Effects of Moisture, Chloropicrin, and Methyl Bromide Singly and in Mixtures on Sclerotia of *Sclerotium rolfsii* and *Verticillium albo-atrum*

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ABSTRACT

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The effects of moisture on the toxicity of methyl bromide (MB) and chloropicrin (CP) to the sclerotia of *Sclerotium rolfsii* and the microsclerotia of *Verticillium albo-atrum* were studied in a laboratory system in which the propagules were exposed to fixed concentrations of the toxicants in a free-flowing mixture in air for varying periods. Moist propagules exposed to the fumigants contained approximately 45% water and saturated propagules contained approximately 150% water, based upon oven-dry weights. The effectiveness of MB was not as greatly affected by the moisture content of the two propagules as that of CP. During short exposures, moist propagules were more sensitive than saturated propagules to MB. However, during long exposures, saturated propagules were more sensitive to the gas. In contrast to MB, the effectiveness of CP was greatly enhanced when the propagules were saturated. Saturated sclerotia of *S. rolfsii* were four to eight times more sensitive than moist sclerotia, depending upon time of exposure to CP. Saturated propagules of *V. albo-atrum* were 40 to 50 times more sensitive than moist propagules. The effect of mixtures of the two gases on saturated sclerotia of *S. rolfsii* was determined by holding MB concentrations constant at 10,200 μl/L and introducing various concentrations (175–825 μl/L) of CP. The mixtures of MB and CP acted synergistically.

Methyl bromide (MB) is commonly used in combination with chloropicrin (CP) to fumigate field soils. The mixtures combine the advantages of the greater soil penetration of MB and higher fungitoxicity of CP. In early work, which directly led to widespread usage in California, Wilhelm et al (14) noted that *Verticillium albo-atrum* Reinke & Berth., which has been known to be relatively tolerant of MB (5), was more effectively controlled by mixtures of MB and CP than by either fumigant alone. Their field results indicated that the two gases acted synergistically, but the relationship was not proven. Kolbezen and Abu-El-Haj (2) devised an apparatus for the quantitative measurements of the effect of fumigants on fungi and other organisms (4); it was modified for use in this study.

The objectives of this study were to determine the effects of moisture on the response of sclerotia of *Sclerotium rolfsii* Sacc. and microsclerotia of *V. albo-atrum* to treatment with MB or CP; and to determine whether mixtures of the two gases act synergistically.

MATERIALS AND METHODS

Apparatus design and operation. The apparatus and method for fumigating with MB-in-air mixtures at constant flow and constant concentration were described previously (2,4). Mixtures of CP in air were produced by bubbling air at a measured rate through warmed liquid CP, then passing this saturated vapor through a helical coil immersed in cold water at constant temperature. Excess CP condensed and yielded a gaseous mixture whose concentration was accurately known from published vapor pressure data. This known CP concentration was mixed with flowing air or MB streams to produce the desired fumigant mixtures. The upper useful CP concentration at room temperature is limited by its vapor pressure and is approximately 25,000 μl/L. The apparatus will be described in another paper (M. J. Kolbezen, *unpublished*).

The concentrations of the gases at the inlet port of the reaction vessels were monitored automatically by gas chromatography every 4 min. Only minor adjustments were necessary during an experiment to maintain exact concentrations. The rates of flow of the gases to each reaction vessel (250-ml Erlenmeyer flask), which were carefully adjusted so they were constant for each flask, varied from 19 to 21 ml/min and averaged 20 ml/min. The gas mixtures were humidified by bubbling them through water (20–22 C) before passage into reaction vessels so that there was no moisture loss from the fungal propagules during a fumigation.

