitive to thiabendazole hypophosphite than to fungicides containing MBC. Thiabendazole hypophosphite was not only generally less fungitoxic at low concentrations than were the MBC compounds, but the colony diameters of some isolates even increased above that of the controls.

Additional key words: chemotherapy, tolerance, benzimidazole, carbendazim.

Lignasan BLP (methyl 2-benzimidazolecarbamate phosphate) (MBC-H_3PO_4) and Arbotect 20-S (2-(4-thiazolyl)benzimidazole hypophosphite) (thiabendazole hypophosphite) are registered for use in the United States for the control of Dutch elm disease (DED) which is caused by *Ceratocystis ulmi* (Buism.) C. Moreau. Methyl 2-benzimidazolecarbamate hydrochloride (MBC-HCl) was used experimentally to control DED in the United States and was registered for commercial use in Great Britain.

The efficacy of the benzimidazoles and their formulations in controlling DED was compared (1,2,4,12,15). Gibbs and Dickinson (5) reported that carbendazim (MBC) was superior to thiabendazole in disease control. Clifford et al (1) found that technical MBC-HCl was distributed better in elms than were the commercial formulations.

Fungicide rates in efficacy studies generally were at or above those registered for disease control (10,11,14). Although high concentrations occur initially at or near points of injection, the amounts of fungitoxicant in the crown vary with translocation rates, and with distances from injection points and decrease with time after injection. Elliston and Walton (3) reported decreases in fungitoxicant levels in American elms from 10–12 μg/ml to less than 1 μg/ml, 12 wk after injection of MBC phosphate. Significant declines in fungitoxicant levels were noted by Clifford et al (1) 55–97 days after injection of MBC-HCl into *Ulmus hollandica.* Thus, information on the fungitoxicity of low concentrations of the benzimidazoles MBC-HCl, MBC-H_3PO_4, and thiabendazole hypophosphite may be helpful in predicting and explaining their performance in disease control.

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MATERIALS AND METHODS

The growth, expressed as an increase in colony diameter, of 27 *C. ulmi* isolates was compared on potato-dextrose agar (PDA) amended with either MBC-H_3PO_4, MBC-HCl, or thiabendazole hypophosphite. Stock solutions of the fungitoxicants were sterilized by filtration through a Gelman Metricel Filter (pore size 0.45 μm) and then thoroughly mixed with molten, sterile PDA. Concentrations based upon the acid salt molecule were formulated at 0.1, 0.3, 0.9, and 2.7 μg/ml a.i.; concentrations based upon the organic moiety of the salt molecule were formulated to give equivalents at 0.3 and 0.9 μg/ml a.i. Plates with unamended agar were used for controls.

C. ulmi isolates obtained in the USA from ND, OH, WI, TN, NY, VA, CO, IL, MA, MO, NC, AL, WV, ME, and from Great Britain were maintained in a culture collection on PDA at 3 C. Conidia from these cultures were grown for 3 days at 22 C in potato-dextrose broth on a Gyrotory Shaker, Model G-10 (New Brunswick Scientific Co., New Brunswick, NJ 08901) and, the resulting suspensions were diluted to 10^6 conidia per milliliter with sterile distilled water. A drop of the diluted suspensions was placed on 1-cm square of moisture-permeable cellophane on PDA plates with 30 squares per plate and incubated at 22 C for 3 days.

After incubation, a cellophane square with the adhering mycelial mat was transferred to the center of a treatment plate containing 25 ml of amended or unamended PDA. These were incubated in the dark at 24 C for 5 days. Colony growth onto the agar was measured (in millimeters) by averaging two diameters of a colony at right angles to one another and subtracting 1 cm for the cellophane square. There were five replications per treatment. The growth in colony diameter, of the isolates of *C. ulmi* on amended agar is expressed as a percentage of that of the same isolate on the unamended PDA controls. Isolates were considered to be tolerant when they grew on chemical amendments in excess of 1 μg/ml.

RESULTS

The growth, expressed as increase in colony diameter, of 27 *C. ulmi* isolates on unamended agar and on agar amended with MBC-HCl, MBC-H_3PO_4, and thiabendazole hypophosphite is shown in Table 1. Isolates grew 13–62 mm on unamended PDA with significant differences in growth among many of them (LSD=4). The average colony diameter of all isolates after 5 days was 43 mm.

Comparisons of the average colony diameter of the isolates indicate that MBC-HCl was the most toxic fungicide at 0.1 and 0.3 μg/ml, followed by MBC-H_3PO_4 and thiabendazole hypophosphite. At 0.9 μg/ml, MBC-HCl and MBC-H_3PO_4 were equally toxic but both were significantly more toxic than thiabendazole hypophosphite. No significant differences were noted among the three chemicals at 2.7 μg/ml. The diameter growth of many isolates, however, varied significantly within a treatment as well as between treatments indicating differences in their chemical sensitivity as well as differences in the fungitoxicity of the benzimidazoles. Isolates tolerant to MBC-HCl and MBC-H_3PO_4, generally, were more tolerant to thiabendazole hypophosphite. One exception was isolate IL3, which was significantly more sensitive to thiabendazole hypophosphite than to MBC-HCl and MBC-H_3PO_4.

