Comparative Toxicity of Gaseous Methyl Bromide to Ten Soilborne Phytopathogenic Fungi

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ABSTRACT

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The fungi were exposed to six concentrations of methyl bromide (MB) in a free-flowing system and the LD_{90} values were calculated. Concentration and time for each LD_{90} value were plotted using log concentration vs. log time, and the resulting plots were linear. Slopes of the curves for such plots varied with each fungus. By comparing mean concentrations per hour required between 3 and 30 hr to obtain LD_{90} values, fungal sensitivities were: most sensitive, *Phytophthora cinnamomi*, *P. citrophthora*, *P. parasitica*, and *Pythium ultimum*; moderately sensitive, *Armillaria mellea*, *Sclerotium rolfsii* (mycelia), and *Rhizoctonia solani*; and least sensitive, *Whetzelinia sclerotiorum* (mycelia and sclerotia), *S. rolfsii* (sclerotia), *Fusarium oxysporum* (mycelia), and *Verticillium albo-atrum* (mycelia and microsclerotia). Sclerotia were more resistant to MB than were mycelia of the same fungus. The response of a given fungus growing in agar was nearly identical to the same fungus growing in roots. The concentrations and times necessary to kill the respective fungi are useful information for evaluating field fumigations.

Additional key word: dosage response.

Methyl bromide (MB) has been used commercially to control soilborne phytopathogenic fungi for approximately 30 yr. We estimate that 3,300 metric tons were used in California in 1976. In spite of extensive usage, accurate data were not available on the actual toxicity of gaseous MB to the common, soilborne phytopathogenic fungi. With the development of methods for applying controlled and monitored concentrations of MB (8, 9) it became possible to measure responses of fungi and nematodes to that toxicant. This is a summation of our studies, believed to be the first on the comparative toxicity of MB to phytopathogenic fungi in which the concentration of gas actually applied was monitored and carefully controlled throughout an experiment. A specific report for the dosage response of *Phytophthora cinnamomi* (14), a generalized account for five of the fungi (15), and an abstract of this paper (16) have been published.

MATERIALS AND METHODS

General.—Fungi were exposed to mixtures of MB and air in a free-flowing system, and the combinations of time of exposure and concentration of MB required to kill 90% of the propagules were determined. Gaseous MB was mixed with compressed air and passed into a 12-outlet manifold (Fig. 1) as reported previously (8). The flow of gases from the manifold was regulated to 20 ml/min by restrictors made of tubes packed with Celite. The gases were bubbled through water, passed into reaction vessels (125-ml Erlenmeyer flasks) containing the fungi, and exhausted into a fume hood. Gas concentrations were monitored by attaching the outlet of a reaction vessel to a gas chromatograph. Concentrations of MB were controlled to approximately ± 1.5% by fine adjustments of the valve adjacent to the manifold. Such adjustments usually were necessary only once or twice in a 24-hr exposure period. Controls consisted of samples of the fungi plated-out at the start of each experiment and after exposure to flowing air in flasks for the duration of each experiment. Viability of these control propagules was 100%. There was no significant variation in response of fungi held in reaction vessels exposed to a given concentration of MB; thus one reaction vessel was used for a single treatment. Duplicated experiments were closely correlated.

The time to reach maximum concentration of MB in a reaction vessel from the start of fumigation was approximately equal to the time required to reach minimum concentration upon aeration. For example, when *P. ultimum* was fumigated with MB (26,500 μliter/liter), the concentration of the effluent from a reaction vessel rose to 80% of the input concentration in 10 min and 95% in 23 min. The curve for aeration was somewhat steeper, decreasing to 5% MB in 18 min. Almost identical curves were obtained when okra stems infested with *V. albo-atrum* were used.

An experiment consisted of one test of the effect of one concentration (C) of MB on one fungus exposed for various times (T), usually at 1 hr intervals (Table 1). Exposures were adjusted to include responses from 0-100% survival of the fungus for each C of MB. Standard linear dosage-response curves for each C were obtained

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by plotting percent survival (probit scale) versus T (log scale). Such curves typically were linear, very steep, and correlation coefficients were uniformly high. The T needed to kill 90% (LD_{90}) of the test propagules for a specific C could be estimated in this manner. In most cases the LD_{90} was estimated by examination of the data in tabular form. At least six different concentrations (5,100 - 50,800 μliter/liter) MB were tested for each fungus (except for *Fusarium oxysporum* f. sp. *apii* where four concentrations were used), giving six or more values for LD_{90}. The individual values of C and T for each LD_{90} were then plotted using log C (μliter/liter) as the ordinate and log T (hr) as the abscissa (Fig. 2). The resulting plots of the LD_{90} values were linear and the slopes of the curves varied with the fungus tested.