Fungi. Sclerotia of *S. rolfsii* were used. They were produced on Difco potato-dextrose agar (PDA) in petri plates and used after at least 12 wk of growth. The sclerotia from 10–12 plates were dislodged by tapping culture plates inverted over a clean paper. Sclerotia were either moist or saturated when fumigated. Moist sclerotia were prepared by drying them on plates, on a laboratory bench for 48 hr, and then rehydrating them by passing water-saturated air over them at 20 ml/min for 16–24 hr. They contained approximately 45% water, based upon oven-dry weight. Saturated sclerotia were collected from PDA plates, and 200–250 were mixed with 100 g of sterile white sand containing 5 ml of sterile water in a 250-ml flask. These sclerotia contained ~150% water based upon oven-dry weight, and they were considered to be saturated. Moist sclerotia were fumigated in empty flasks, whereas saturated sclerotia were fumigated in moist sand in flasks. Two replicate flasks were used for each exposure period; there rarely was a significant difference in response of the propagules in duplicate samples.

After fumigation the flasks were quickly aerated by flushing with air for 15 sec. Moist sclerotia were retrieved and individually plated on PDA containing 30 μg of streptomycin sulfate per liter and using 20 sclerotia per plate and five plates per treatment from each reaction vessel. Saturated sclerotia were retrieved after aeration by adding 20–30 ml of sterile water to the sand, swirling it, and pouring off the floating sclerotia into a cheesecloth screen. They were plated out as described for the moist ones. Data on viability of the sclerotia were taken daily until readings for fungus germination for 3 consecutive days were the same. Usually this occurred within 10–15 days after fumigation.

Suitable propagules of *V. albo-atrum* were difficult to obtain. The best method consisted of growing the fungus on filter paper. Conidia from each of four test tube cultures of the fungus growing on PDA were suspended in 10 ml of sterile water, mixed with 10 ml of double-strength Czapek broth, and used to saturate sterile Whatman No. 1 filter papers. The papers were drained of excess liquid, placed in sterile petri plates, and incubated 2–4 wk at 24 C.

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After this treatment, the filter papers contained 10,000–20,000 microsclerotia per square centimeter. When used, the filter papers were removed, air-dried 40–48 hr prior to fumigation, cut into 1-cm squares, and five squares were skewered approximately 1 cm apart on a steel wire. Since there was not enough time to prepare inoculum and fumigate on the same day, the pins, with the papers affixed to them, were enclosed in a plastic bag at 23 C for 20–24 hr to avoid contamination before fumigating.

When fumigated in the moist condition, the papers were placed in a flask without further treatment; for the saturated condition, the papers were saturated with sterile water before enclosing them in the flasks. After fumigating and aerating, each paper was cut into four equal squares and aseptically placed upon water agar containing 30 μg of streptomycin sulfate per liter and sterilized okra straw. Okra straw was prepared by taking stems 20 cm long from greenhouse-grown plants, chopping into pieces approximately 4 cm long, drying, grinding in a mill, and sterilizing with propylene oxide (1 ml propylene oxide per 0.5-L volume for 24 hr). Approximately 100–200 pieces were added to each petri dish and mixed with molten agar containing streptomycin. Each small square of paper containing the inoculum was considered to be a propagule unit even though it contained thousands of microsclerotia. Plates were observed daily until there was no change in numbers of propagule germination for 3 successive days. The straw in the medium was useful in the identification of *V. albo-atrum* since the microsclerotia that form on the straw could be recognized easily.

The effectiveness of treatments was assessed by plotting standard dosage response curves with data obtained by exposing fungus propagules to a constant concentration of gas and varying times of exposures as described earlier (6). The LD_{90} values for each concentration and its corresponding time of exposure were linear when plotted on log-log paper, using concentration (C) and time (T) as coordinates.

RESULTS

Effect of moisture on response of fungi. The nature of the responses of both fungi to MB are in Fig. 1A and C. Individual variations were small even when saturated propagules were fumigated. The effectiveness of MB was not greatly affected by the moisture content of the fungus propagules, especially *V. albo-atrum*. The response of *S. rolfsii* to MB was more pronounced, however. The CT curves for the LD_{90} values of moist and saturated propagules for each fungus intersected (Fig. 1A). This demonstrated that the effect of moisture content of the propagules on effectiveness of MB was different during short, than during long exposures. During short exposures, moist propagules were more sensitive to MB than were saturated propagules. During long exposures, however, the situation was reversed and saturated propagules were more sensitive to MB.

