Effect of Methyl Bromide or Carbon Disulfide on Armillaria and Trichoderma Growing on Agar Medium and Relation to Survival of Armillaria in Soil Following Fumigation

Donald E. Munnecke, M. J. Kolbezen, and W. D. Wilbur

Professor of Plant Pathology, Chemist, and Staff Research Associate IV, respectively, Department of Plant Pathology, Univ. of Calif., Riverside 92502.

We wish to thank F. Abu-El-Haj for assistance in these studies.

Accepted for publication 20 April 1973.

ABSTRACT

The in vitro responses of *Armillaria mellea* and *Trichoderma viride* to methyl bromide or carbon disulfide were studied by determining the effects on linear growth of the fungi on an agar medium. Visible growth of *A. mellea* ceased almost immediately upon exposure to methyl bromide at 600, 1,200, or 2,400 ppm (v/v) for periods up to 24 days, and did not resume again for varying periods after the gas was removed. For treatments above 1,000 ppm, and exposures of 1 to 12 days, the lag period (which includes duration of exposure to the toxicant plus the period after treatment before growth resumed) was approximately 20 days with few exceptions. *T. viride* was more resistant than *A. mellea* to methyl bromide, and was capable of growing during treatment with concns of 600 and 1,200 ppm of methyl bromide. At 2,400 ppm growth ceased, but resumed almost immediately upon removal of the gas.

The responses of the two fungi to treatment with carbon disulfide were similar to those observed with the methyl bromide treatments. Both fungi were more tolerant of carbon disulfide than of methyl bromide.

In postulating how *T. viride* may become antagonistic to *A. mellea* after fumigation, the supposition is made that *A. mellea* must be weakened in some way. The lag period observed for *A. mellea* following fungicide application may be an indication of such weakening. It is feasible that *T. viride* is able to exploit this period and to exert its antagonistic action toward *A. mellea* at that time.

Phytopathology 63:1352-1357

Additional key words: antagonism in relation to fumigation, growth lag following fumigation.

There is considerable evidence that *Armillaria mellea* (Vahl) Quel. is killed by some sort of biologic activity which occurs following treatment of soil with carbon disulfide or methyl bromide; whereas, in natural soils, *A. mellea* may exist for many years, presumably in the presence of its antagonists. Various authors (1, 2, 3, 4, 5, 7, 9) have reported that *Trichoderma* spp. are intimately involved in the

destruction of *A. mellea* in soil, particularly following treatment of soil with carbon disulfide. Recently evidence (9) has been adduced that *Trichoderma* spp. are highly correlated with the decline in viability of *A. mellea* following exposure to sub-lethal concn of methyl bromide and burial in nonsterile soil.

If an antagonist, presumably *Trichoderma* spp, is to be active against *A. mellea* in the field following fumigation, it must be more resistant to the fumigant than is *A. mellea*. The experiments reported herein were designed to test this hypothesis by determining the response of *A. mellea* and *T. viride* growing on agar medium to carefully controlled doses of methyl bromide or carbon disulfide. An attempt is made to relate the responses of the two fungi to the toxicants and to the antagonistic mechanisms that presumably exist in fumigated soil harboring the two fungi.

MATERIALS AND METHODS.—*Cultures used.*—Isolate D-73, *Armillaria mellea* (Vahl) Quel. and isolate A-19, *Trichoderma viride* Pers. ex Fr. were used. Both cultures have been used in laboratory and field experiments by us for many years (10).

Medium.—Citrus agar medium was used for all experiments. The medium was prepared by boiling 200 g dried ground citrus sucker in one liter of water for 20 min, filtering, and using the filtrate to dissolve 20 g sucrose and 15 g agar. The concoction was diluted with distilled water to make one liter and sterilized in an autoclave.

Fumigant gas mixtures.—A method for preparing desired concn of methyl bromide in air at continuous flow, and the analysis of the input and output concn to each set of tubes by gas chromatography has been described by Kolbezen et al. (6). Carbon disulfide concn were prepared by bubbling nitrogen through liquid CS_2 at room temperature in a pressurized system to obtain a mixture presumably saturated with CS_2. This was passed through helically wound copper tubing and a condensate collecting reservoir both maintained at 5 C in a water bath. The rate of flow of the resulting concentrate of CS_2 of constant composition (ca. 14%, v/v) was controlled by restrictors and the mixture introduced into a manifold system to mix with streaming air as described for methyl bromide (6). A detailed description of the system is in preparation. Methyl bromide or carbon disulfide at the predetermined concn in air was passed over the surface of the fungal colonies and exhausted into a hood. The rate of flow was 20 ml per min. Controls consisted of similar arrangements with air-only being passed over the cultures. Concns of methyl bromide used were approximately 600, 1,200, or 2,400 ppm in air, or as otherwise stated in the text or figures.

