Dosage Response of Phytophthora cinnamomi to Methyl Bromide

Donald E. Munnecke, James L. Bricker, and M. J. Kolbezen

Professor of Plant Pathology, Staff Research Associate III, and Chemist, respectively, Department of Plant Pathology, University of California, Riverside 92502.

We wish to thank Howard Ohr and Steve D. Campbell for their assistance.

A portion of this research was supported by the California Avocado Advisory Board.

Accepted for publication 21 February 1974.

ABSTRACT

Dosage responses of *P. cinnamomi* to methyl bromide (MB) were determined in 48 experiments by fumigating the fungus in a free-flowing system at concns from 4,000 to 38,700 μliters/liter (vol) for exposure times 1-46 h. The various LD_{90} values were linearly correlated by plotting the log concn (μliters/liter) vs. log time (h). In this way concn × time products (CT) for all LD_{90} values, within limits, were obtained. The slopes of the CT regression lines obtained for *P. cinnamomi* growing on agar, in living roots, or existing as chlamydospores in soil were almost identical, indicating that the response of *P. cinnamomi* to MB was uniform, regardless of the type of test material used. Analysis of the three CT curves indicated that chlamydospores were the most sensitive to MB.

Phytopathology 64:1007-1009.

There are obvious gaps in our knowledge of the behavior of gaseous fungicides in the soil. One of these is that we do not know the accurate dosage responses of soil-borne plant pathogens to various fumigants. With the advent of the development of equipment and methods for accurately and continuously applying carefully controlled and monitored concns of methyl bromide (MB) to an experimental system (3, 4), it has become possible to accurately measure dosage responses of fungal (7) and nematodal (10) pathogens to MB. This study is a continuation of our work along these lines, using *Phytophthora cinnamomi* Rands, an ubiquitous root pathogen, which can persist in soil in dead avocado roots for many years (11). The study was designed to show the dosage response of *P. cinnamomi* when it was growing on agar, in infected roots or as chlamydospores in soil during fumigation.

MATERIALS AND METHODS.—*Application of MB.*—The apparatus used to fumigate materials is shown in Fig. 1. The details for applying MB and monitoring the system have been described previously (3, 8). A regulated flow of MB gas was mixed with compressed air and passed into a manifold (visible at top right in Fig. 1). The flow of gas from the manifold was controlled at 20 ml/min by restrictors of Celite packed in tubes. The mixed gases were humidified by bubbling through water and led into 125-ml Erlenmyer flasks which served as reaction vessels. The gas escaped from the vessels to another manifold and was exhausted through the fume hood. Gas equilibration was rapid, hence sorbtive factors were not a problem in attaining the desired concn of MB. Monitoring was done by connecting a vessel outlet directly to a gas chromatograph. There were 12 outlets for MB and 12 for air only, which served as controls. For clarity, only four reaction vessels are displayed in Fig. 1: in some experiments up to 15 vessels were used. At times the contents of three reaction vessels were used as a sample for each time interval, but no significant variation was observed between vessels, so one flask per time period was adequate. Controls always were made at times corresponding to the beginning, middle, and last exposure. Passage of humidified air did not affect viability of *P. cinnamomi*.

Method of testing P. cinnamomi.—*P. cinnamomi* culture PC 40 obtained from G. A. Zentmyer was used for in vitro studies, while naturally infected avocado roots collected from a commercial grove near Fallbrook, Calif. were used for in situ studies.

On agar medium (19 experiments).—Twenty 3-day-old disks of *P. cinnamomi* (5 mm in diam), obtained from growing margins of cultures on glucose-potato agar supplemented with yeast extract (GPAYE), were placed in individual reaction vessels and exposed to concns of MB ranging from 9,200 to 36,600 μliters/liter for periods up to 27 h. Usually treatments were separated by 1-h intervals. After exposure 20 disks were plated-out on GPAYE plates at 24 C and observed for signs of visible growth for 1-7 days. Disks not showing growth after exposures corresponding to the LD_{90} dose, as well as those not growing after exposure to the next highest dose, were removed, aseptically split in half, and incubated in nutrient broth for several days to test viability. Usually no growth of *P. cinnamomi* was observed upon re-culturing, but in the few cases when growth did occur, LD_{90} values were recalculated accordingly.