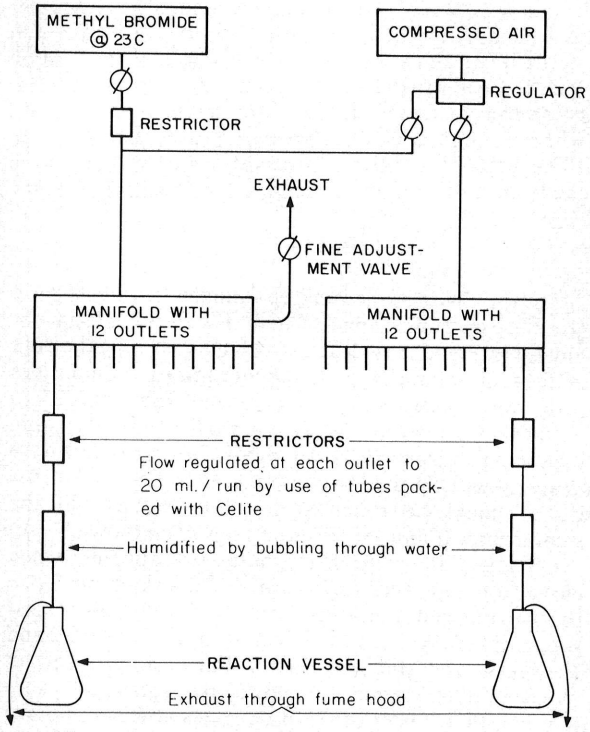

Fig. 1. Diagram of the free-flowing system used to fumigate various fungi.

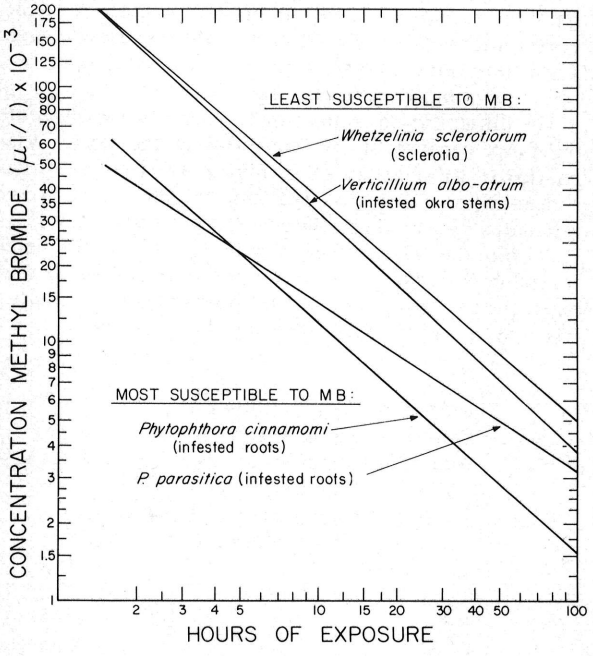

Fig. 2. Concentrations and times necessary to kill 90% of propagules. For clarity, points are not included; the regressions were calculated using six to 23 points per curve.

Specific methods (Table 1).—The fungi tested and the number of experiments with each were as follows: *Armillaria mellea* (Vahl) Quel., six; *Fusarium oxysporum* f. *apii* (R. Nelson and Sherb.) Snyd. & Hans., four; *Pythium ultimum* Trow, seven; *Phytophthora cinnamomi* Rands, 32: *Phytophthora citrophthora* (Sm. and Sm.) Leonian, eight; *Phytophthora parasitica*

TABLE 1. Concentrations (C) and times (T) used in experiments and values of C when T equals 1 and 100 hr. These data are useful for plotting LD_{90} values[a]