Growth tubes.—The fungi were grown on citrus agar (CA) medium in glass tubes 350 X 25 mm, open at each end. An experimental unit consisted of four tubes containing the growing fungal cultures linked in series for each concn/time. Rubber stoppers were fitted onto each end of the tubes with 5-mm glass tubing protruding through them to allow gas exchange. Sterilized growth tubes were half-filled with 85 ml sterile melted CA which was cooled in a horizontal position, so that the inlet and outlet tubes were not covered by the solidified agar. The medium filled approximately half of the cross-sectional area of the tubes. Pieces of fungus inoculum were placed on the agar at one end of the tubes and the rhizomorphs or mycelia were allowed to grow about 100 mm linearly until the daily rate of growth was uniform. The growth rates were approximately 10 mm/day for *A. mellea* and 12 mm/day for *T. viride*. The fungi were then exposed to methyl bromide or CS_2 at carefully regulated concn. After various treatment periods, filtered air was passed through the tubes. Linear growth of the fungi was measured during and following fumigation. The experiments were terminated when growth rates equalled initial rates, or when cultures reached the opposite end of the growth tubes.

RESULTS.—Experiments showed that passage of methyl bromide through the tubes in a series left no harmful residues in the agar, and that there were no detrimental effects to *A. mellea* subsequently grown on the treated agar medium. There was no difference in response between the first and terminal tubes in a series. Analysis of agar after a fumigation and air purge showed no residual bromide ion or absorbed methyl bromide.

Input concn of the gas mixtures were measured periodically throughout each experiment. The output concn of terminal tubes in a train were measured frequently at the start of an experiment, thereafter periodically. With carbon disulfide at 3,000 ppm input, the output of a terminal tube reached 80% of that concn in 1 hr and 92% in 3 hr. After several more hours, the output reached 98% of the input and maintained this concn thereafter. At the end of a treatment period, the tubes were purged by passing filtered air through them. After 1.5 hr, the output decreased to 300 ppm. Since CS_2 bled slowly from the tubes, purging was continued for 24 hr. Methyl bromide attained in-out equilibrium more rapidly and desorbed more completely with a 2-hr air purge.

Growth was determined by measuring the linear extension of rhizomorphs or mycelium. In this medium, *Armillaria* grew as rhizomorph-like mycelium which was white and flat with widths of 1 to 20 mm, and thus differed from rhizomorphs normally found on the exterior of roots in the field, where they tend to be round and black. There were some difficulties in measuring growth of this mycelium. Rhizomorphs occasionally broke through the agar surface and protruded into the air. Growth ceased until new rhizomorphs were formed under the surface again, when linear growth resumed. Sometimes, new growth in fumigated cultures originated within the main mass of mycelium instead of from the margins of the culture. Fortunately, these occurrences were rare, and when observed, the tubes were discarded.

Effect of methyl bromide on A. mellea.—Eight experiments on the effect of methyl bromide on growth of *A. mellea* were made. The concns used ranged from 600 to 2,400 ppm and times of exposure varied from 0.5 to 24 days. The most striking results

were that viable growth of *A. mellea* ceased almost immediately upon application of the gas with all treatments. The combinations of concn and times used were not lethal, and growth resumed at varying times following aeration of the cultures. These responses are shown in Fig. 1 (top) for treatments for 6 days.