Some treatments and isolates interacted to increase colony diameter from about 20–33% above that on the unamended controls. In all but one instance, stimulatory treatments involved thiabendazole hypophosphite and were generally restricted to concentrations of 0.1 and 0.3 μg/ml although thiabendazole hypophosphite at 0.9 and 2.7 μg/ml stimulated the growth of CO3T and WIT, two tolerant isolates.

Comparisons of the average growth of *C. ulmi* isolates on amendments based on concentrations of either the acid salts or of the organic moiety portions of MBC-HCl, MBC-H_3PO_4, and thiabendazole hypophosphite at 0.3 and 0.9 μg/ml appear in Table 2. MBC-HCl and MBC-H_3PO_4 were more toxic than was thiabendazole hypophosphite at both 0.3 and 0.9 μg/ml based on concentrations of either the salt or the moiety. MBC-HCl was more toxic than was MBC-H_3PO_4 at 0.3 μg/ml based on the concentration of the salt. Both were equally toxic based on the concentrations at either 0.3 μg/ml of the moiety or at 0.9 μg/ml of either the moiety or the salt. A concentration of 0.3 μg/ml of the salt or moiety of thiabendazole hypophosphite was not toxic, whereas the moiety was more toxic than the salt at 0.9 μg/ml.

DISCUSSION

The efficacy of benzimidazoles in the control of DED was largely determined on the basis of concentrations equal to or above those recommended on the label. Fungitoxicant concentrations found in elm branches were significantly lower than the concentrations injected into the tree. Rapid decline in fungitoxic activity in leaves and twigs of injected trees, or incomplete chemical distribution was observed (1,3,8). Thus, chemical efficacy at lower concentrations may be important in control of DED.

TABLE 1. Growth of *Ceratocystis ulmi* on potato-dextrose agar (PDA) amended with MBC-HCl, MBC-H_3PO_4, or thiabendazole hypophosphite

		Growth (percent of controls) on PDA amended with acid salts (μg/ml)											
		0.1			0.3			0.9			2.7		
C. ulmi isolate	Growth (mm) on unamended PDA[a]	MBC-HCl	MBC-H_3PO_4	Thiabendazole hypophosphite	MBC-HCl	MBC-H_3PO_4	Thiabendazole hypophosphite	MBC-HCl	MBC-H_3PO_4	Thiabendazole hypophosphite	MBC-HCl	MBC-H_3PO_4	Thiabendazole hypophosphite
ND[b]	41	68	107	119*	9	30	126*	0	0	42	0	0	0
OH	44	72	109	117*	7	24	111	0	0	41	2	0	1
WI	49	72	99	125*	0	19	119*	0	0	27	0	0	0
TN	13	91	97	118*	18	38	120*	0	0	73	4	0	0
NY	44	76	99	119*	3	18	118*	0	0	26	0	0	0
VA	13	100	88	82	108	85	87	69	66	···	56	54	62
MA-B	39	64	80	101	9	21	97	0	0	26	0	0	0
IL3	40	71	82	105	10	23	117*	0	0	32	0	0	0
CO1	35	78	80	96	0	11	106	0	0	15	0	0	0
COFM	21	108	126*[c]	110	13	45	109	0	0	38	0	0	0
MO	47	68	81	101	5	18	103	0	0	28	0	0	0
CHT6	60	60	78	101	13	23	93	0	1	24	0	2	0
FG39	39	88	96	131*	24	40	133*	3	5	76	1	0	8
NC	48	101	103	99	30	60	99	0	2	86	0	0	8
C. Buism.	33	86	104	134	18	51	116	1	2	71	0	0	0
MA-NH	62	67	89	81	11	22	76	0	1	14	0	1	1
MA-N	42	82	95	92	25	42	88	1	8	48	0	4	9
AL-NA	48	79	81	105	12	33	98	0	3	55	0	0	4
SK-12	52	61	84	103	0	13	96	0	0	6	0	0	0
AL-A	55	60	77	92	9	21	92	0	0	19	0	4	0
TOOLE	44	70	87	98	13	25	97	0	0	34	0	0	0
WV	59	59	85	98	0	16	93	0	0	18	0	0	0
ME	57	62	80	93	1	16	86	1	1	16	0	0	0
W6	64	63	75	108	8	15	104	1	0	27	0	0	0
WIT[d]	49	100	112	121*	100	88	116	103	86	116	98	114	123*
CO3T	41	89	97	100	90	102	124*	93	101	131*	107	100	120*
IL3	40	91	109	100	93	102	74	75	96	24	29	46	0
AVG	43	78	93	103	26	39	103	15	17	42	12	13	13
LSD	4	8	8	10	8	9	8	6	5	8	7	9	4

[a] Colony diameters were measured after 5 days of incubation in the dark at 24 C. One centimeter, the size of the initial square of inoculum, was subtracted from each diameter measurement. Growth measurements represent an average of five replications per isolate.