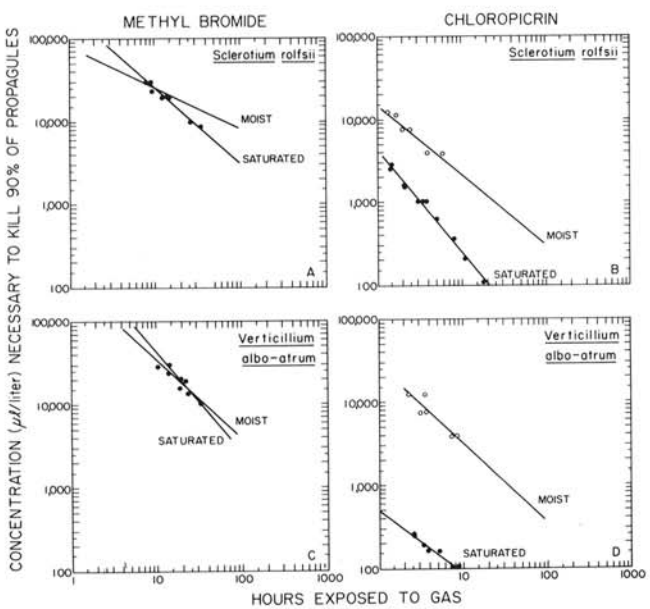

Fig. 1. Effect of moisture content of fungus propagules on their response to fumigation with methyl bromide (MB) or chloropicrin (CP). Moist propagules containing approximately 45% water (oven-dry weight basis). Saturated propagules containing 150% water (oven-dry weight basis) were fumigated in a slight excess of water. **A,** MB vs sclerotia of *Sclerotium rolfsii*; **B,** CP vs sclerotia of *S. rolfsii*; **C,** MB vs microsclerotia of *Verticillium albo-atrum*; **D,** CP vs *V. albo-atrum*. Note: regression lines for moist propagules in Fig. 1A and C are from data in Table 1 (4); figure points are for saturated propagules only.

In contrast to MB, the effectiveness of CP was greatly affected by the moisture content of the fungus propagules (Fig. 1B and D). In fact, it was difficult to obtain reproducible data when moist propagules were fumigated, because dosage response curves frequently were saw-toothed, rather than linear, and it was necessary to use high concentrations and short exposures to obtain reproducible data, such as reproduced in Fig. 1B and D. The curves (Fig. 1B and D) of the moist and saturated propagules were not parallel, but they did not intersect under the conditions used, as was the case with MB. Saturated propagules, especially those of *V. albo-atrum,* were much more sensitive to CP than moist propagules. Saturated sclerotia of *S. rolfsii* were approximately 4.5 to 7.7 times (at 2 and 7 hr exposures, respectively) more sensitive to CP than to their moist counterparts. With *V. albo-atrum*, saturated propagules were approximately 40–50 times (7 and 2 hr exposures, respectively) more sensitive than moist ones. The extreme sensitivity of *V. albo-atrum* in the saturated condition made it imperative to use very short exposure periods for the studies of MB and CP mixtures.

Comparison of effectiveness of MB and CP (Fig. 1). The two fungi were much more sensitive to CP than MB. Against moist sclerotia of *S. rolfsii* CP was approximately 7–11 times (2 and 10 hr exposures, respectively) more effective, and in the saturated state it was approximately 100 times (2 or 10 hr of exposure) more effective than MB. It was more difficult to make comparisons of the effects of the gases on *V. albo-atrum* because of the extreme sensitivity of the fungus to CP when fumigated in the saturated condition. Conversely, its high insensitivity to MB when exposed in either the moist or saturated condition further complicated the problems. When the responses for 7-hr exposures were compared, CP was approximately 11 times more effective than MB against moist propagules of *V. albo-atrum* and over 500 times more effective when they were saturated.