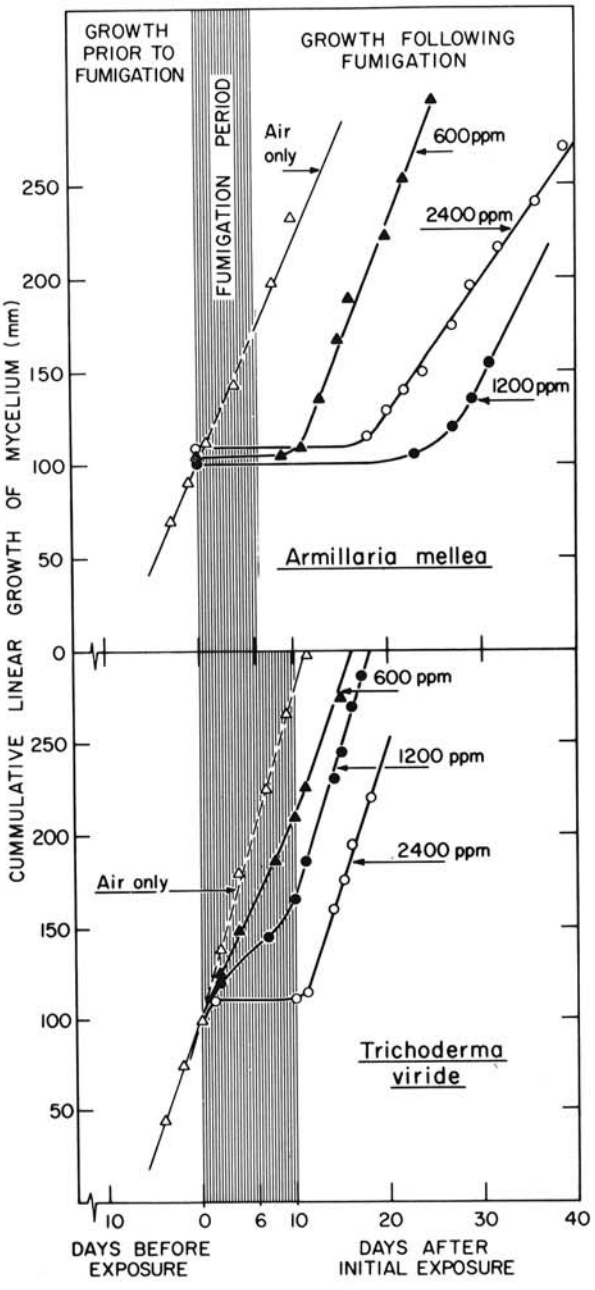

Fig. 1. Inhibition of linear growth of fungi treated with methyl bromide applied in an air stream continuously passing over the colony at the rate of 20 ml per min. **Top.** Effect on growth of mycelia and rhizomorphs of *Armillaria mellea* treated for 6 days. **Bottom.** Effect on growth of *Trichoderma viride* treated for 10 days.

The lag period, consisting of the time of fumigation plus the time following fumigation before growth resumed, was somewhat unusual. This is shown in Fig. 2 for experiments involving treatment with five concns of methyl bromide exposed for periods of less than 1 day to 24 days. The length of the lag period was proportional to the duration of the treatment only once (1,066 ppm). This proportionality was not evident with the other concn used. Instead, with concn above 1,066 ppm methyl bromide, the lag periods were quite similar, the mean being approximately 20 days. The two largest deviations from the mean occurred when the two longest times of exposure were used for concns of 1,200 and 2,400 ppm. The response to the lowest concn (600 ppm) were erratic and did not fit the patterns obtained from the other tests. This correlates with results obtained when fumigating infested root pieces; exposures to concn less than 600 ppm produced erratic results in biological kill of *Armillaria* (8).

These data clearly indicate that linear growth of mycelium and rhizomorphs of *A. mellea* is stopped by a 2-day exposure to a concn of methyl bromide as low as 600 ppm. Growth did not resume for a significant period after the toxicant was removed.

Effect of methyl bromide on T. viride.—In preliminary experiments, cultures of *A. mellea* and *T. viride* were exposed to very high concns of methyl bromide for 3 days. *A. mellea* was killed, but *T. viride* survived, indicating that *T. viride* was considerably more tolerant of methyl bromide. Also, when *T. viride* was subjected to 500 ppm methyl bromide for 10 days, growth was almost identical to growth of the fungus in air. In more refined experiments, the effect upon linear growth of *T. viride* treated with 600, 1,200, or 2,400 ppm methyl bromide for 10 days was measured (Fig. 1, bottom). Only slight inhibition of