Fungus	Type of test material[a]	Range of: Concn. (μliter/liter $\times 10^{-3}$)	Range of: Exposure (hr)	Value of C in Fig. 2 when: T=1	Value of C in Fig. 2 when: T=100
Armillaria mellea	Mycelia	12.8-33.7	5.6-17.1	140	2.8
Fusarium oxysporum f. *apii*	Mycelia	15.6-34.0	8.8-18.7	134	2.8
Pythium ultimum	Mycelia	11.7-33.4	2.5-14.7	55	4.4
Phytophthora cinnamomi	Mycelia	8.3-38.1	1.5-18.5	69	2.7
	Infected roots	5.1-39.2	2.8-27.5	96	1.8
	Chlamydospores	5.1-40.0	1.4-22.0	51	1.8
P. citrophthora	Mycelia	16.5-32.3	3.3- 8.6	74	2.8
P. parasitica	Mycelia	10.3-33.7	3.5-17.9	85	3.0
	In roots	10.7-39.4	1.5-11.8	65	3.3
Rhizoctonia solani	Mycelia	11.2-34.3	3.2-27.5	74	5.6
Sclerotium rolfsii	Mycelia	11.0-39.4	3.7-26.5	79	5.0
	Sclerotia	11.4-32.9	7.9-62.0	84	8.2
Verticillium albo-atrum	Mycelia	15.6-38.8	6.0-25.7	128	6.3
	Infested stems	6.1-50.8	6.7-58.0	304	3.8
Whetzelinia sclerotiorum	Mycelia	11.8-33.9	6.8-27.0	165	4.3
	Sclerotia	11.8-39.4	8.5-25.5	280	3.8

[a] "Mycelia" refers to cultures growing on agar media. Usually various spore forms were avoided by culture practices; see text for exceptions.

(Dast.), 12; *Rhizoctonia solani* Kuehn, seven; *Sclerotium rolfsii* Sacc., 15: *Verticillium albo-atrum* Reinke and Berth., 30; and *Whetzelinia sclerotiorum* (Lib.) Korf & Dumont sunsu Purdy, 13. All of the fungi were grown on potato-dextrose agar (PDA) for 5 mm-diameter disks were cut from the advancing margins of the mycelia. Twenty disks were used for each treatment. After exposure to MB, the mycelial disks were aerated rapidly by attaching the reaction vessels to the air manifold and then tested for viability on PDA. Propagules were observed microscopically up to 5 wk and the percentage of the dead propagules was determined. In some cases, propagules presumed to be nonviable were split and added to nutrient broth and observed further.

The effect of MB also was determined on *P. cinnamomi* and *P. parasitica* in infected avocado or pine roots, respectively. Roots (1-2 mm diameter) from infected trees were agitated in water 10 min, cut into 1-cm lengths, dipped in 70% ethanol, placed on a selective agar medium ($P_{10}VP$), and incubated for 2 days at 24 C (23). Twelve roots positive for the fungus were retrieved and buried 1 cm deep in 50 ml of sandy loam soil in each reaction vessel. The soil contained 10-12% water (w/w). After fumigating, the aerated roots were washed in sterile water, plated-out on $P_{10}VP$ agar medium and incubated at 24 C. Observations of viability were made for up to 10 days or until they were unchanged for 3 successive days. Roots exposed to the LD_{90} dose and the next higher dose not showing evidence of growth of *P. cinnamomi* were removed, minced, plated on fresh medium, and observed 1 additional week.

Okra (*Hibiscus esculentus* L.) stems infected with *V. albo-atrum* were used to test the response of microsclerotia to MB. Stems of glasshouse-grown okra 2-3 mm in diameter were dried and sterilized with propylene oxide. Spore suspensions of the fungus were poured over stem pieces 5 cm long, which were steeped 30-45 min; the pieces then were removed and placed on moist filter paper in glass petri dishes, and incubated 2 wk at 24 C. Microsclerotia were formed throughout the stem pieces. The pieces were air-dried for 24 hr and then humidified in a reaction vessel by passing moist air from the manifold over them for 24 hr to attain approximately 20% moisture at each fumigation. After fumigation of four pieces per vessel, the stems each were cut into five pieces 1 cm long and placed on agar containing sterile chopped okra stems and streptomycin (30 μg/ml). Readings were made until there were no changes in counts of viability for 3 successive days.

Chlamydospores of *P. cinnamomi* were obtained from cultures 14 days old grown in V-8 juice broth (12). The resulting mycelia were washed, ground for 2 min in a blender, and filtered through cheesecloth. Chlamydospores were separated by low speed centrifugation, counted, and mixed with autoclaved sandy loam soil to provide 100-250 chlamydospores per gram of soil. The water content of the soil was adjusted to 15% (w/w). After fumigation, 23 g of the soil was shaken in 200 ml water and 1-2 ml of the suspension was added to each of five dishes and swirled in cooled liquid $P_{10}VP$ agar medium. The number of colonies that developed following treatment with MB was compared with the number of colonies that developed following exposure to air alone.