In infected avocado roots (19 experiments).—Avocado roots, approximately 1.5 - 3.5 cm × 1-2 mm, naturally infected with *P. cinnamomi*, were treated with 4,500 - 38,700 μliter/liter MB for 1-46 h. Before being exposed to MB, roots were agitated in water for 10 min, cut into 1-cm lengths, dipped in 70% ethanol, plated-out on a selective medium, and incubated for 2 days at 24 C. The cornmeal base medium, ($P_{10}VP$) contained: Pimaricin, 10 μg/ml; Vancomycin, 200 μg/ml; and PCNB, 100 μg/ml (9). Twelve roots positive for *P. cinnamomi* on the medium were removed and added to each reaction vessel containing 25 ml sandy loam soil, making a depth of 1 cm. An additional 25 ml soil was added so that the roots were buried 1 cm deep in the flasks. The soil contained 12% moisture. MB was introduced above the soil. After treatment roots were retrieved, washed in sterile water, plated-out on $P_{10}VP$ medium in petri dishes, and incubated at 24 C. Observations for 1-10 days were made until viability readings for at least three successive days were unchanged. Those roots not showing evidence of *P. cinnamomi* after 10 days at the LD_{90} and the next

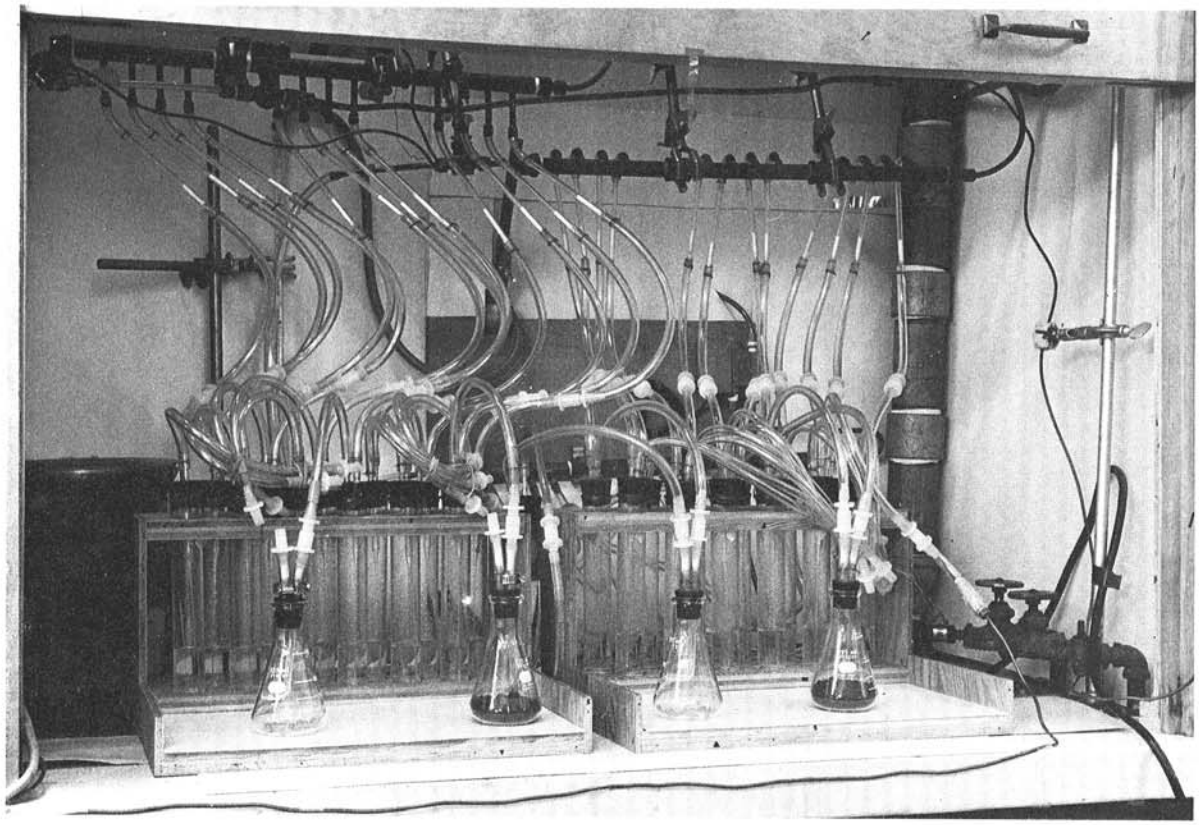

Fig. 1. Apparatus used to continuously apply controlled and monitored concns of MB in humidified air (for clarity, only four reaction vessels are connected to the apparatus). MB was mixed with air at the top right, passed through restrictors into tubes containing water, and then into reaction vessels (125-ml Erlenmyer flasks) containing *Phytophthora cinnamomi* prepared in various ways. Exhaust gases were led by tubes to the exhaust port of the fume hood. Air only was passed through the manifold system at the left. Gas concn was monitored directly by connecting an inlet or outlet of a reaction vessel to a gas chromatograph (not shown).

successive treatment were removed, minced with a scalpel, plated on fresh P_{10}VP agar, and observed another week for growth of the fungus. LD_{90} calculations were made on the basis of both platings.