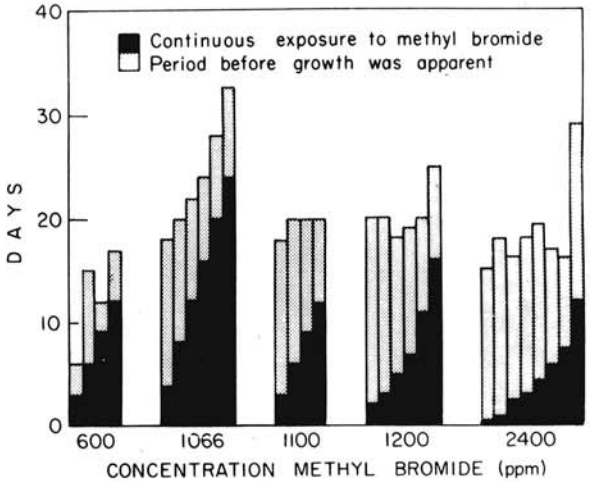

Fig. 2. Effect of methyl bromide concentration and length of exposure on the time required from start of fumigation before growth of *Armillaria mellea* resumed (lag period). Growth ceased with all treatments immediately upon application of the gas.

growth occurred at 600 ppm, and growth continued unabated in the presence of methyl bromide. It was particularly significant that, even at 1,200 ppm, growth occurred in the presence of methyl bromide, although the rate was less than that in air. At 2,400 ppm, growth ceased during the exposure period, but it resumed almost immediately upon removal of the gas.

Effect of carbon disulfide on A. mellea.—Three experiments were performed on the effect of carbon disulfide on linear growth of *A. mellea*. Once, 6,000 ppm was used for seven periods ranging from 18 hours to 18 days; and twice, 3,000 ppm was applied for five periods ranging from 1 to 15 days. With all combinations used, visible growth stopped during the treatment, and there was a pronounced post fumigation period before growth resumed. The response of *A. mellea* to exposures for 8 days is presented in Fig. 3 (top). In contrast to the data obtained using methyl bromide, the lag periods for exposure to carbon disulfide were fairly proportional to the concn and time of exposure of the gas. These relationships are shown in Fig. 4. These studies confirm data obtained earlier, using a less accurately controlled concn of carbon disulfide, that *A. mellea* stopped growing in the presence of carbon disulfide, that a period of delayed growth occurred following treatment, and that the length of the lag period was proportional to the product of the concn and time of exposure.

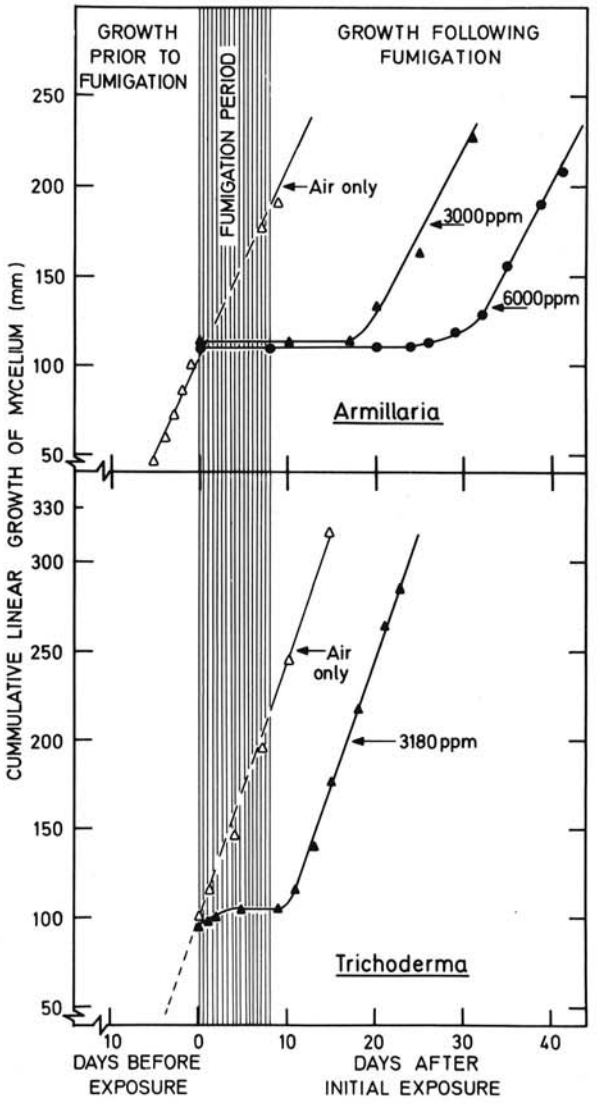

Fig. 3. Inhibition of linear growth of fungi treated with carbon disulfide applied in an air stream continuously passing over the surface at the rate of 20 ml per min. **Top.** Effect on growth of *Armillaria mellea* treated for 8 days. **Bottom.** Effect on growth of *Trichoderma viride* treated for 8 days.