Sclerotia of *W. sclerotiorum* and *S. rolfsii* produced on PDA were mixed in 50 ml of sandy loam soil containing 11-14% water (w/w) and fumigated with MB. Usually 50 sclerotia of *W. sclerotiorum* in autoclaved soil, or 100 sclerotia of *S. rolfsii* in nonautoclaved soil were used per treatment. After fumigation, the sclerotia were washed in sterile water and sclerotia of *S. rolfsii* were plated-out on PDA containing streptomycin (50 μg/ml), chlortetracycline (50 μg/ml), and benomyl (15 μg/ml), whereas sclerotia of *W. sclerotiorum* were plated-out on PDA without additives. Observations of viability were made until there were no changes for 3 successive days.

RESULTS

The responses of the fungi to fumigation with MB are given in Fig. 2 and Table 1. Curves for the other fungi not plotted in Fig. 2 may be constructed by plotting the data in Table 1. All of the plots for concentrations and times (CT) were linear, with slopes varying from -0.5025 to -1.0400. Correlation coefficient values (Table 1) were high, ranging from -0.922 to -0.996. When the data were analyzed with the aid of a computer-assisted program, the curves could be classified in three groups but the significance, if any, of the groupings is unknown.

Obvious differences in response were apparent when curves for fungi such as *P. cinnamomi* and *P. parasitica* (both in infected roots) were compared with those for less susceptible fungi such as *W. sclerotiorum* (sclerotia) and *V. albo-atrum* (infested stem). For example, for an exposure of 5 hr, approximately 23,000 μliters/liter were required to kill 90% of the propagules of *Phytophthora* spp., but approximately three times more MB was required to kill 90% of the propagules of *W. sclerotiorum* and *V. albo-atrum*. Such comparisons are easily made when the curves for the various fungi are somewhat parallel, but not when they intersect. In cases where the curves intersect, the relationships of the responses at high C are reversed compared to those at low C (compare *P. cinnamomi* and *P. parasitica* in Fig. 2).

The difficulties in comparing the relative responses of the fungi because of the variations in the slopes was partially alleviated by comparing the average concentration ($\bar{C}$) for all the fungi tested. The $\bar{C}$ were calculated by integrating the curves, such as shown in Fig. 2, within the 27-hr time interval (3 to 30 hr) where reproducible LD_{90} data were obtainable. Most of the experiments were performed in this range of exposures. Each curve can be represented by the equation:

$$\log C = a \log T + \log b,$$

then $C = bT^a$ or $CT^{-a} = b$

where C = concn in μliters/liter

T = time in hours

a = slope (d log C/d log T)

b = C-intercept at T = 1

$$\text{Then } \bar{C} = \frac{b}{27} \int_3^{30} T^a dT = \frac{b}{27(a+1)} \left[T^{a+1} \right]_3^{30}$$

The a and b values were obtained by electronic calculator using the method of least squares.

The $\bar{C}$ values in Fig. 3 provide a basis of comparison for the susceptibility to MB of the various fungi, since $\bar{C}$ encompasses the entire region of effective time and concentration used in these experiments. The $\bar{C}$ of the Phycomycetes (*P. cinnamomi*, *P. parasitica*, *P. citrophthora*, and *Pythium ultimum*), the most susceptible fungi, were very similar, whereas the $\bar{C}$ for *W. sclerotiorum* (sclerotia) and *V. albo-atrum* (infested stems), and *F. oxysporum* var. *apii* (mycelia), the most resistant of the fungi, was approximately 2.5 times greater. Preparations of the other fungi had $\bar{C}$ which fell in the range between these groups.

Another basis of comparison is available since $\bar{C}$ interceps curves in Fig. 2 for all the fungi at approximately 12 hr. The values in Fig. 3 therefore have added interpretive value since they are the concentrations needed to yield LD_{90} in a 12-hr fumigation for all the fungi.

The sclerotia of a fungus always were more resistant to MB than its mycelium. With *P. cinnamomi*, mycelia were more resistant than chlamydospores to MB. However, with *P. cinnamomi* and *P. parasitica*, the responses of mycelia and infected root tissues were nearly identical.