Test for synergism between MB and CP. Saturated sclerotia of *S. rolfsii* were used in studies of synergism since results for that system were the most reproducible. Dosage responses were obtained for MB or CP fumigations alone and for mixtures containing a constant concentration of MB, but various concentrations of CP. A test for synergism was made as follows.

Dosage response curves were obtained for a range of concentrations of CP (112–2,680 μl/L) (Fig. 2). The responses of the sclerotia were predictable, accurate, and the regression lines for each concentration were parallel. Data for exposure to CP at 2,680 μl/L are included even though only a few valid data points were obtained experimentally because of the extreme susceptibility of the fungus, even for short exposure periods. The slope of the curve was estimated based upon data obtained from lower concentrations of CP.

The effect of mixtures of gases was determined by maintaining MB concentration at 10,200 μl/L and mixing various concentrations of CP with it. CP concentrations varied from 175 to 825 μl/L. Dosage response data from two series wherein CP at 386 or 616 μl/L was combined with MB at 10,200 μl/L were used are presented in Fig. 3. Two curves for CP alone (365 or 620 μl/L) and one for MB alone (10,200 μl/L) are included for comparison. The slope of the dosage response curve for MB was very high, which is typical for fungal responses to MB. The slopes of the dosage response curves for CP alone were lower than that for MB, and the slopes of the dosage response curves for the mixture of the two gases were similar to those for CP alone. Addition of CP to MB resulted in dosage response curves that had lower intercept values

than those of the corresponding response curve for CP alone.

A test for synergism between the two gases was made by use of the CT plots of LD_{90} data obtained from dosage response curves of Figs. 2 and 3. In Fig. 4 curve 1 represents the response to MB concentrations ranging 9,000–30,000 μl/L. Curve 2 is for the response to CP over a range of 100–2,900 μl/L, and curve 3 is for the responses to mixtures of MB at 10,200 μl/L and CP at 175–825 μl/L. Since curve 3 was parallel and had a lower intercept value than curve 2, it indicated that the mixtures were more toxic to water-saturated sclerotia than was either gas used alone.

The data were analyzed by the method of Wadley (12). In this method the effect of one toxicant is expressed as the equivalence of the other and the sum of equivalences is compared graphically with effects of the toxicants used singly. We selected the data for CP at 620 μl/L for calculations because it most closely approximated the mixture containing CP at 616 μl/L in MB at 10,200 μl/L (Fig. 3). We adapted Wadley's (12) method as follows: With MB alone it required 26.3 hr of exposure to MB at 10,200 μl/L to obtain a LD_{90} dose. The concentration × time (CT) product was 268.3×10^3 μl/L per hr. With CP alone at 620 μl/L the CT product was 2.79×10^3 μl/L per hr. The equivalence ratio of CP/MB was 2.8 divided by 288.3, or approximately 0.01. That is, MB was approximately 100 times less effective than CP under these specific conditions. When this factor is used to calculate the dose of the mixture of CP at 620 μl/L and MB at 10,200 μl/L in terms of CP equivalences, the mixture is equal to CP at 722 μl/L (CP at 620 μl/L + [MB at 10,200 μl/L × 0.01] = 722 μl/L). By entering curve 2, Fig. 4 with CP at 722 μl/L, a value of 4.2 hr is shown to be needed for the corresponding LD_{90}. However, experimental data shown on curve 3 indicated that a dose of 722 μl/L only required an exposure of 3.1 hr to obtain the LD_{90}. Hence, the mixtures of CP and MB acted synergistically.