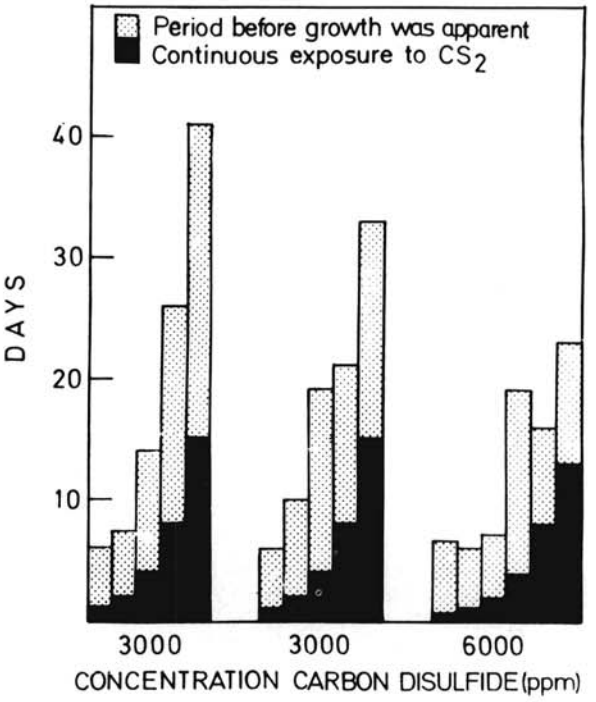

Fig. 4. Effect of carbon disulfide concentration and length of exposure on the time required from start of fumigation before growth of *Armillaria mellea* resumed (lag period). Growth ceased with all treatments immediately upon application of the gas.

Effect of carbon disulfide on Trichoderma viride.—The effect of exposure of mycelia and spores of *T. viride* for 1-10 days to 3,180 ppm carbon disulfide in a moving air stream was studied. Growth was slowed by exposure for 1 day, but a pronounced lag was not evident. With exposure for 2-10 days, growth ceased after exposure for 2 days and resumed again within 1 day after the toxicant was removed. The response of *T. viride* to exposure for 8 days is shown in Fig. 3 (bottom).

DISCUSSION.—The difference in the response of *A. mellea* and *T. viride* to methyl bromide or carbon disulfide has great significance in attempting to

explain how the two organisms interact in soil following soil fumigations. *A. mellea* inocula (infested root pieces) survived fumigation in air with low concns of methyl bromide or carbon disulfide, provided that the inoculum pieces were either kept in air or buried in sterile soil after fumigation. But if the inocula were buried in nonsterile soil immediately after fumigation, then *A. mellea* was not viable after 21 days. The ease with which *Trichoderma* becomes antagonistic, following fumigation, to *Armillaria* growing on a wood substrate is somewhat anomalous (9). *A. mellea* survives for many years on roots and stems deep in soil amongst *Trichoderma*, as well as other soil microorganisms. The paradox is confounded by discoveries (Wilbur & Munnecke, *unpublished*) that *T. viride* easily eliminates *A. mellea* from mixed cultures on agar media, but only massive concns of spores of *T. viride* added to *A. mellea* growing on wood will kill otherwise nontreated *A. mellea*. Also, if *T. viride* is growing as a monoculture using previously sterilized soil as a medium, and large quantities of *T. viride*-infested soil are applied, *A. mellea* may be killed, even though it is growing on a natural wood substrate (1, 4). In this regard, neither Garrett nor we have been able to increase the concn of *Trichoderma* in natural field soils to levels that are antagonistic to *A. mellea* without accessory use of chemicals such as carbon disulfide or methyl bromide. As a result of these observations and experiments, we believe that the evidence indicates that *A. mellea* must be weakened or stressed before *Trichoderma* may effectively exert its antagonistic action on it. We believe that the delayed growth period following fumigations in these experiments is indicative of a weakening effect resulting from the fumigations. In the experiments reported herein we have shown that *T. viride* is capable of growing in the presence of methyl bromide at concn at which *A. mellea* does not visibly grow for periods of approximately 20 days. Perhaps it is during the delay period that *T. viride* is capable of parasitizing *A. mellea*, but not at other times. This phase of research is being currently pursued in this laboratory.