As chlamydospores mixed in soil (10 experiments).—Chlamydospores of *P. cinnamomi* were obtained from cultures growing 2 wk in the dark at 24 C on 25 ml clarified V-8 juice broth (6) in 100 × 15 mm glass petri dishes. Four mycelial mats were washed four times in water, ground in a Waring Blendor, and filtered through four layers of cheesecloth. After two slow-speed centrifugations for 30 sec each (0-400 *g*), the chlamydospores were concd in the pellet fraction, mixed with water, and counted using a nematode counter. Sufficient chlamydospore suspension was thoroughly mixed in freshly autoclaved sandy loam soil to yield 100-250 spores per g soil. At the same time the moisture content of the mixture was adjusted to 15% by wt. Twenty-three g of chlamydospore-infested soil was added to each reaction vessel and treated with MB. After fumigation, the soil was shaken in 200 ml sterile water for 5 min on a rotary shaker. Depending upon the number of chlamydospores in the soil, 1-2 ml were added to each of five petri dishes and swirled in cooled, liquid P_{10}VP agar medium. After 3-7 days at 24 C the number of colonies growing in the agar medium were counted. Each colony was presumed to have arisen from one chlamydospore. Evaluation of effect of MB was done by comparing the number of colonies in MB-treatments with those arising in air-only treatments. An average of 7.7 colonies were produced per plate in the air-only treatments.

Assessment of data and expression of dosage responses (LD_{90}, CT).—The main objective of the study was to determine the product of the concn (μliters/liter) × time of exposure (h) necessary to kill 90% (LD_{90}) of the test propagules. This was called the CT value and was expressed in thousands (K). To do this, individual experiments were made using a single concn of MB for varying times. LD_{90} values for each concn of MB were obtained by inspection of the data or by graphical means. When plotted, the curves were so steep that arithmetic plots instead of the usual semi-log plots were used. This is portrayed in Fig. 2-A where the curve is nearly linear rather than more typically sigmoidal, as is usual with other systems. When enough values for LD_{90} were obtained from separate experiments involving a range of concns of MB, data were expressed by plotting log concn (μliters/liter) vs. log time (h) for each LD_{90} value. Within limits, response was linear and the CT was obtained directly from the graph.

RESULTS.—Some of the results of 48 experiments are presented in Fig. 2. Response of *P. cinnamomi* in vitro to

MB nearly was identical to that of *P. cinnamomi* growing in infected roots (Fig. 2-B, C). This is indicated by the fact that both the slopes and positions of the dosage response curves were similar, resulting in nearly identical CT values as follows: in roots, 105K-148K; on agar, 105K-156K. Chlamydospores of *P. cinnamomi* reacted to MB similarly to the other two preparations of the fungus, but they were more sensitive to the fumigant (Fig. 2-D). This is indicated by the fact that the slope of CT was the same, but CT values were lower, being 84K-116K.

DISCUSSION.—In determining dosage responses for pathogens to gaseous fungicides such as MB, it is obvious that response will not be linear at all time-concns used. This has been shown previously by Hague (1, 2). Probably the only way that such departure from linearity may be determined is by experimentation with very high and very low doses, but these entail problems in interpretation of data (for example, high concns are difficult to apply and assay properly, low concns are complicated by metabolic effects due to the long exposures involved). It is for these reasons that no effort has been made to extrapolate our data in Fig. 2-B, -C, and -D beyond actual experimental data obtained.

By plotting LD_{90} values as in Fig. 2-(B-D), useful curves are obtained, since they enable us to calculate the CT (concn × time) necessary to produce a lethal (LD_{90}) dose to *P. cinnamomi*. Since the slope of the curves is not 1.0, this CT varies, being lowest at the highest concn and highest at the lowest concn. By using the standard CT curve, however, CT are easily obtained for the ranges of 3,000 - 35,000 μliter/liter for 3-30 h. Such CT values have both practical and theoretical applications. In practical use, it is possible to assay commercial field fumigation using MB (5) and to determine CT obtained at various profiles in the soil. By referring to CT curves, such as these for *P. cinnamomi,* one can quickly estimate whether a field fumigation would be adequate to control the pathogen before planting. As more facts are accumulated on DR of various pathogens to fumigants, it is likely that such data will be put to use to provide quality control of field applications.