DISCUSSION

In these experiments the effect of moisture content on effectiveness of CP and MB was measured quantitatively in a laboratory assay. It has been shown (1,6,7–10) that organisms are more susceptible to these fumigants when wet than when dry, but we were surprised to discover how much more susceptible the test fungi were to CP when the propagules were fumigated in the saturated condition. In initial experiments, we used propagules that were considerably drier than reported in these experiments but dosage response curves were erratic and saw-toothed rather than linear. To avoid this variability, plugs of mycelia of the fungi growing on agar were fumigated and CP so effectively killed the fungi in the plugs that it was almost impossible to get a combination of time and concentrations low enough to allow the plotting of valid dosage response curves. This led to the use of water-saturated propagules; however, *V. albo-atrum* was so sensitive to CP that we could not use it in our experiments on synergism. Probably the reason why saturated propagules were so sensitive to CP as contrasted to MB is because CP is so much more soluble in water than MB.

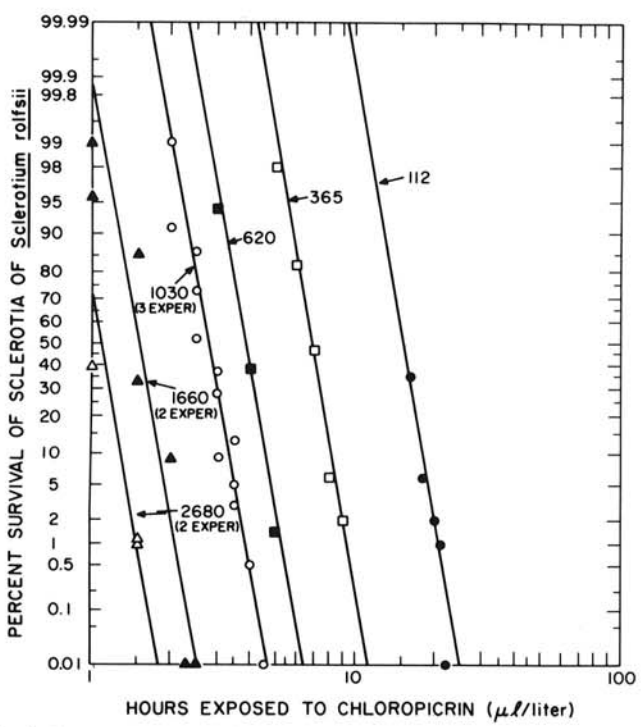

Fig. 2. Dosage responses of sclerotia of *Sclerotium rolfsii* to chloropicrin (CP). Water-saturated (150% water, oven-dry weight basis) sclerotia were exposed for various times to moving air containing CP at constant concentrations as indicated. Numbers in the figure refer to microliters of CP per liter of air.

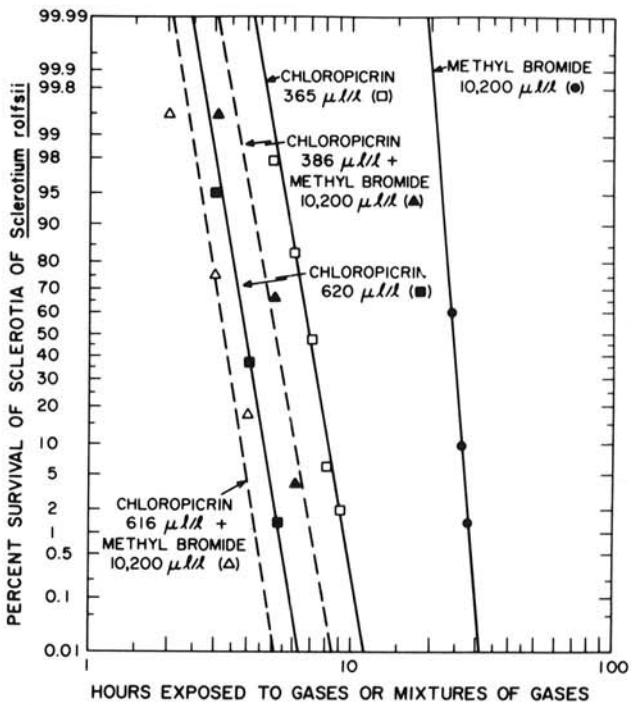

Fig. 3. Dosage responses of water-saturated (150% water, oven-dry weight basis) sclerotia of *Sclerotium rolfsii* to methyl bromide (MB) only, chloropicrin (CP) only, and mixtures of MB and CP.