These data correlate well with our earlier report on the dosage response of *A. mellea* to methyl bromide (8). The concns used are within the accurate ranges of the dosage response curves derived earlier, 600-2,400 ppm for 3-10 days. Coincidentally, the data are within the ranges of measurements, wherein concn of methyl bromide deep in field soils are frequently of the order of 600-1,000 ppm for over 10 days (Kolbezen, Munnecke, & Wilbur, *unpublished*).

A. mellea, as contrasted to *T. viride*, was much more susceptible to methyl bromide or carbon disulfide. Mycelial growth of *A. mellea* was stopped by application of either methyl bromide or carbon disulfide and growth did not resume for long periods following aeration of the cultures. In contrast, growth of *T. viride* was unaffected, or only temporarily inhibited, by much higher combinations of concn X time. Also, growth of *T. viride* resumed almost immediately upon removal of the toxicants.

Both fungi used in these experiments were more tolerant of carbon disulfide than of methyl bromide. It is impossible from these experiments to assess accurately these relationships, but the toxicity of carbon disulfide is of interest, since it is generally less fungitoxic than methyl bromide. The reasons for this may be, in part, because the concn were maintained at a constant level and so sorbtive effects were minimized. Also, the fungi were directly exposed to the vapors, so problems due to poor permeation through extraneous materials were avoided. Conversely, carbon disulfide has been used for decades to attempt to control *A. mellea*, with varying degrees of success, and it may be that *A. mellea* is particularly susceptible to it as compared to other plant pathogenic fungi.

We realize that only one isolate of each fungus was used and that the results obtained possibly may be atypical of what might happen in the field. This is a common limitation to such studies since variation among organisms is the rule, rather than the exception. However, the isolates used here formed an actual *Armillaria-Trichoderma* complex obtained from an infested citrus grove; furthermore, the reported responses fit well with our other laboratory and field experiences and thus add credibility to these data. For example, Ohr et al. (9) showed that the LD_{50} value for two *Armillaria* isolates was approximately half that for the mean of four *Trichoderma* spp. isolates, and that there was very little variation in response within the two genera. Further, *Trichoderma* spp. are invariably isolated from *A. mellea*-infested roots following field fumigations and it takes approximately 3 weeks of storage in nonsterile soil following fumigation before *A. mellea* is killed by its antagonists, predominantly *Trichoderma* spp. (9). The length of the storage period following fumigation agrees closely with the lag period reported in this paper.

Studies are being continued to attempt to accurately assess the defense mechanisms of *A. mellea*, and the attack mechanisms of *Trichoderma* spp. in relation to soil fumigation within this intriguing complex system.

LITERATURE CITED

1. BLISS, D. E. 1951. The destruction of Armillaria mellea in citrus soils. Phytopathology 41:665-683.
2. DARLEY, E. F., & W. D. WILBUR. 1954. Some relationships of carbon disulfide and Trichoderma viride in the control of Armillaria mellea. Phytopathology 44:485 (Abstr.).
3. EVANS, E. 1955. Survival and recolonization by fungi in soil treated with formalin or carbon disulfide. Trans. Brit. Mycol. Soc. 38:335-346.
4. GARRETT, S. D. 1957. Effect of a soil microflora selected by carbon disulfide fumigation on survival of Armillaria mellea in woody host tissues. Canad. J. Microbiol. 3:135-149.
5. GARRETT, S. D. 1958. Inoculum potential as a factor limiting action by Trichoderma viride Fr. on Armillaria mellea (Fr.) Quel. Trans. Brit. Mycol. Soc. 41:157-164.
6. KOLBEZEN, M. J., & F. J. ABU-EL-HAJ. 1972.

Fumigation with methyl bromide. I. Apparatus for controlled concentration, continuous flow laboratory procedures. II. Equipment and methods for sampling and analyzing deep field soil atmospheres. Pestic. Sci. 3:67-80.

7. MUGHOGHO, L. K. 1968. The fungus flora of fumigated soils. Trans. Brit. Mycol. Soc. 51:441-459.

8. MUNNECKE, D. E., W. D. WILBUR, & M. J. KOLBEZEN. 1970. Dosage response of Armillaria mellea to methyl bromide. Phytopathology 60:992-993.

9. OHR, H. D., D. E. MUNNECKE, & J. L. BRICKER. 1973. The interaction of Armillaria mellea and Trichoderma spp. as modified by methyl bromide. Phytopathology 63:965-973.

10. WILBUR, W. D., D. E. MUNNECKE, & E. F. DARLEY. 1972. Seasonal development of Armillaria root rot of peach as influenced by fungal isolates. Phytopathology 62:567-570.