The CTs of nine of the fungus preparations tested (Fig. 4, Table 2) were relatively constant over the ranges of exposures of 5,000-30,000 μliters/liter. With others, particularly *S. rolfsii* (sclerotia), *R. solani*, and *S. rolfsii* (mycelia), and *V. albo-atrum* (mycelia), great differences in CTs occurred, depending upon the concentration applied. This group, which is typified by *S. rolfsii* (sclerotia), was relatively insensitive to MB at 5,000 μliters/liter but relatively sensitive to the gas at 30,000 μliters/liter. The responses of the remainder of the fungi were similar to those for *W. sclerotiorum* (sclerotia) and *P. parasitica* (roots) in Fig. 4. Values of CT for all fungi were highest when low concentrations of methyl bromide were used.

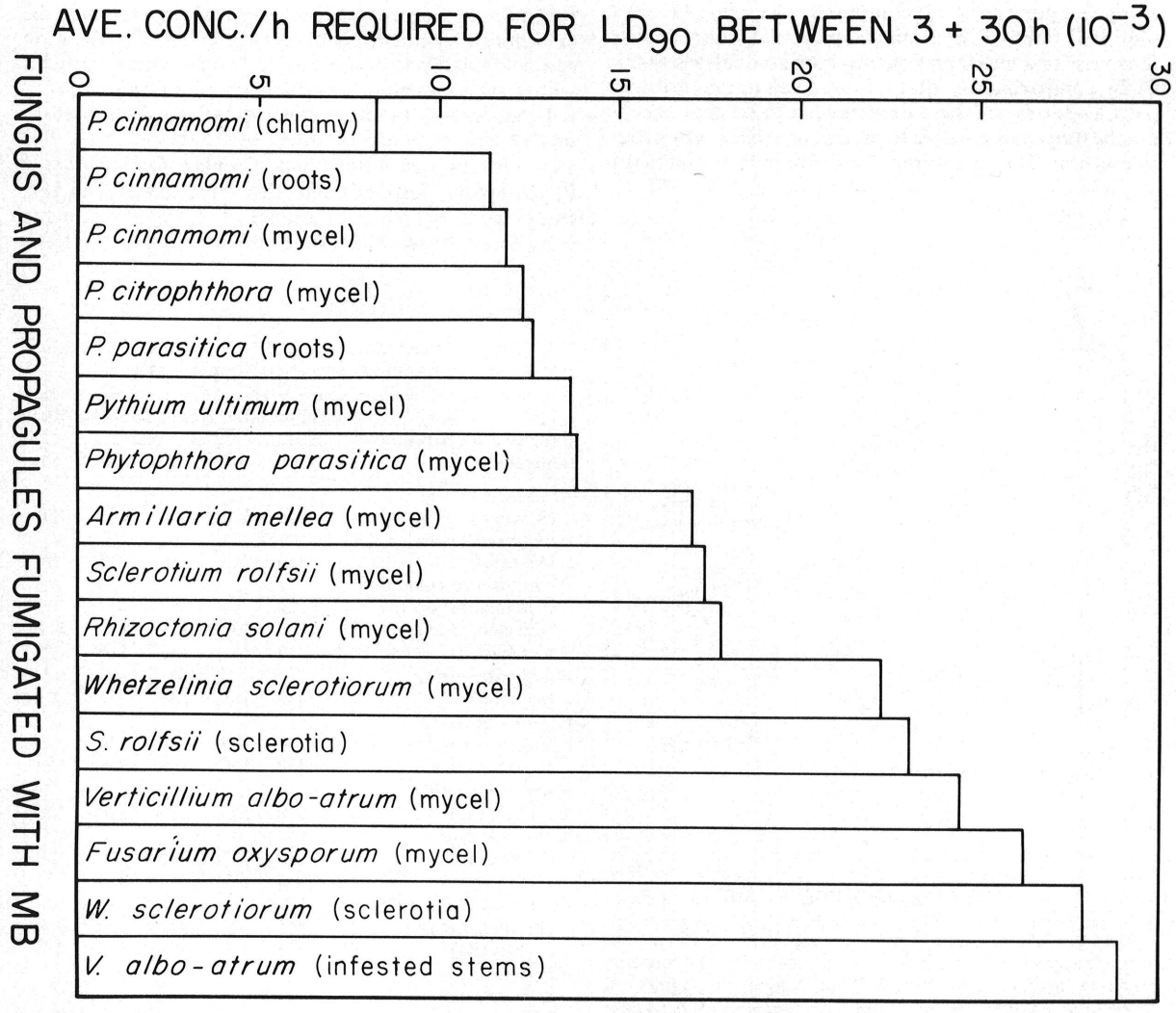

Fig. 3. Average concentration per hour ($\bar{C}$) of methyl bromide required between 3 and 30 hr to kill 90% of the propagules of 10 fungi showing relative sensitivites of the different fungi and different propagules of a given fungus to methyl bromide.