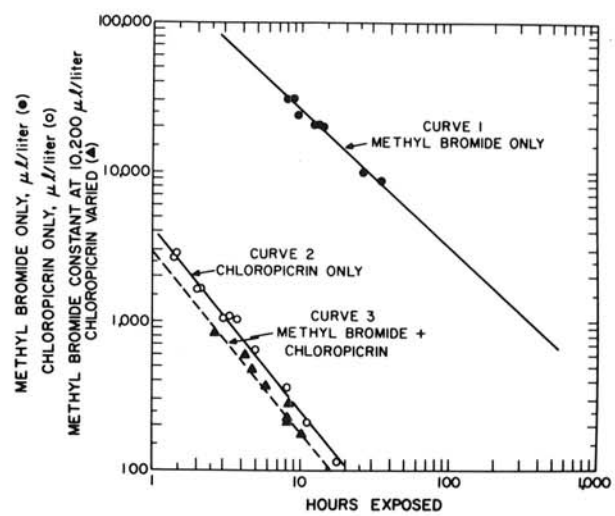

Fig. 4. Concentrations × times (CT) necessary to kill 90% of water-saturated (150% water, oven-dry weight basis) sclerotia of *Sclerotium rolfsii* exposed to methyl bromide (curve 1), chloropicrin (curve 2), or mixtures of methyl bromide and chloropicrin (curve 3).

It is common knowledge that CP is a much better soil fungicide than MB under ideal soil conditions. Even so, we were surprised to find that CP was approximately 100 times more effective than MB against water-saturated sclerotia of *S. rolfsii*. Caution should be taken in trying to apply these laboratory studies of water-saturated sclerotia to practical application in the field. It is unlikely that similar conditions would occur in a field because high soil moisture impedes the gaseous diffusion of both MB and CP. Presumably if a sclerotium in a field soil were as saturated as in these experiments, the soil would be too wet for distribution of the fumigants. The responses of moist sclerotia probably would be nearer those encountered in a field with soil moisture optimum for fumigation. Probably the value of 7–11 times greater effectiveness of CP than MB is more nearly indicative of what to expect in the field.

Sclerotia of *S. rolfsii* produced in soil react to toxicants differently than sclerotia produced on laboratory media (3). We experimented with sclerotia produced according to the method of Linderman and Gilbert (3), but we could not use such sclerotia for our purposes because of low germination and high contamination by other organisms. Since we needed large quantities (over 170,000 sclerotia used) for 65 experiments over a 5-yr period, we relied upon laboratory-produced sclerotia exclusively for these studies.

The question then is, why not use greater proportions of CP in the MB/CP mixtures (usually 2:1, occasionally 1:1, and rarely a higher proportion of MB to CP) used in commerce? Some of the reasons are that MB, a good fungicide, diffuses through soil much more rapidly than CP, it penetrates unrotted tissues more readily, it is a more effective herbicide, and it dissipates from the soil more rapidly.

Wilhelm (13) showed that the preponderance of field data and experiences indicated that MB/CP mixtures acted synergistically, as measured by the effects on soilborne pathogens, particularly as they affected strawberry diseases. Later, Van Aasche et al (11) also claimed synergism for the mixtures. However, the published evidence for the claims was not sufficient to prove the point, so we attempted to investigate the phenomenon by using our quantitatively accurate techniques. It is a credit to the acumen of Wilhelm et al (14) that their intepretations were correct and that mixtures do indeed act synergistically against fungi.

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