CT determinations have scientific usage as well. In experiments involving combinations of chemicals with biocontrol mechanisms, they may be especially pertinent. For example, it is possible to treat *P. cinnamomi* with concns of MB which are known to be sub-lethal. Such sub-lethal doses might predispose the pathogen to attack by saprophytes and result in killing the fungus with doses of fungicide far less than is required normally. In this manner less use of a toxicant might be possible. This sort of application has been used successfully by members of this laboratory with *Armillaria mellea* and MB (8).

It appears significant that CT of *P. cinnamomi* growing on agar was nearly identical to that obtained from roots. Since infected roots are more difficult to work with experimentally than agar cultures of *P. cinnamomi,* this allows a researcher to use in vitro materials with a reasonable assurance that results are akin to those found in nature.

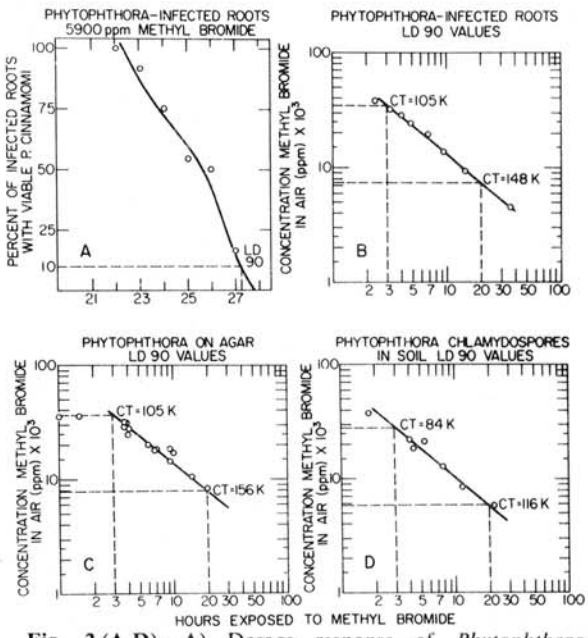

Fig. 2-(A-D). **A)** Dosage response of *Phytophthora cinnamomi* in avocado roots when exposed to 5,900 μliters/ml (vol) MB for 22-27 h; **B)** concn × time products (CT) for LD_{90} values for *P. cinnamomi* exposed while in avocado roots. **C)** CT for LD_{90} values for *P. cinnamomi* exposed while growing on a laboratory medium, **D)** CT for LD_{90} values for *P. cinnamomi* exposed while existing as chlamydospores in soil.

LITERATURE CITED

1. HAGUE, N. G. 1959. Effect of methyl bromide fumigation on the potato eelworm. Plant Pathol. 8:68-72.
2. HAGUE, N. G., and U. SOOD. 1963. Soil sterilization with methyl bromide to control soil nematodes. Plant Pathol. 12:88-90.
3. KOLBEZEN, M. J., and F. J. ABU-EL-HAJ. 1972. Fumigation with methyl bromide. I. Apparatus for controlled concentration, continuous flow laboratory procedures. Pestic. Sci. 3:67-71.
4. KOLBEZEN, M. J., and F. J. ABU-EL-HAJ. 1972. Fumigation with methyl bromide. II. Equipment and methods for sampling and analyzing deep field soil atmospheres. Pestic. Sci. 3:73-80.
5. KOLBEZEN, M. J., D. E. MUNNECKE, L. H. STOLZY, W. D. WILBUR, F. J. ABU-EL-HAJ, and T. E. SZUSZKIEWICZ. 1974. Factors affecting deep penetration of field soil by methyl bromide. Hilgardia (In press).
6. MIRCETICH, S. M., G. A. ZENTMYER, and J. B. KENDRICK, JR. 1968. Physiology of germination of chlamydospores of Phytophthora cinnamomi. Phytopathology 58:666-671.
7. MUNNECKE, D. E., W. D. WILBUR, and M. J. KOLBEZEN. 1970. Dosage response of Armillaria mellea to methyl bromide. Phytopathology 60:992-993.
8. OHR, H. D., D. E. MUNNECKE, and J. L. BRICKER. 1973. The interaction of Armillaria mellea and Trichoderma spp. as modified by methyl bromide. Phytopathology 63:965-973.
9. TSAO, P. H., and G. OCANA. 1969. Selective isolation of species of Phytophthora from natural soils on an improved antibiotic medium. Nature 223:636-638.
10. VAN GUNDY, S. D., D. MUNNECKE, J. BRICKER, and R. MINTEER. 1972. Response of Meloidogyne incognita, Xiphinema index and Dorylaimus sp. to methyl bromide fumigation. Phytopathology 62:191-192.
11. ZENTMYER, G. A., and S. M. MIRCETICH. 1966. Saprophytism and resistance in soil by Phytophthora cinnamomi. Phytopathology 56:710-712.