DISCUSSION

Since these are laboratory tests, some caution must be used in comparing the relative toxicity of MB to various fungi as they cannot be expected to be typical of all field conditions. The degree of maturity of sclerotia and chlamydospores, and thickness and age of infected roots are obvious conditions to consider. Also, moisture content and types of soil and host tissues profoundly affect fungal responses to MB (18, 19). We have attempted to standardize our tests as much as possible to avoid some of these errors. Field observation and other experiments have confirmed some of our data; i.e., *V. albo-atrum* is very resistant to MB (17), and buried sclerotia of *S. rolfsii* have been observed by us to survive very high concentrations of MB in field experiments. Obviously, when fungi exist as sclerotia or similar structures, the response to MB may be quite different from mycelium. Such factors must be considered when attempting to apply these data to the field.

We fumigate fields and assay the soil air at various depths to determine the MB concentration (10). While C at a given point in soil is continually changing, usually a plot of C versus T after initiation of fumigation shows a sharp rise to a maximum, then a more gradual decrease in C. By comparing the total CTs attained in the field with the CTs shown by these experiments to be necessary to kill the fungi, it is possible to predict whether a fumigation is a success. The data in Fig. 3 are especially useful in this regard, since they are generalized comparisons of the fungi to MB.

The fact that the fungi were uniformly more sensitive to MB when exposed to high concentrations than to low concentrations (Fig. 4) is very significant in the strategy of the use of MB for control of fungi in field soils. Interpretation of the data demonstrates that it is important to use sufficient amounts of MB initially to obtain as high a concentration of MB as possible in the soil profile.

Although we can predict a kill of 90% of the propagules of a fungus by treatment with MB, we can not be positive that the remaining 10% of the propagules would be unable to cause disease. The slopes of the dosage response curves are very steep and the time required to kill 99% of the propagules at a given concentration of MB is only a few minutes longer than the time required to kill 90%. Therefore, if the total CTs in a field fumigation were in excess of those required to give the LD_{90} values, it is probable that most of the population would be killed and commerical control of the disease would be attained. With organisms such as *S. rolfsii* and *W. sclerotiorum*, the number of propagules (sclerotia) in soil may be determined by direct counts, so the effect of fumigation can be bioassayed. It appears to be much more difficult with a successful soil inhabitant such as *P. ultimum*.

In laboratory practices these data have been used to predict the response of other organisms to MB. For example, by reasoning that *Glomus fasciculatus*, a Phycomycete, responds like the Phycomycetes in this study, we were able to adjust CTs in initial experiments very close to those which were needed to obtain the LD_{90}

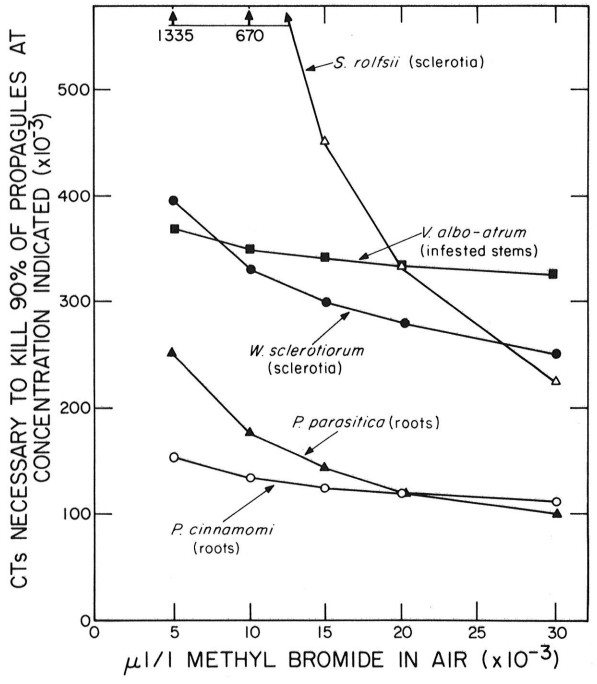

Fig. 4. Relationships between concentration of methyl bromide applied and the concentrations (μliters/liter) × time (hr) products necessary to kill 90% of fungal propagules of *Sclerotium rolfsii*, *Verticillium albo-atrum*, *Whetzelinia sclerotiorum*, *Phytophthora cinnamomi*, and *Phytophthora parasitica* at the various concentrations used.