[b] Origin of *C. ulmi* isolates: WI (Wisconsin); OH, C. Buism., Toole (Ohio); ND (North Dakota); TN (Tennesee); NY (New York); VA (Virginia); CO (Colorado); MA (Massachusetts); IL (Illinois); MO (Missouri); CHT6, FG39, SK-12, W6 (Great Britain); NC (North Carolina); AL (Alabama); WV (West Viringia); and ME (Maine).

[c] Percentages followed by an asterisk represent increases in colony diameter significantly greater than controls as determined by analyses of variance ($P = 0.05$).

[d] Tolerant isolates CO3T, WIT, and IL3 were selected, in vitro, from sensitive isolates following exposure to MBC-HCl.

TABLE 2. Average growth of 27 isolates of *Ceratocystis ulmi* on potato-dextrose agar amended with MBC-HCl, MBC-H_3PO_4, or thiabendazole hypophosphite based on concentrations of the acid salt molecule or of the organic moiety

Concentration[b] (μg/ml)	Average growth (percent of controls)[a]					
	MBC-HCl		MBC-H_3PO_4		Thiabendazole hypophosphite	
	Salt	Moiety	Salt	Moiety	Salt	Moiety
0.3	35[c]	33	46	32	101	97
0.9	27	27	28	27	49	39

[a] Amendments based upon concentrations of either the acid salt or organic moiety of the molecule.

[b] Two colony diameters were averaged after 5 days of incubation in the dark at 24 C. One centimeter, the size of the initial square of inoculum, was subtracted from each diameter measurement.

[c] For determining significant differences, LSD ($P = 0.05$) = 5.

Many of the *C. ulmi* isolates we tested varied significantly from one another in increase in colony diameter in response to the same treatment indicating differences in chemical sensitivity between isolates. The diameter growth of most isolates was inhibited at chemical concentrations at or above 0.3 μg/ml. The growth of other isolates was either not inhibited or was significantly increased above that of the controls by these treatments. These findings suggest that variability in chemical sensitivity of the isolate may play a role in the level of disease control achieved by chemotherapy. Work by Schreiber and Gregory (11) reported that disease control with MBC-H_3PO_4, at concentrations equal to or above label recommendations, may be ineffective in elms infected with chemically tolerant isolates.

The chemical nature of the benzimidazoles affected its fungitoxicity. Comparisons of MBC and thiabendazole based on concentrations of either the acid salt or organic moiety indicated greater fungitoxicity for MBC than for thiabendazole. This coincides with findings that MBC fungicides, in general, provide higher levels of disease control (5,6). Also, in all except a single instance, increase in the diameter of colonies of individual isolates was in response to thiabendazole. However, at 2.7 μg/ml, no significant differences were noted in the average fungus growth between any of the benzimidazoles. This indicates sufficiently high concentrations of thiabendazole were present to compensate for differences in activity between the benzimidazoles.

The acid form of the cardendazim also influenced fungus growth. MBC-HCl was more active than MBC-H_3PO_4 at 0.1 and 0.3 μg/ml but not at 0.9 or 2.7 μg/ml. Gibbs and Dickinson's (5) results suggest greater disease control with MBC-HCl than with MBC-H_3PO_4. Based on equal concentrations of the respective salts, solutions of MBC-HCl contain more MBC than do solutions of MBC-H_3PO_4. Thus, observed differences in fungitoxicity can be explained. This is substantiated by the equal fungitoxicity of amendments of the two formulations when concentrations were based upon the organic moiety rather than upon the total acid salt. In contrast, Kondo et al (7), found MBC-H_3PO_4 more toxic than MBC-HCl at 250 μg/ml.

Positive cross-tolerance between benzimidazoles occurs frequently (14,16). We found it to occur in three of four tolerant isolates. Nishijima and Smalley (9), however, found only one of four carbendazim-tolerant isolates tolerant to thiabendazole. Ruppel (10) found the degree of cross-tolerance in *Cercospora beticola* to be related to the fungus isolate, and the fungicide and its concentration. If benzimidazole-tolerant isolates reported in nature (11,13) occur in increasing numbers in the *C. ulmi* population, the usefulness of benzimidazoles will be limited. The proportion of positive to negative cross-tolerance among *C. ulmi* isolates, however, will influence the impact of chemical tolerance on control.

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