TABLE 2. Concentration-time (CT) required for LD_{90} values at specific concentrations of methyl bromide (MB).

Fungus + preparation fumigated	CT required at concn of MB ($\times 10^{-3}$)				
	5	10	15	20	30
Armillaria mellea					
(Mycelia)	250	220	206	196	180
Pythium ultimum					
(Mycelia)	385	215	150	118	84
Phytophthora cinnamomi					
(Chlamydospores)	120	94	80	72	63
(Infected roots)	153	137	129	122	114
(Mycelia)	210	155	132	116	99
P. citrophthora					
(Mycelia)	230	165	138	122	105
P. parasitica					
(Infected roots)	250	175	141	120	99
(Mycelia)	250	190	168	148	126
Rhizoctonia solani					
(Mycelia)	610	350	255	200	150
Sclerotium rolfsii					
(Mycelia)	510	317	240	200	150
(Sclerotia)	1,335	670	450	334	225
Verticillium albo-atrum					
(Infested stems)	365	350	345	330	330
(Mycelia)	725	490	398	350	276
Whetzelinia sclerotiorum					
(Mycelia)	395	330	300	280	252
(Sclerotia)	370	353	345	340	330

response (11). This saved time and materials and avoided the need for performing numerous preliminary experiments. In the same manner, knowing that nematodes are much more sensitive to MB than are fungi, initial CTs were chosen that came close to actual values needed (24).

Many have formulated equations showing that the effect of a poison is a function of C and T. Busvine (1) discussed this and mentioned a first approximation formula CT=W (Haber's formula) but gave no reference. In 1921 Quayle and Knight (21) commented that percentage of dosage multiplied by time gave a constant, and later Knight (7) stated more precisely that "...time × concn equalled a constant." The concept was expounded by toxicologists working with insects (6, 20, 22), nematodes (2, 4, 5, 24),and fungi (3, 14). Some researchers ignored the fact that CT plots for lethal doses were not linear over the whole range of C and T. Busvine (1) and Sun (22) discussed this at length. Presumably, at low C, the poison is neutralized or excreted, and, if the rate is constant, the CT curve would asymptotically approach the highest tolerated C. Flury [cited by Busvine (1)] proposed the equation $(C-C_o)T = W$ to introduce the C_o term for this toleration effect. The toleration effect does not hold with all poisons and, since we did not use these extremes in our experiments, the response of fungi to MB is unknown. The other deviation from linearity occurs with short exposures to high doses. Peters [cited by Busvine (1)] regarded this deviation as due to saturation of tissues of the organism and proposed the variation, $(C-C_s)T = W$ of Haber's formula. The variable C_s represents the excess of concentration over the saturation of tissue and is therefore not a constant. Later Kenaga (6) made a graphical analysis similar to ours (Fig. 2) and cautioned that plots of exposures less than 1 hr or greater than 24 hr should not be used in his system. We also found extreme exposures to be unreliable, which was one of the reasons for selecting exposures of 3-30 hr as being most typical.

Others have developed special equations to better represent the relations between data obtained when high concentrations and short exposures or low concentrations and long exposures are included. Moore (13) proposed that $CT^x = K$ fitted the relationship, whereas Busvine used $C^nT = W$. The two equations are identical because $x = 1/n$ and $K = W^{1/n}$, where x = slope d log C/d log T and n is the reciprocal. Our equation, $CT^{-a} = b$, considers that the plots of log C, log T are linear, within limits, as verified by the high correlation coefficients. Then a is the slope and b the intercept of C at unit time, T. Thus, by this treatment of the log C vs. log T plot of Sun's data (22, Table 13) for *Tribolium confusum* larvae fumigated with CS_2, the correlation coefficient of linearity was −0.999, the slope was −1.1222 and the calculated intercept at 1 hr was 254 mg/liter.

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